DETERMINATION OF OXYTETRACYCLINE RESIDUES IN THE BLOOD AND OTHER TISSUES OFCHICKEN (Callus domesticus L)

BY

S. 0. AMUDA-GIWA (DVM)

DECLARATION

This is to certify that I carried out the work reported in this thesis partly in the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University Zaria, under the supervision of Professors Y.O. Aliu and N. Nwude; and partly at the Area Chemical Laboratory, Food & Drug Administration and Control, Federal Ministry of Health, Kaduna under the supervision of Mr. P. O. Anyameluhor.

The work of other investigators is acknowledged and referred to. No part of this thesis has been submitted elsewhere for a degree or diploma.

Sulaiman Omotesho Amuda-Giwa

Ahmadu Bello University, Zaria

November, 1992.

CERTIFICATION

This thesis entitled "DETERMINATION OF OXYTETRACYCLINE RESIDUES IN THE BLOOD AND OTHER TISSUES OF CHICKEN (Gallus domesticus L.) by SULAIMAN OMOTESHO AMUDA-GIWA meets the regulations governing the award of the degree of Master of Science (Veterinary Toxicology) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Internal Examiners

Prof. Yusuf. O. Aliu, DVM, Ph.D.
(Major Supervisor)
Department of Physiology and
Pharmacology,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria - Nigeria.

Prof. Nwankwo Nwude, Dr. med. vet., PhD.
(Member, Supervisory Committee)
Department of Physiology and
Pharmacology,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria - Nigeria.

Dr. Abdullahi Mustapha, B.Sc., Ph.D.
(Member, Supervisory Committee)
Department of Pharmaceutical Chemistry,
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University Zaria-Nigeria.

External Examiner

Dr. Patrick A. Onyeyili, DVM, Ph.D.
Dept. of Physiology & Pharmacology,
Faculty of Veterinary Medicine,
University of Maiduguri
Maiduguri.

Date: 20-7-94

ACKNOWLEDGEMENTS

My thanks go to my wife for her consistent assistance and encouragement during this study. I am grateful to Mr. Patrick O. Anyameluhor for his orientative assistance on the instrumental Chromatography and Spectroscopy. I want to thank all members of staff, Department of Veterinary Physiology and Pharmacology who assisted me on this work: especially Professors Molokwu E.C.I.; Nwude, N. and Dr. Adaudi, A.O. for their accommodation. I further thank Prof. Nwude, N. for the constructive criticism and valuable suggestions made on the report-format. I acknowledge the guidance, useful suggestions and assistance of Dr. Abdullahi Mustapha of the Department of Pharmaceutical Chemistry, and also the analytical methods proffered by Dr. Daniel Ngur of the Department of Pharmacology and Clinical Pharmacy. I am grateful to most especially Professor Y.O. Aliu and Dr. J. Amupitan whose trainings and counselling were found invaluable to obtain the analytical preparedness required by this research. I also thank a number of staff/lecturers of the Department of Chemistry, A.B.U., Zaria for their technical assistance. I extend profound gratitude to Dr. Patrick Onyeyili (my external examiner) for his diligent read-proving of the thesis, and the expression of useful suggestions.

Acknowledgement is made to the Food & Drug Administration and Control, Federal Ministry of Health and Human Services for the Study Leave granted to pursue the course, and also thankful for the material support. I commend the part-financing of the research by Wellcome Nigeria Fund. Last but not the least, the typing assistance of Messrs: Sabo Bature and Sunday O. Unwuchola is thankfully acknowledged.

ABSTRACT

Due Co the oxytetracycline's usefulness and availability, its effect in a local food animal (Chicken: Gallus domesticus L) was investigated, and the residual drug level in the tissues, assessed.

Seventy-two young broiler-type chick (of both sexes) were used for the experiment. They were herded in batches of 12 as groups A - F. The groups were given feed (Sanders (R) miixed with the Terratnycin (^{R}) poultry Formula at 300, 200, 150, 50 and 0 g/ton for the groups A - F, respectively, and served water: at lib. They were housed in a slat-litter partitioned illuminated cages, and sacrifised at 3, 6, 9 and 12 weeks intervals. Analysis for the oxytetracycline levels in the muscle, kidney, liver, fat, intestinal contents and the blood were done; using ultraviolet-visible spectroscopy. The tissue samples were also evaluated by thin layer chromatography and colour-spot-tests.

The spectroscopic assay were used for eggs from 6 mature layers, also grouped A - F. From the spectroscopy, the highest drug residue obtained in the muscle, liver, kidney and fat were:- 1.70, 2.65, 4.0 and 2.85u/g respectively at 300g/ton dosage level after 6 weeks of feeding. The least concentrations were recorded in the above tissues at three weeks of feeding 50g/ton ration. Two days drug withdrawal resulted in abscence of drug residues in birds fed 50, 100 and 150 g/ton rations respectively. The eggs - test also were negative for residues during medication and at drug withdrawal period: at all dosage levels, for the one week medication period.

The R_f value (Retardation factor) obtained by the thin layer chromatography of 40 paired samples, averaged 0.08 ($P = 0.95$) for the oxytetracycline antibiotic in the specified stationary and mobile phases. Colour Spot Tests evaluation of the samples and standards, showed that the B. Vet. C. (1953) positive colour reaction (crimson) was found often with 0.5mg/ml contained in media preparation of 0.25ml and 0.5ml volumes.

The animals gained weight at all drug-feeding-levels: the highest being 93.0% after feeding 200 g/ton for 12 weeks, the lowest was 14.3% when 0g/ton was fed for 3 weeks. Various mortality rates were also observed per group. Pathologic lesions were observed in the viscera of animals sacrifised or posted. The lesions present in the liver of some of the birds ranged from mottled appearance, to necrotic foci: and appear to be dose dependent. Some of the birds also had Eimeria tenellosis, taeniasis, and/or ascariasis. The oxytetracycline prophylactic and curative dosages, were reviewed in-line with the existing regulatory measures, the 100g/ton dosage level, on account of "group-average" gain-in-weight, toxicity/liver lesions, the tendency of drug residue levels falling in proportion to drug concentration in feed, was consequently evaluated to be optimal for prophylaxis, or as growth-promoter in the chicken. The regulatory guidelines necessary for drug use in food animal production were suggested, as a factor for production of food, and proper nutrition: for the attainment of the goals of primary health care in this country.

TABLE OF CONTENTS

		PAGE
Title Page	:	i
Declaration	:	ii
Certification	:	iii
Acknowledgement	:	iv
Abstract	:	v
Table of Contents	:	viii
List of Tables	:	xi
List of Figures	:	xii
List of Plates	:	xii
List of Appendices	:	xii
Abbrevations, Symbols and Definitions	:	xiii

INTRODUCTION

Chapter 1.

1.1	Discovery of Tetracyclines	:	1
1.2	Susceptible Organisms	:	2
1.3	Extent of Usage	:	2
1.4	Commercial Availability and Purity		7
1.5	Research Objective	:	7

Chapter 2. LITERATURE REVIEW

2.1	Chemistry	:	14
2.2	Pharmacology and Toxicology of Oxytetracycline	:	25
2.3	Pharmacokinetics and Bioavailability		28
2.4	Drug Residues in Animal-Derived Foods		32

PAGE

Chapter 3. MATERIALS AND METHODS

3.1 Materials : 34

3.1.1 Test Drugs and Standards : 34

3.1.2 Test Animals and Management : 35

3.1.3 Laboratory Glasswares : 36

3.1.4 Analytical (Grade) Reagents : 36

3.1.5 Analytical Instruments : 37

3.2 Methods : 38

3.2.1 Study Design : 38

3.2.2 Medication of Experimental Animals 39

3.2.3 Sampling Methods for Blood and Other Tissues : 40

3.2.4 Stock and Working Solutions Prepared 41

3.2.5 Preparation of OTC Standards : 42

3.2.6 Ultra-Violet-Visible Spectroscopy 44

3.2.7 Colour Spot Tests : 50

3.2.8 Thin Layer Chromatography : 51

Chapter 4. RESULTS

4.1 Observations made after Medication of Experimental Animals : 53

4.2 Levels of OTC Residues in Blood and Other Tissues from: Spectroscopy : 55

4.3 Levels of OTC Residues in Blood and Other Tissues from: Colour Spot Tests : 62

4.4 Levels of OTC Residues in Blood and Other Tissues from: Thin Layer Chromatography 70

PAGE

Chapter 5. DISCUSSIONS AND CONCLUSION

5.1 Management and Medication of Animals 74

5.2 Levels of OTC Tissue Residues from Colour Spot Tests, Chromatography and Spectroscopy 76

5.3 Aspects of Regulations on Drug Residues in Human Foods : 80

5.4 Conclusion : 82

BIBLIOGRAPHY : 85

APPENDICES : 94

LIST OF TABLES

Table 1: Percentage Weight Gain of Birds fed with various concentrations of OTC.

Table 2: Average Oxytetracycline (OTC) concentrations ($\mu g/g$) In the Blood and Other Tissues of chicks, after Administration in Feeds at Various concentrations and Time Intervals.

Table 3: Quantities of Oxytetracycline Residues Found in Layers' Blood and Eggs at various Sacrifice Intervals and Drug Feeding levels: Evaluated from the Regression Analysis.

Table 4: Tissues' Oxytetracycline Recovery: Evaluated from the Regression Analysis.

Table 5: Evaluation of Oxytetracycline content of Terramycin Poultry Formula.

Tables 6: (a-c) Colour Spot Tests on Oxytetracycline Standards

Tables 7: (a-d) Colour Spot Tests for Oxytetracycline Residues in Tissues.

LIST OF FIGURES

Figure 1 Octahydro analog of naphthacene

Figure 2 Tetracycline

Figure 3: Oxytetracycline

Figure 4: Chlortetracycline

Figure 5: Three acid groupings in tetracycline nucleus

Figure 6: Tetracycline (Pharmacophore)

Figure 7: OTC configuration for N M R.

Figure 8: Extraction of Oxytetracycline from the Biologicmatrix

LIST OF PLATES

Plate I: The Domestic fowl (chicken): Gallus domesticus L.

Plate II: Experimental Animals: Starting stock

Plate III: The Tissue Samples

Plate IV: Colour spot test on ten preparations of constant 1ml volumes having OTC concentrations from 1.0mg to 10.0mg.

Plate V: Colour-change from crimson (Plate IV) to yellow

LIST OF APPENDICES

Appendix 1: Absorbance Readings of Working Solutions A-D.

Appendix 2: Absorbance Readings of Oxytetracycline Standard solutions: Used for constructing Calibration Curve.

Appendix 3: Least Square Regression Analysis of UV-Visible Spectrophotometry's Data of Oxytetracycline standards

Appendix 4: Oxytetracycline calibration curve: Derived by Least Square Regression Analysis.

Appendix 5: Quantities of Oxytetracycline (OTC) Residues in the Broiler-type Tissues at various Feeding Duration: Evaluated from the Regression Analysis.

Appendix 6: Chromatography of Broiler-type Tissues and Oxytetracycline Standards.

ABBREVIATIONS, SYMBOLS AND DEFINITIONS

ABBREVIATIONS:

ad lib/ad libitum	:	At pleasure
A.E.R.L.S.	:	Agricultural Extension & Research Liaison Service
A.U.C.	:	Area Under the Curve
B. Vet. C.	:	British Veterinary Codex
B.V.M.	:	Bureau of Veterinary Medicine
b.w.	:	Body weight
C.R.D.	:	Chronic Respiratory Disease
C.S.F.	:	Cerebrospinal Fluid
C.T.C.	:	Chlortetracycline
D.C.	:	Doxycycline
E.D.T.A.	:	Ethylene diamine tetraacetic acid
ED_{50}	:	Effective dose for 50% of the test animals
eg.	:	For example
FAO	:	Food and Agricultural Organisation of the United Nations
HCl	:	Hydrochloric acid
hr	:	hour(s)
ie.	:	that is
i/m	:	intramuscular
in status quo	:	in the same state (as formerly)
in utero	:	in the womb
in vivo	:	in living tissue
i/p	:	intraperitoneal
i/v	:	intravenous

LD_{50}	:	Lethal dose for 50% of the test animals
≮	:	Not less than
M	:	Molar/Molarity
MC	:	Methacycline
N	:	Normal/Normality
NaOH	:	Sodium hydroxide
OTC	:	Oxytetracycline
OTC-C	:	Oxytetracycline conventional
OTC-LA	:	Oxytetracycline Long acting
OTC.HCl	:	Oxytetracycline hydrochloride
$OTC.2H_2O$	:	Oxytetracycline dihydrate
PEG 400	:	Polytethylene glycol 400
Qty	:	Quantity
(R)	:	Registered/Trade Mark
rpm	:	Revolutions per minute
RTC	:	Rolitetracycline
S/N	:	Serial number of animal/sample/solution
TC	:	Tetracycline
TC.HCl	:	Tetracycline hydrochloride
TI	:	Therapeutic Index
t.i. d.	:	ter in die/Three times daily
US/USA	:	United States of America
USFDA	:	Food and Drug Administration of the United States of America
USP	:	United States Pharmacopeia
UV-Vis/UV-Visible	:	Ultraviolet - Visible Spectrophotometer/ Spectrophotometry.

v/v	:	Volume-to-Volume
WHO	:	World Health Organisation of the United Nations
w/v	:	Weight-to-volume

SYMBOLS:

A	:	Absorbance of UV-Vis Spectrophotometer
b	:	Pathlength of the light through the solution
c	:	Concentration of substance of interest in the solution of pathlength "b".
°C	:	Degree(s) centigrade
C$	:	Canadian dollar(s)
g/gm	:	Gram(s)
K	:	Constant parameters: ξ and b
Kg	:	Kilogram(s)
ξ	:	Not less than
M	:	Meter(s)
mg	:	milligram(s)
ml	:	millilitre(s)
mm	:	millimeters
μ	:	micron (ie 10^{-6})
μg	:	micrograms
μL	:	microlitre
nm	:	nanometers (ie 10^{-9}m)
pH	:	Hydrogen ion concentration
ppm	:	Parts per million
$\propto$	:	Proportional to
ton	:	metric ton(ie 1,000kg)
U$	:	United States (US) dollars

DEFINITIONS:

Absorbance	:	UV-Vis Spectrophotometers absorbance reading at 353 nm.
Sample	:	A portion (of Chemical Preparation or animal tissue) taken from a specified type-quantity/batch to be used for chemical analysis.
Tissues	:	Sample of the broiler-type components, ie blood, intestinal contents, muscle, kidney, liver and fat; or the eggs.
Musculus biceps femoris	:	One of the posterior femoral (thigh) muscles. It originates from the pelvis (tuber ischii) and lines the length between it and the knee bone (the knee joint) where it inserts. It is used for extension and flexion of the limb, and also for propulsion.
Musculus longissimus dorsi.	:	One of the muscles of the trunk (back axial ordinate).
Musculus semimembranosus.	:	One of the posterior femoral (thigh) muscles. It originates from the pelvis (tuber ischii) and lines the length between it and the femur and tibia (the knee joint) where it inserts. It is used for hip extension.
Musculus semitendinosus.	:	One of the posterior femoral (thigh) muscles. It originates from the pelvis (tuber ischii) and lines the length between it and the leg bone (tibial crest) where it inserts. It extends and flexes the limb, and also augments propulsion.

All the muscles described are for cattle.

DEDICATION

This work is dedicated to my wife, Binta Iyabo (Nee Bello).

CHAPTER 1

INTRODUCTION

1.1 Discovery of Tetracyclines:

The tetracycline antibiotics were discovered in 1948. Up till today, they remain one of the most widely dispensed therapeutic agents. They have wide antimicrobial spectrum and clinical application, convenience of administration, and relative lack of toxic effects. Hundreds of the drugs' derivatives have been reported, and at least five semi-synthetic analogs are in clinical use.

Chlortetracycline (CTC) was the first member of the family to be discovered. It was isolated by Duggar (1948) from Streptomyces aureofaciens during Lederle Laboratories' soil screening programme. Kane , Finlay and Sobein of Pfizer Laboratories' reported in 1949, the discovery of oxytetracycline (OTC) in fermentation liquors of Streptomyces ramosus (Mitscher, 1978). Experiment performed to elucidate the structure of these first two substances (CTC and OTC) led to the production of a third member, Tetracycline (TC), which retained the useful activity spectrum of both CTC and OTC. However, TC was subsequently isolated from Streptomyces aureofaciens, Streptomyces ramosus and Streptomyces viridofaciens. Methacycline (MC), Doxycycline (DC), and Rolitetracycline (RTC) are semi-synthetic members of the tetracycline group brought relatively recently into medical use.

1.2 Susceptible Organisms

Tetracyclines in general are bacteriostatic, but large doses can elicit bactericidal activity. They are active against both gram-positive and gram-negative bacteria; and those other pathogens unaffected by some other antibiotics (Booth, 1986a). Examples of such pathogens include: Rickettsia, Mycoplasmas, Spirochetes and Actinomycetes. Oxytetracycline can in addition, exhibit antiprotozoal activity at high doses. The susceptibility of a particular micro-organism to each of the tetracyclines is quite similar, although individual strain differences do occur. Bacterial Q^+ factor resistance to tetracyclines was reported to readily develop, and persists for longer period of time than many other antibiotics. OTC has little effect on Mycobacterium tuberculosis, and has no effect on pathogenic fungi. It is active against certain viruses, but effective against only certain strains of Proteus vulgaris and pseudomonas pyocyanea.

1.3 Extent of Usage

1.3.1 Clinical Spectrum:

Clinically, OTC is indicated for use in the treatment of Coliform-Salmonella group infections (eg. calf scours, enteritis in pigs, Salmonella pullorum infection in chickens, Salmonella infection in turkeys), virus pneumonia of pigs, and strangles in horses. OTC is used for the treatment of other poultry diseases including blue comb in turkeys, chronic respiratory disease in chicks (caused by PPLO), infectious sinusitis complex in turkeys,

contagious catarrh in pullets and cockrels, and erysipelothrix infection in turkeys. Other applications include treatment of mastitis (especially those associated with Staphylococci), Escherichia coli infections, anthrax, pasteurellosis in calves and young chickens, Leptospirosis, Foot rot in sheep, Fusiformis infections (sheep and cattle) Feline influenza, Actinomycosis, Actinobacillosis, and Anaplasmosis. There would be good response when used to treat pnuemonia or bronchitis in all species. It is effective against cystitis in small animals, tonsilitis in dogs, metritis in cattle, mare, dogs and cats. It can be used to control secondary infections in cases of equine influenza, virus pneumonia of pigs, infectious panleucopenia of cats (cat distemper), and the distemper complex of dogs. OTC has been used successfully to restrict infections following abdominal surgery. It is also used at 20µg/ml of diluted semen for antibiotic sterilization of bull semen. It is used topically for infectious keratoconjunctivitis in cattle and sheep, external otitis in dogs, pyogenic skin infections, and infected wounds and burns.

1.3.2 Agricultural Usage:

Antibiotics are known to be extensively used in modern animal production. In 1972, ten billion doses of antibiotics, representing some 2.4 million kilograms of these substances were approved for use in the United States of America by the Food and Drug Administration. In 1971, one hundred and sixty million prescriptions were written for antibiotics much of which were for tetracycline. A survey of

1.05 billion prescriptions in 1967 also showed that antibiotics represented the single most prescribed category (12.3%). Whereas among the 200 most prescribed drugs in USA, tetracycline is second only to ampicillin as the most frequently prescribed generic drug (Mitscher, 1978). It remained in the top 50 since the listing began in 1966.

The antibiotics mostly used by themselves in commercial feedstuffs are chlortetracycline, procaine penicillin and oxytetracycline (Goldberg, 1959). In USA, the monetary value of antibiotics added to animal feed in 1969 was estimated to be U$ 90.6 million (US Dollars);a considerable proportion of which was CTC and OTC. Mitchell (1984), viewing the highly competitive economic system of livestock production in North America, estimated the value of veterinary drugs marketed by Canada Pharmaceutical Manufacturers in 1982 to the tune of C$ 36.3 million (Canadian Dollars).

1.3.3 OTC Usage in Research,Space Biology, and as Diagnostic Aid:

Published work on the use of OTC in health and livestock maintenance include the chemoprophylactic immunization of cattle with the drug in combination with Theileria parva protozoans, against East Coast Fever and related tick-borne diseases (Radley et al, 1980). The practical advantage and economic savings using 5mg/kg (b.w) of Oxytetracycline Long acting i/m, was reported by Sanusi and Aliu (1986), following the pre-immunising infection of cattle with

Anaplasma marginale as against the recommended dosage of 20mg/kg in the treatment of bovine anaplasmosis.

Radio-labelled oxytetracycline was used in conjunction with histomorphometry to investigate the effects of selective skeletal unloading (decreasing weight-bearing) on bone formation in inbred strains of rats: by hind limb elevation during the neonatal first two weeks. Globus et al.(1986) observed the results of the investigation to have a profound implication for understanding bone dynamics during space flight, immobilization, or bed rest. It was viewed as an opportunity to study also the hormonal/mechanical factors that regulate bone formation. The extent of incorporation of tetracycline by osteoid was used by Harrison (1980) to determine the differencies between the effects of phosphate and vitamin D deficiencies on bone metabolism. The radio-labelled tetracycline was also used by Wong (1983) to diagnose and evaluate in vivo bone resorption of mineral and matrix among the micro-ophthalmic mice and their normal littermates in utero. Therapeutic potential and localization of tetracyclines were also investigated in rodent tumors by Davis et al. (1977). Induced remission of the development of symptoms using tetracycline has been employed to diagnose plant diseases associated with mycoplasma-like organisms (McCoy, 1982). It was also used for field control of some tree diseases. It is used to treat lethal yellowing in palm trees (Windholz et al.,1976).

1.3.4 Food Preservation:

Up to 50% of field crops get spoilt due to bacterial action before reaching consumers' tables. Spoilage organisms had been controlled in

poultry using CTC and OTC as the most effective compared to other antibiotics such as chloramphenicol, erythromycin, procaine penicillin, magnamycin, polymyxin B (Goldberg, 1959). The shelf-life of the low-concentration-antibiotic solution-treated poultry was found to be about double that of the untreated controls. Steady supply at reasonable cost is made possible in the fishing industry through the use of antibiotics to control losses due to spoilage. By that method, boats could stay out longer and range farther to ensure profitable catch. This possibility was supported by an experiment using various OTC concentrations, in brine to preserve fish fillets (Goldberg, 1959). In the meat industry, antibiotics have also been used to tenderize carcasses. OTC at concentrations between 30 - 50 ppm was infused into musculus semimembranosus prepared as 1.9cm steaks from the rounds of beef carcasses. They were vacuum-sealed in plastic film, conditioned at various temperatures, and cooked. The tenderness was found to increase by all the conditioning procedures over that of controls. In another study, high temperature conditioning was applied to intact beef cuts from animals which had been injected pre-slaughter with OTC. The tenderness of musculus longisimus dorsi, musculus semitendinosus, and musculus biceps femoris from the animal, was greater after 14 days at 2°C than after 1 day at 43°C. The musculus semimembranosus showed a greater increase in tenderness at the higher temperature of conditioning, suggesting that the latter (temperature)

might be more effective with intrinsically less tender muscles (Lawrie,1975). In the preservation of soft drink beverages, OTC and CTC were used. This is apart from benzoic acid (as sodium or potassium salt) and certain antioxidants that are commonly used (Amuda-Giwa, 1987).

1.4 Commercial Availability and Purity:

Veterinary preparations of Oxytetracycline available for oral administration or injection are found to be relatively pure (Ashworth,1985), but they still contain low levels of impurities. As such, it was asserted that it is difficult to purchase CTC, TC or OTC that are totally pure. For example, purity of commercial CTC is claimed not to be greater than 95%. Whereas the purity of the TC in feed additives is even less. This was adduced to the fact that such TC was sourced from the residual tetracycline found in the dried ground mycelial powder harvested from fermentation tanks (Ashworth, 1985). Also, it was reported that there were low concentrations of TC in both OTC-containing and CTC-containing materials. TC on the other hand does not appear to be contaminated with other tetracyclines. The veterinary oxytetracycline preparations commercially available in Nigeria are however either imported or repacked here in the country. Their purities therefore should be of the standard obtained in the manufacturing/exporting countries: if not less.

1.5 Research Objective

1.5.1 Poultry as a Source of Animal Protein:

In normal physiological state, all body fluids (except bile and urine) and every living cell contain protein. Nearly half of the dry weight of the human body is protein (Longenecker,1963). Protein deficiency manifests as a complex syndrome. This is because diet low in proteins may also supply inadequate amounts of vitamins.

Protein deficiency in man, like most other nutritional deficiencies may result amongst other factors from defective intake (either in quantity or quality), poor absorption, or faulty synthesis.

Idusogie (1971) estimated that for an adult in Nigeria, daily minimum protein requirement is between 65 and 72 gm per person. An adult male in the USA was estimated to require 70gm, while the female required 58gm (Pascual et al.,1976). The Food and Agricultural Organization (FAO, 1966) recommended that 35gm (about 50%) of this (72gm) minimum requirement should be obtained from animal products. A large proportion of Nigerians cannot presently afford the "luxury" of daily animal protein in their diets, even at quantities below the minimum requirement. This would be due to scarcity and high cost. Of the total poultry available in Nigeria as at 1983, 84.974×10^6 were from local traditional source, while 3.409×10^6 were from modern poultry keeping (commercial) source. This shows that the modern poultry keeping constituted a mere 3.86% of total available poultry meat in Nigeria that year (Federal Office of Statistics, 1983). On similar down-turn, it was noted from internally generated data, that total commercial poultry population dropped from 40.4 millions in 1983 to 14.5 million in 1988; and to 8,6 million by june, 1989. These gave corresponding total tonnage of poultry meat as 159, 58 and 46 tonnes in the respective years (Livestock Feeds Limited Ikeja, 1989). Poultry meat and eggs in unit-price

consequently rose from ₦4.60/kg and ₦3.50/crate (of 30 eggs) respectively in 1983 to ₦15.00 and ₦30.00 by June, 1989.

1.5.2 Protein Potential from Local Chicken:

The local chicken (Gallus domesticus) was recognized as villager's most efficient converter of feed, the contributor to the villager's income and to the supply of animal proteins for Nigerian citizens (Agricultural Extension and Research Liaison Service - AERLS 1982). It, however, lays very few eggs per year and grows too slowly. AERLS consequently proposed stock-improvement steps such as:-

(i) Upgrading with improved breeds

(ii) Supplementary feeding

(iii) Selection and culling, and

(iv) Chemotherapeutic control of diseases and parasites

to enhance the performance of the stock.

Okon (1975) observed that the local chicken though being of tropical origin (Nesheim et al, 1979) has since spread throughout the world and have been developed into many different "breeds" and varieties in different countries. They were given breed names generally based on the locality where the breed was first raised, or on the plumage and other features of the fowl. In Nigeria, there are at least three common such "breeds" namely: the Harco, the B 390, and the B 300 (Okon, 1975). The local domestic fowls are preferred for food by many families in Nigeria due to their taste and texture .

They are known to be more resistant to diseases than some of the imported breeds, and also adapted against predation.

1.5.3 Poultry Husbandry and the Use of Drugs:

The use of less than 1.0 ppm antibiotic supplement in animal feed improved feed conversion ability (Goldberg, 1959). Oleandomycin and erythromycin for example promoted growth in chicks and poults (Goldberg, 1959) and when the antibiotics were withdrawn from the livestocks' diets, loss of accelerated growth advantage was reported. At 5 to 7.5 g/ton, OTC was used to increase rate of gain in poultry and 10 to 50 g/ton feeding levels had been used to aid increased egg production in laying hens. Poultry was observed to grow twice as fast on one-half as much feed today as they did 20 years ago: one of the reasons being the effective use of antibiotics (Goldberg, 1959). Through various well recognized vehicles (meat, milk or egg), veterinary drugs and/or metabolites get into foods for humans (Amuda-Giwa, 1985). Also, the plasma concentration, metabolism or excretion of drugs administered to livestock, are influenced by certain factors. Such factors include animals species, age, sex, body weight, disease state and biorhythms (Radeleff, 1970). As such, the use of drugs predispose the animals to leaving drug residue in the tissues and the by-products. Such residues may pose some hazards to the consumers. Potential health hazards therefore from drug residues in foods are categorized into:

(i) Toxicological:- This includes aplastic anaemia, thyrotoxicosis, carcinogenicity, deposition in bones and teeth, etc. Radeleff (1970) however remarked that it is possible to kill an animal with little residue appearing in the fat or even build-up relatively high tissue residues without inducing intoxication. In a way, chemical residues in animal tissues represent a failure of the body to eliminate or completely metabolize the substance. A phenomenon attributable to species differences or the individuals' physiologic or pathologic dispositions (Clarke and Clarke, 1967). Prolonged chloramphenicols (CAP) topical ophthalmic administration at the rate of less than 100mg per patient had caused aplastic anaemia. In one case, the lowest total dose to cause death in man was 82mg of CAP administered over 40 days. But this low level of CAP administered was considered by Allen (1985) to be of public health significance, since such levels may occur in edible tissues of clinically treated food-producing animals.

(ii) Microbiological:- This is in relation to transmittable drug resistance. There were reports of rising incidence of mastitis due to penicillin-resistant Staphylococci possibly due to subtherapeutic exposure.

A study conducted in Zaria on dried fish, dried beef, fried fish, roasted beef (Tsuya), and fried chicken; showed that the preparations contained Staphylococcus aureus resistant to penicillin, trimethoprim, chloramphenicol, tetracycline and streptomycin (Kwaga and Adesiyun, 1984). The development of resistant strains of the human pathogenic bacteria has been demonstrated in vitro to concentrations of penicillin, stroptomycin, chlortetracycline and oxytetracycline as low as the antibiotic residue levels obtained in foods (Eagle, et al, 1952). Studies (in the USA) also showed that the subtherapeutic use of antibiotic is implicated in the transmission of antibiotic - resistant bacteria pathogenic to man from animals (Amuda-Giwa, 1984).

(iii) Immunological:-- Immunopathological mechanisms were involved in documented cases in which veterinary drug residues had demonstrably led to harm in humans (FAO, 1984). Penicillins were cited as the aetiological agents uniformly involved. They are all known for their allergenic properties in both veterinary and human clinical medicine. Following ingestion by a previously sensitized person, milk containing levels 0.5 units penicillin per ml had caused severe anaphylaxis. In certain conditions,

however, virtually all drugs can serve as antigens (FAO/WHO, 1984).

In summary therefore, recognising the ready availability of veterinary drugs in the market in this environment, the detection and quantitation of such administered drugs residues in the tissues of food animals, is the objective of this research: using Gallus domesticus as the experimental animal, and Oxytetracycline as the test drug.

CHAPTER 2

LITERATURE REVIEW

2.1 Chemistry:

The tetracycline group antibiotics in general contain a common structure which may be regarded as an octahydro analog of the hydrocarbon naphthacene (Figure 1). Taking the structure of tetracycline

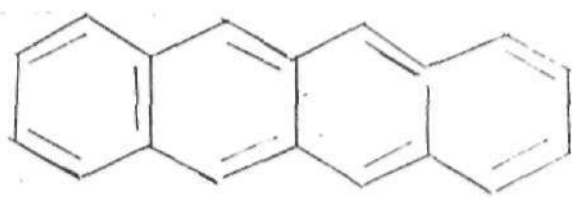


Figure 1: Octahydro analog of naphthacene

(Figure 2) as the nucleus, oxytetracycline (Figure 3) contains this (tetracycline) nucleus with a hydroxyl group instead of a hydrogen atom at carbon 5 in benzene ring B, (Figure 3). Chlortetracycline contains a chlorine atom on carbon 7 in ring D (Figure 4). As with chlortetracycline and tetracylcine, oxytetracycline is amphoteric and will form salts with either acids or bases.

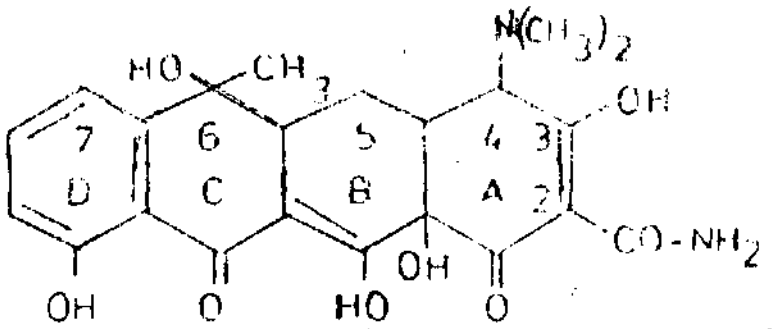
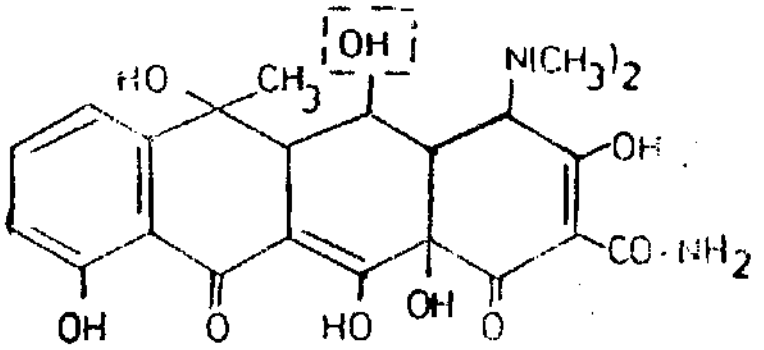
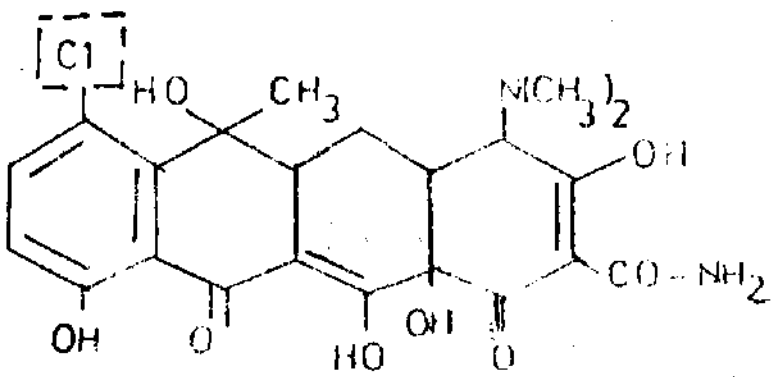


Figure 2: Tetracycline: 4-dimethylamine-1,4,-4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-di-oxo-naphthacene carboxamide



igure 3: Oxytetracycline 4-dimethylamine-1,4,-4a,5,5a,-6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphacene carboxomide



igure 4: Chlortetracycline 7-Chloro-4-dimethylamino-1,4,4a,5,5a,6,11-12a-octahydro-3,6,10,12,12a-pentahydroxy-6 methyl-1,11-dioxo-2-naphthacene carboxamide

(Goldberg 1959).

Oxytetracycline (Oxytetracycline dihydrate) base and its salt (oxytetracycline hydrochloride) are crystalline powders, yellow, odourless and have bitter taste. The base is slightly soluble in water while the hydrochloride salt is readily soluble. Tetracycline, oxytetracycline and chlortetracycline contain three

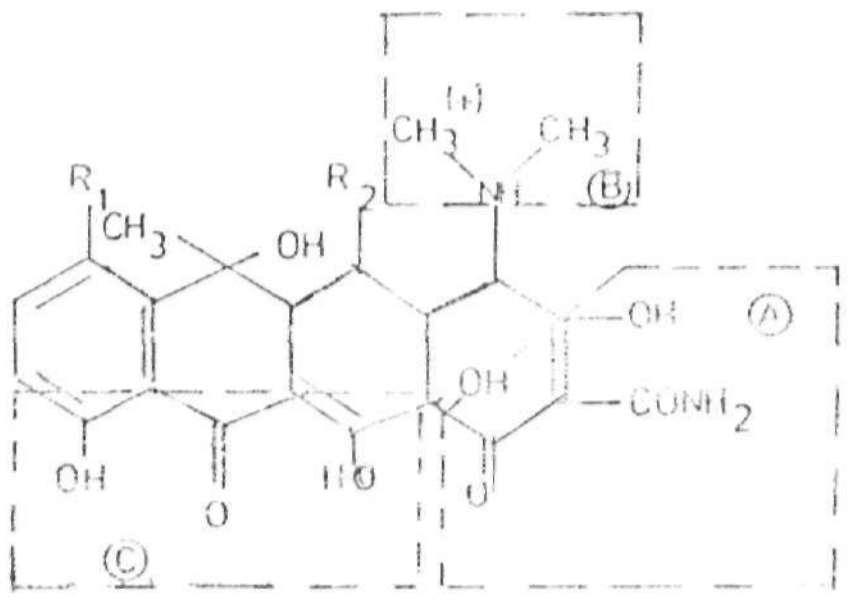


Figure 5: Three acid groupings (A,B & C) in tetracycline nucleus.

Figure 5a: Oxytetracycline: R_1=H, R_2 = OH

" 5b: Chlortetracycline: R_1 = Cl, R_2 =H

" 5c: Tetracycline : R_1 =R_2 = H

acid groupings of different types. The general formula written as an acid salt (Figure 5) can be partitioned to show three distinct acid groups: the tricarbonyl methane system A, the ammonium cation B, and the phenolic diketone system C.

Oxytetracycline dihydrate has a melting point of 181 - 182°C, while that of the hydrochloride salt is 190 - 194°C. The polycarbonyl system of oxytetracycline prompts the ready formation of chelate complexes with metallic ions. The 6 - deoxy - 6 -demethyl tetracycline is the simplest known tetracycline with essentially complete

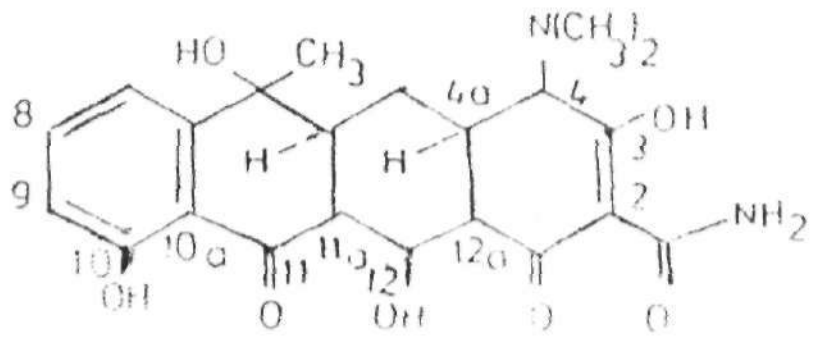


Figure 6: Tetracycline (Pharmacophore)

pharmacological properties. This structure may be regarded therefore as the "pharmacophore" (Figure 6). The tetracycline ring is required for full bioactivity. The tetracyclines form highly insoluble complexes with Ca (II), Mg (II), Fe (II) and Al (III) ions; especially at neutral pH's in the presence of amino acids. This not only diminishes their apparent potency in vitro, but impedes their absorption from gastro intestinal tract on oral administration.

2.1.1 Analytical Methods

Chromatography:- Chromatographic method (of all types) is a technique of separating a mixture into its component parts, which could subsequently be identified by classical methods. The technique requires two phases - the stationary and the mobile phases. The techniques mode is operated on any of the following combination of *two-phase systems*:

- Solid/Liquid: eg. Paper chromatography , Thin layer chromatography.
- Liquid/Liquid: eg. "Liquid chromatogram" of Steiger and Reichstein (1938) used for fractionation of suprarenals' steroids.
- Gas/Solid:i.e. Gas Solid Chromatography - The stationary phase is solid. Its use almost entirely is restricted to the analysis of permanent gases (Clarke, 1974).
- Gas/Liquid:i.e. Gas Liquid Chromatography - The stationary phase is liquid. It is used to analyse many categories of substances.

For substances being separated by chromatography, the speed of their progress is dependent on the relative degree of absorption and/or adsorption of the components by the surface of the stationary phase. Such situation confers a retardation factor (R_f) which is peculiar to such components, and can be used for their identification.

Paper chromatography is the oldest of the modes, dating back to the work of Runge (1795 - 1867). "Runge pictures" appeared as early as 1850 (Machery-Nagel & Co. Ed'.74). Izmailov and Shraiber(1938)

described the use of thin layer of adsorbent on glass plates for the separation of galenicals. Kirchner et al (1952) designed equipments for the preparation of chromatostrips and used the technique for the investigation of terpenoids. Mottier and Potterat (1952) worked out a method for analysing food dyes on layers of nonbonded alumina and also separated amino acids by this technique. Stahl (1964) devised a system using the standardized glass plate (5cm x 20cm and 20cm x 20cm), a new apparatus for the preparation of layers, and a standard adsorbent (silica-gel: Plaster of Paris). Stahl et al (1956) studied the variables of the technique in a thorough manner and subsequently called the method "Dunnschicht chromatographie" or Thin Layer Chromatography (TLC). Most TLC work has been done in clinical chemistry where speed is an important factor. Precision in TLC was assessed to be 5 to 10% while it was 2 to 5% in GLC (Sadee and Beelen, 1980). TLC can separate mixture in microgram quantities. The resolved components of a spotted sample can be examined/identified or quantitated by reflectance or transmittance densitometry, spot area measurement, using radio active tracers, or removed for spectrometric analysis.

The stationary phase in TLC is usually a polar solid: e.g. silica-gel or alumina. Chemically modified microparticulate silicas used in high pressure liquid-chromatography (HPLC) have become available. Whereas, production of plates with layers of very small and uniform particles which result in more compact sample

spots and improved resolution has given rise to the term "high performance TLC" or "HPTLC". In TLC work, the mobile phase used on silica gel however should be that with as low a polarity as possible to achieve a satisfactory separation. It can be said that quantitative TLC is a versitile, sensitive, and specific technique that can replace hplc or glc assays in research and clinical laboratories if proper instrumentation is available (Sadee and Beelen, 1980). The greater speed and versatility of TLC has given it a rapid widespread use in all fields especially for the separation of organic materials. It is ideally suited to following the course of complex reactions, quality control, purity checks, clinical diagnosis and forensic tests.

Spectroscopy:

Ultraviolet (UV) Absorption/Spectra of Tetracyclines:

(i) The beta-tricarbonyl system of ring A contributes a strong absorption at about 260 nm (nanometre).

(ii) The aromatic ring D conjugates with the beta-diketone moiety in rings B and C (Figure 2) to produce a visible band at about 360nm - accounting for the pale to bright yellow colour of the tetracycline (Mitscher, 1978).

(iii) OTC in alcoholic 0.01 N hydrochloric acid gives UV spectra at 357nm, 267nm.

After boiling for five minutes in 0.2 N sodium hydroxide, the characteristic absorption spectrum changes with a sharp visual increase in yellow-orange colouration (Monastero et al,1951). Such a caustic treated OTC solution after acidification to pH 1.7 shows no maximum at 353mu (millimicron); i.e. 353nm. The differential absorption measured at 353mu can be used for the spectrophotometric analysis of OTC. The change from yellow to a yellow-orange colouration after caustic treatment is specific for OTC relative to the other common antibiotics and has been made the basis for its quantitation.

Infra-red (IR) Spectra:

IR spectra are usually determined in potassium bromide (KBr) discs or mineral oil mulls. For identification purposes, the IR spectra are virtually identical for almost all the tetracyclines for the followings:-

(i) a) amide band at about 6 microns

b) large carbonyl envelope near 6.2 microns

(ii) The differences are mostly in the finger print regions below 6 microns

(iii) The TC nitriles have a typical C-N at 4.5 microns.

(iv) The OTC C_{12a} has a formate ester band at about 5 - 8 microns.

In each of these cases TC and OTC (iii & iv) above, the band has identification value because it lies in region which is otherwise transparent amongst tetracyclines.

Nuclear Magnetic Resonance Spectra (NMR):

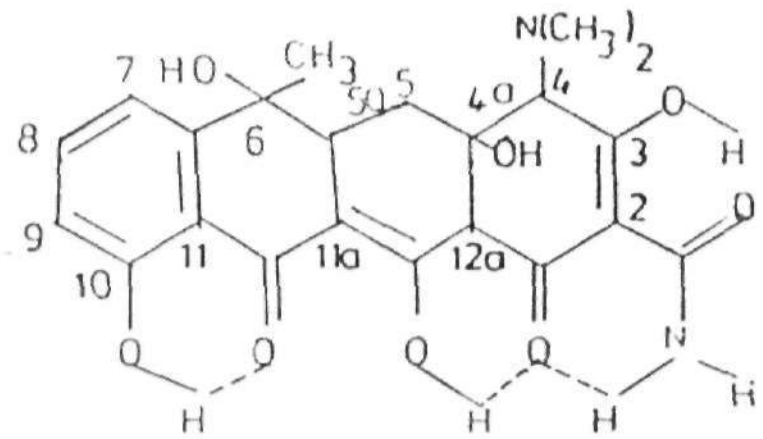


Figure 7: OTC configuration for NMR

NMR appropriate coupling constants for OTC:-

J_{4-4a} = 9 HZ; J_{4a-5} = 0-2 HZ

J_{5-5a} = 4 HZ.

Mass Spectrometry: The tetracyclines are relatively non-volatile and are thermally unstable. So their electron-impact mass spectra are rich in fragment ions and weak in molecular ion intensity. The identity of the various peaks in the fragmentation scheme is supported by high resolution mass measurements, substituent labelling and metastable ions. Just like most tertiary amines, this function dominated the fragmentation. In the highest mass region, there would be m/e 426 for dehydration; followed by ions 409 and 391 from loss of NH_3 and water from the carboxamide function; as well as 381 for loss of the whole group as $CONH_3$. In addition, one sees m/e 366 and 364 from loss of CH_3NCH_2, and $(CH_3)_2NH$ respectively from 409. Most of the fragments seen involve rings A and B because rings C and D become naphthalenoid quickly (Mitscher, 1978).

Review of Other Analytical Methods Used in OTC Assay

Microbiological Methods:

Schifferli et al (1982) described the use of a microbiological cylinder plate bio-assay procedure which utilized the organism, Bacillus cereus var. mycoides (ATCC 11778) to analyse OTC in veal calves. They developed a modification of the technique and achieved a limit of sensitivity of 0.125 µg/ml. Ragheb et al (1973) reported an analysis in feed: consisting of plate assay, and manual turbidimetric methods. They used Klebsiella pneumoniae (ATCC 10031) for the turbidimetric method and get a detection range between 0.04 and 0.08 µg/ml in the assay tubes. An alternative microbilogic assay method which has legal status is the Turbidimetric Method. The test organism is Staphylococcus aureus (ATCC 6538-P), the cell density of which, is read photoelectrically after 4 hours of incubation. An automatic system, AUTOTURB$^{(R)}$ system (turbidimetric) for the analysis of antibiotics was introduced in 1971 by Kuzel and Kavanagh. They used a flowcell to minimize inaccuracy of turbidity readings that could arose due to the flow birefringence phenomenon exhibited by rod-shaped organisms. The system also had an automatic diluter. It had been used to analyse monesin and tylosin in premixes and finished feeds, and also for OTC in premixes.

Fluorimetric Method:

Determination of OTC in premixes was reported by Katz and Fassbender (1973). The method was observed to be rapid (took less

than 2 hr) and reasonably specific for OTC as regards interference from chlortetracycline (CTC). This was because of significant difference between excitation and emission wavelenghts for these compounds: 390 and 520nm for OTC as against 350 and 420 for CTC.

Other Methods:

Monastero et al (1951) reported a colorimetry method. TLC was reported by Ascione et al (1967), and Ashworth (1985); while paper chromatography was reported by Addison and Clarke (1963). Gel Filteration Chromatography was reported for OTC analysis by Griffiths (1966), and also by Ascione et al (1967). HPLC works on OTC were reported by Sharma et al (1977a), Sharma et al (1977b), Nillson-Ehle et al (1976), and Ashworth (1985). Petz (1984) published a review of chemical analysis of residues of veterinary drugs in foods (Language: German). He noticed that published works on GLC analysis of OTC was almost non-existent. This he asserted, could probably be due to non volatile nature of OTC and the challenging task of its derivatisation to augment its stability at high oven temperatures. In the samples work-up procedure, he mentioned the use of Subtilisin Carlsberg (Sigma P. 5380) to homogenize animal-derived food samples and also collagenase and hyaluronidase enzymes to separate-out the drug in the tissue substances. He made reference to the work of Hamaan et al (1979) in Milchwissenschaft of 1979, No. 34:357. The method was successfully used for the detection of tetracycline, chlortetracycline and oxytetracycline.

2.2 Pharmacology and Toxicology of Oxytetracycline

In chemotherapy, chemical agents act by giving specific antagonistic effect on the disease organisms (bacteria, viruses, parasites, etc). In 1897, Paul Ehrlich postulated the principle of chemotherapy. Later, he differentiated between immunotherapy (the use of vaccines) and chemotherapy. He did a lot of work on the modern concept of Therapeutic Index (TI): which stipulates that, chemotherapeutic substances should be maximally parasitotropic and minimally organotropic. Explaining that:-

$$TI = LD_{50}/ED_{50}$$

Where LD_{50} is the lethal dose for 50% of the test animals, and ED_{50} is the effective dose for 50% of the test animals.

However, the main purpose of antimicrobial usage is to kill or inhibit pathogenic micro organisms in the infected animal or man. In this regard, the tetracyclines (OTC, TC, CTC, etc.) act by modifying the molecular mechanisms of replication and protein synthesis by their effect on ribosomes. They exert a bacteriostatic effect by inhibiting protein biosynthesis after binding to the 80s ribosomal subparticle (Franklin and Snow, 1971). Cessation of protein synthesis results and the cells stop multiplying and will eventually die. The 80s ribosomes of eukaryocytes, including yeasts and man, are much less sensitive to the effect of tetracyclines. This accounts for the useful selective toxicity of these drugs. Pharmacologically, the oxytetracycline dihydrate (OTC. $2H_2O$) and oxytetracycline hydrochloride

(OTC. HCl) are the same. As the($OTC.2H_2O$) is relatively insoluble, it is therefore unsuitable for parenteral injection. But its taste is less bitter than that of OTC.HCl and so, makes it more suitable for oral administration (Brander and Pugh, 1971).

Oxytetracyclines therapeutic concentrations in the cerebrospinal fluid for example, will normally be achieved only when the meninges are inflamed (Brander and Pugh, 1971). Intrathecal route of administration is therefore recommended to initiate treatment for cases involving the central nervous system (CNS). OTC passes placental barrier readily, although foetal blood concentration is usually low (sometimes as low as 25%). But it may reach those of the maternal circulation upon continuous administration of sufficient therapeutic dosages. Therapeutic levels in the pleural and peritoneal fluids (serous membrane barrier) can readily be achieved from even oral dosage of OTC, although parenteral administration (intraperitoneal: i/p, or intravenous:- i/v) is recommended to establish initial levels in cases of severe and acute inflammations. Single i/v doses can give therapeutic milk levels, as OTC readily diffuse into milk. The drug (OTC) also occurs in bile, and could reach a level in excess that of concurrent serum level. OTC from bile increases that in the intestine, some may be reabsorbed to begin the cycle again: intrahepatic circulation (Mercer et al:, 1978b; Brander and Pugh, 1971). Whereas streptomycin and neomycin are not absorbed into the systemic circulation in therapeutic concentrations when given orally. When injected, very

little (Streptomycin) is excreted into the intestinal lumen except through the biliary system. In such cases therefore, only parenteral injection is suitable for treating systemic infections and, oral route for intestinal infections. Oxytetracycline is not absorbed to appreciable extent from the intestines of poultry (Brander and Pugh, 1971).

Biliary excretion from high doses of OTC was postulated to cause a disturbance of the horses intestinal flora: thus leading to proliferation of Clostridium perfringens and possible toxin production. Usage of OTC.HCl (Syn. Terramycin (R)) at 2mg/1b (b.w) had been reported to produce post-surgical or postanesthetic colitis in horses. Bocker et al (1984), observed that hepatotoxicity is a potential side effect of the tetracyclines. Their studies showed that in some species including man and mouse, females are more prone to develop liver damage than males. In goats, intravenous administration of large doses of OTC.HCl had caused adrenocortical hyperplasia and decreased thyroid follicular cell size. Since the tetracyclines pass the placental barrier and could appear in mother's milk, so also moderate doses lead to a definite yellowing and reversible slowing of bone growth in fetuses, infants and young children. The deposition is essentially permanent and it is dose related (Mitscher, 1978). Excess doses can lead to weakness in bone structure and teeth. It is not preferable to administer tetracyclines to children before about the age of eight. Sixty-four babies given TC

soon after birth had 50 of them develop yellow or brown discolouration of the teeth with enamel hypoplasia in some of the cases. Duration of treatment appeared to be less important than the total dose (Wallman and Hilton, 1962). Likewise, sixty-one adults receiving lengthy courses of TC, all showed deposition in the nails of the fingers and toes. By using doses of 1gm daily, deposition was visible in 3 weeks (Douglas, 1963). Todd (1967) described the general toxic effects of oxytetracycline hydrochloride "as for tetracycline hydrochloride". When OTC.HCl was administered at a dose of 500mg to an adult (t.i.d) for 3 days, the person developed photosensitization, redness, swelling and itching of the body. The tetracyclines delay blood coagulation, and may potentiate the effect of dicoumarol anticongulant. Over-aged (expired) tetracycline preparations degrading to 4-epianhydro tetracycline, showed toxicity resulting in damage to renal tubular function (Fellers and Lindquist, 1964). The dys-function would produce the symptoms of nausea, vomiting, glycosuria and albuminuria in human beings.

2.3 Pharmacokinetics and Bioavailability:

When tetracyclines are administered by routes requiring absorption, on getting into the blood stream, they enter into the enterohepatic circulation. They undergo excretion and reabsorption in the small intestine. They penetrate readily but unevenly into most organs of the body. Substantial concentrations are achieved in liver, spleen, kidney and lung. Tetracyclines are bound reversibly

to serum proteins: globulins, albumins and lipoproteins (Powis, 1974). Serum binding of TC, CTC and OTC are 24%, 47% and 20%, while their protein binding are also 55 - 64%, 55% and 27 - 35% respectively (Baggot, 1977). Most authorities however believe that bound drugs in general are biologically inactive, hence tetracyclines, bound to plasma proteins act as a reservoir of the drug. The biological half-life and the tissue penetration of tetracyclines are strongly influenced by the protein binding. Half life of oxytetracycline in the dog is 6.0 hr. (Baggot, 1977).

Pharmacokinetic studies of OTC have been reported in a number of animals: Cows and ewes (Ziv and Sulman, 1974), dog (Baggot, 1977), horses (Teske et al, 1973) and pigs (Mercer et al, 1978a), while that of TC in chicken was studied by Anadeon et al, (1985), and CTC in turkeys: by Pollet et al (1985). No pharmacokinetic study for OTC in chicken was seen during the literature search. The pharmacokinetic studies of oxytetracycline long acting (OTC-LA) in relation to conventional oxytetracycline (OTC) showed that the OTC-LA formulation had a longer serum half life and a significantly greater "area under the curve" (AUC i/v) value than the conventional-OTC (Davey et al, 1985). It also had a lower serum peak level with a significant extension of drug serum concentration than the conventional formulation. From the studies in Pigs (Xia et al, 1983), intramuscular injection of OTC-LA gave a lower maximum concentration (C_{max}) compared to conventional OTC. The initial absorption rate was faster

for OTC-LA and the maximum plasma concentration was reached within 1 hour, while the maximum for OTC-C was achieved after 4 hr. Calculation of the AUC i/v showed that this was almost identical for the two preparations, indicating an equal bioavailability.

In man, about 15% OTC is excreted in the first 24 hr. after reaching a maximum in about 4 hour. But as much as 50% may be recovered ultimately in active form (Brander and Pugh, 1971). It appeared that there was no interference with absorption of OTC when mixed with either neomycin or bacterin for the treatment of pastuerellosis in turkeys (Skeeles et al, 1985). Two forms of OTC (OTC. HCl and OTC.$2H_2O$) administered intravenously to dairy cows were reported not to have statistical differences between their pharmacokinetic parameters derived from three-compartment body model.

Pharmacokinetics of drugs however are known to change in diseased states (Pollet et al, 1985). This is due to alterations in plasma perfusion, hepatic function, drug binding, drug transport and/or renal function. The administration of CTC to fowl-cholera-infected turkeys resulted in an increase in plasma levels due to increased intestinal permeability and lowering of the hepatic and/or renal clearance (Pollet et al, 1985). Miller and Wyatt (1985) in their study with aflatoxicosis in chicks, observed a decreased CTC plasma concentration.

This was explained to be due to the decreased binding of OTC to plasma proteins, which resulted in increased elimination of drug through the kidney, other than the liver route.

Except a given drug is injected directly into the vascular system, it would be erroneous to assume that such drug would have a rapid and complete access to the systemic circulation. A drug product may have a relatively high systemic availability, but a slow rate of absorption from the site of administration may give subtherapeutic plasma (or serum) levels of the drug. Administering usual dosage of such drug could give the impression of ineffectiveness; but dosage readjustment, or modification of the drug-product may just be the need for therapeutic efficacy. The biological performance of a drug product therefore, can be effected by its bioavailability. Bioavailability is defined as the rate and extent to which a drug administered as a drug product, enters the systemic circulation in an unchanged form (Baggot, 1977). For example, dissolution of the solid drug formulations in the stomach is necessary for adequate absorption in the duodenum. Some formulations have been reported to give poor blood levels due to inappropriate particle size and dissolution rates (WHO, 1974). Dissolution rate can be however enhanced by using the salt form of a drug or by decreasing the particle size and as such increase the bioavailability of the therapeutic agent. But despite good dissolution and absorption properties (dosage form factors),

bioavailability could also be affected by physiologically modified factors, such as drug absorption interactions. Biotransformation by enzymes in the intestinal mucosa or liver, or both (preceding drug entry into the systemic circulation i.e. "first pass effect") may also significantly reduce bioavailability. Categories of drugs requiring priority testing for bioavailability (WHO, 1974) include: drugs having relatively poor solubility in water, coated drugs, sustained release dosage forms, and solid oral dosage forms of various systemic anti-infective agents.

2.4 Drug Residues in Animal-derived Foods

Quantities of drug residue vary considerably from tissue to tissue: generally higher in the tissues of storage, excreting or metabolizing organs. The injection site of cattle given oxytetracycline was positive for the presence of the drug, eight days post administration. In a USFDA (United States Food and Drug Administration) surveillance studies of bovine intramammary infusion antibiotic preparations, 67% of the products tested, left residues in the milk from 12 to 140 hours beyond the milk discard time. Whereas 96 hours was the maximum allowed by law for disappearance of drug residue in milk following intrammammary treatment. About 10,000 samples of red meat and poultry were analysed for drug residues in 1971, four hundred and thirty-five were reported positive (Kerr, 1972). Whereas as little as 40 units of benzylpenicillin may evoke an allergic reaction in highly sensitized people (Siegel, 1959), hence penicillin tolerance for human food was set at zero (FAO/WHO,1969).

Public health consideration for drug residues is not limited to sources from treated animals. Some individual States in the USA for example permit use of recycled animal waste without regulatory control. Some advantages of such usages had been ascribed to reduction of environmental polution; when fed to livestock, it served as an economic source of energy in food animal production. The animal wastes so used included: meat meal tankage, blood meal, hydrolysed poultry feathers, poultry by-products-meal, and dehydrated paunch products (Booth, 1986b). Several States in USA however have specific regulations for animal waste feed-products, and set withdrawal times if the waste is from poultry, or animals that have been fed drugs. Human health risk from drug or chemical residues was evaluated by Blair when animal waste was fed to food animal (Booth, 1986b), he observed the risk as being greater than the risk from pathogens. Apart from drugs or chemicals from treated animals, or usage of re-cycled waste posing health hazard to consumers of animal-derived foods, consumption of products from animals eating poisonous plants have been reported to have toxicological effects (Schoental, 1965).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Test Drugs and Standards:

The test drug used for this research was the oxytetracycline hydrochloride manufactured by Pfizer Products Limited, Animal Health Division, Ikeja, Nigeria: Under Authority of Pfizer Inc. New York, USA. The brand-name was "Terramycin$^{(R)}$ Poultry Formula with Antigerm 77". The stated composition was terramycin hydrochloride activity 55g, and Benzethonium chloride 55g per kilogram-weight of the product. They were packed in aluminium-polyethylene pouches of 50g each, net content. The batch number was 401 11 93.

Oxytetracycline dihydrate (OTC. $2H_2O$) was the reference drug standard used in this work. Two preparations were obtained. The first was in a glass ampoule with net content of 100mg OTC.$2H_2O$. It was manufactured by the World Health Organization (WHO) International Laboratory for Biological Standards. National Institute for Biological Standards and Control, Holy Hill Hampstead London. This was used in the preliminary study to evaluate the analytical methods intended for use in the research. The second preparation was in an amber-coloured glass bottle with net OTC.$2H_2O$ content of 10gm. It was manufactured by Sigma Chemical Company, St. Louis, Missouri, USA. The batch number was 521-06261. This was used as the OTC drug standard for quantitative calibration curve, and also as internal standard for assessing OTC contents of various tissues and media. The two drug standards described above were provided by the Food & Drug Administration and Control, Federal Ministry of Health & Human Services, Central Drug Control Laboratories, Yaba, Lagos.

3.1.2 Test Animals and Management

The domestic fowl (chicken) Gallus domesticus L was used for the drug residue tests. It is the most common and most important poultry bird. The local strain of the domestic fowl was used for this study (Plate I).

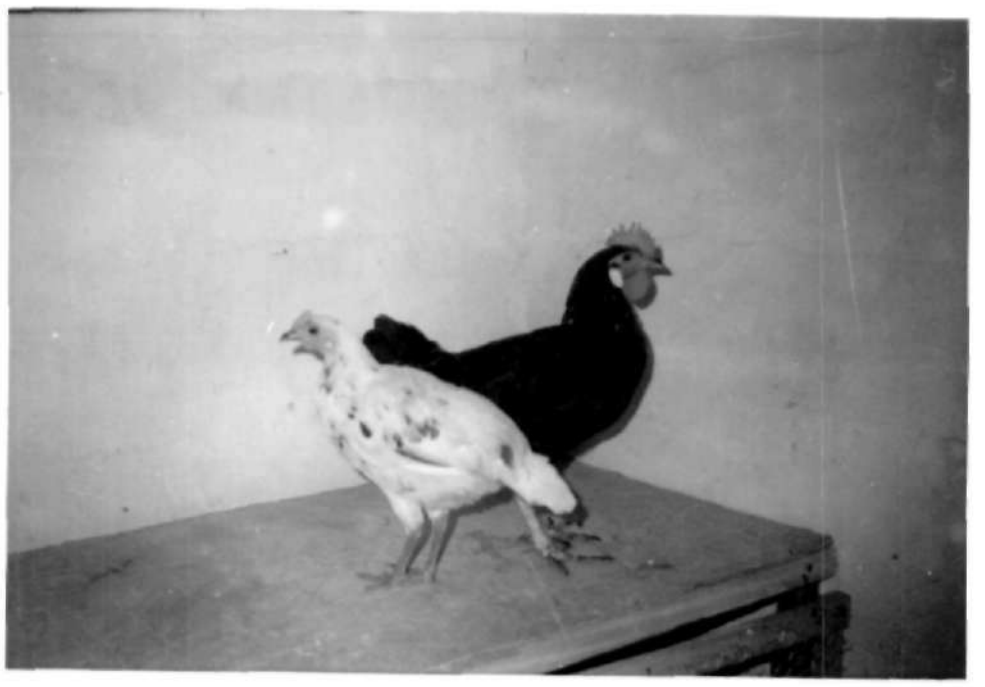


Plate I: The Domestic Fowl (Chicken) - Gallus domesticus L.

The animals were housed in partitioned poultry holding, provided with drinkers, feeding troughs and commercial poultry feed (Sanders (R)). Sufficient potable water was given adlib. The housing was fitted with incandescent electric lights, while wood-shavings served as litter. Antiprotozoan and antibacterial preventive measures were taken. Weighing balance was used for periodical animals' weight measurement.

3.1.3 Laboratory Glasswares:

Erlenmeyer flasks (various sizes)

Volumetric flasks (polyethylene stoppered: various sizes, plain transparent/amber coloured)

Graduated cylinders (various sizes)

Beakers (various sizes)

Centrifuge tubes (glass round bottomed/plastic conical)

Heavy wall (Pyrex) ignition tube, rimless.

Test tubes (Pyrex) and racks

Burettes

Graduated pipettes (10ml, 5ml, 1ml)

Bulb pipettes (25ml, 10ml)

Pasteur pipettes

Funnels(Glass/Plastic)

Glass syringes (Hamilton$^{(R)}$ Precision)

Separatory funnels (various sizes)

Petri dishes

Glass pestle & mortar

Serum sample bottles (Screw capped)

Retort/Burette stands and clamps.

3.1.4 Analytical (Grade) Reagents:

di-Sodium Ethylene diaminetetra acetic acid (EDTA)

Methanol

Trichloroacetic acid (TCA)

Sodium Chloride

Ethyl acetate

di-Sodium Hydrogen orthophosphate (anhydrous)

di-Sodium Hydrogen phosphate (anhydrous)

Potassium hydroxide

Ammonium hydroxide

Conc Hydrochloric acid

Conc Sulphuric acid

Propylene glycol

Polyethylene glycol 400

Glycerin

Modified Silica Gel H_6F (254) (without C_aSO_4): BDH

Whatman Filter papers (Nos 1, 5 & 7)

3.1.5 Analytical Instruments:

Set of Thin Layer Chromatographic Kit: spotting guide, leveller, spreader, development tank, layer's thickness gauge, etc. CAMAG Muttens/Switzerland

Ultra-Violet Lamp. MINERALIGHT$^{(R)}$ multiband UVSL-58 Ultraviolet Product Inc. San Gabriel Calif. USA.

Transparent Tracing papers and graph sheets

SARGENT - WELCH pH meter: Model IP. Sargent Welch Scientific Company.

PYE UNICAM pH Meter: Model 291 MK 2.

IEC HN - S II Centrifuge DAMON/IEC Division

Fisher Flexa - Mix: Magnetic stirrer

pbi - Sterilmixer: Homogeniser

Dissecting Instruments: Sainik Biology Set.Bombay Surgical,India No. 277077

Mettler balance: Type H16 Mettler Instruments Corp. Highstown N.J.

SAFETY-FLOW Laboratory Fume Hood: Fisher Scientific Co. Instrument Div. Indiana P.A.

Ultraviolet-Visible Double beam Spectrophotometer. Unicam SP 1800

HAMILTON Microsyringes: Hamilton Micromeasure. Bonaduz Switzerland.

Air pump. Charles Ansten Pumps Ltd. Weybridge England Ser. No.K16854.

3.2 Methods

3.2.1 Study Design:

The experiment was performed with 72 grown-up chicks, of 1 - 2 weeks of age (Plate II).

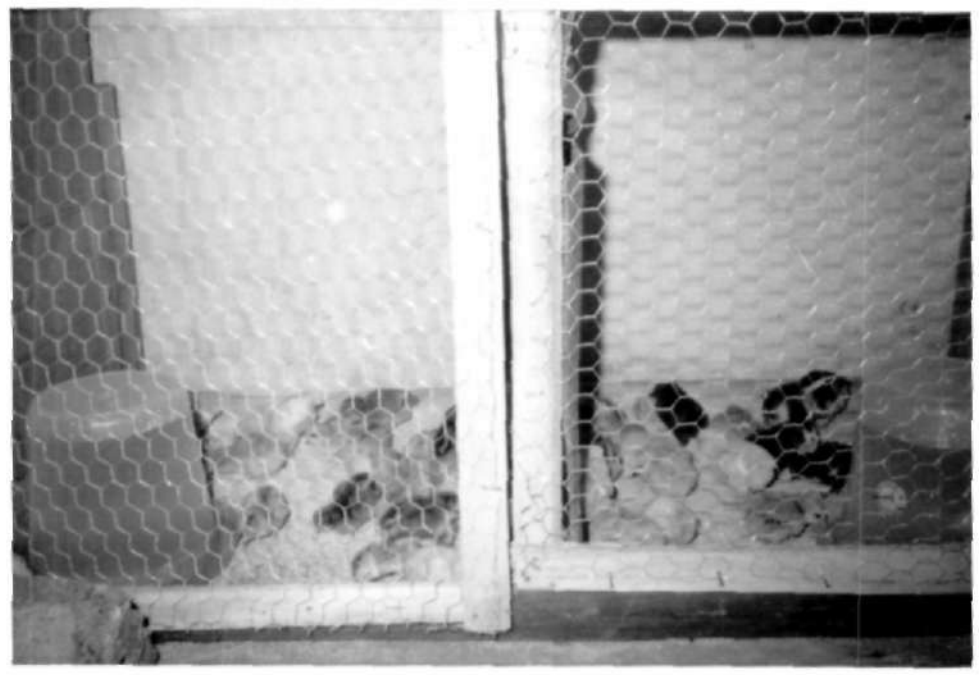


Plate II: Experimental Animals Starting Stock.

They were sourced from various litter-mates. They were separated into 6 groups (A-F) of 10 chicks each with two extra chicks per group, as replacement stock in case of mortality. The chicks were leg-banded for positive identification. The chicks were group-adapted in an open space in front of their partitioned cages for 1 week. During this period, they were served chick mash (Sanders Feeds Seepc Nig. Ltd.).

3.2.3 Sampling Methods for Blood and Other Tissues

Upon sacrifice, 1 - 3gm of whole blood was carefully collected into a previously weighed petridish containing 10ml of phosphate buffer (pH 4.5). The weight of the blood eventually used for analysis was noted as a requirement for OTC drug quantitation per gram of sample.

The chickens were each dissected fresh by skinning-off the feathers. No hot water was used. One tigh was randomly sampled per bird and for young small chicks, the two thighs were sampled. The abdominal cavity was dissected and the liver, kidney, intestinal contents and fat (if present) were each separately collected into polyethylene bags (Plate III).



Keys

A - Thigh muscle

B - Kidney tissue

C - Liver tissue

D,E&F - Fat tissue from various locations

G - Intestinal contents.

Blood sampled into the bottle (top right corner)

Plate III: The Tissue Samples.

The animals vary in ages and sizes at the various times of sacrifice. Therefore, 0.5 - 3.0gm of the specimens were obtained as available. All samples from each bird were labelled and immediately kept frozen at 0°C until analysed.

For the layers, the last two eggs in a week were sampled/ collected (if laid) during the first two weeks duration. The egg drug residue study lasted three weeks: made up of premedication, medication and drug withdrawal week respectively. All eggs laid in the third week (from days 15 to 21 i.e. after withdrawal of drug medication) were collected and analysed for the residual OTC level. Premedication blood sample and concurrent blood sample for each egg dropped for all the experimental weeks were obtained for analysis.

All the tissues and eggs collected were analysed within one week of sampling.

3.2.4 Stock and Working Solutions Prepared

Ammonium hydroxide (absolute)

Ethylene diamine tetraacetic acid (0.1 M)

Ethylene diamine tetraacetic acid buffer (pH 7)

Ethylene diamine tetraacetic acid (pH 7 buffer) in Ethylacetate (Ratio 1:6 v/v:- mobile phase for TLC).

Hydrochloric acid (0.17 N)

Hydrochloric acid (0.1 N)

Hydrochloric acid (0.01 N), alcoholic

Oxytetracycline Reference Drug Standard: Stock I
(10 mg/ml in 100ml ethylacetate)

Oxytetracycline Reference Drug Standards (10 ethyl acetate preparations: 1mg/ml to 10 mg/ml).

Oxytetracycline Reference Drug Standards 10 ethyl acetate preparations: 1 µg/ml to 5000 µg/ml).

Oxytetracycline Reference Drug Standards (13 ethyl acetate Preparations: 0.05 µg/ml to 20 µg/ml).

Oxytetracycline Reference Standards (10 ethyl acetate Preparations: 10^{-8} to 10^{1} mg/ml)

Oxytetracycline Reference Drug Standard: Stock II
(10 mg/ml in 100 ml 0.1 N HCL diluent).

Oxytetracycline Reference Drug Standard (10 Preparations of 0.025 - 10 mg/ml in alcoholic 0.01 N HCL).

Sodium Hydroxide (1.0 N)

Sodium Hydroxide (0.4 N)

Sodium Hydroxide (0.2 N)

Trichloroacetic acid (20% w/v)

3.2.5 Preparation of OTC - Standards:
Stock and Working Solutions

The Oxytetracycline dihydrate powder (Sigma Chemical Co. USA), 1.0gm was accurately weighed. This amount was dissolved in a 250ml erlenmeyer flask with 1ml propylene glycol. It was then quantitatively transferred into a 100-ml volumetric flask with 50ml ethyl acetate. The stock solution was made up to 100ml mark with

ethyl acetate to give a final concentration of 10mg/ml. Various concentrations of 4 working solutions A-D were prepared from the "Stock Solution I" (SS.I). "Solutions A" were prepared to provide 10 concentrations of OTC ranging from 1mg/ml to 10mg/ml ethyl acetate. "Solutions B" were prepared to give ten OTC concentrations ranging from 1µg/ml to 5000 µg/ml. "Solutions C" were prepared to give OTC concentrations ranging from 0.05 µg/ml to 20.0µg/ml. "Solutions D" consisted of 10 (ten) OTC concentrations ranging from 10^{-8} to 10^{1} mg/ml. In preparing Solutions B,C & D, "SS.I" was further diluted 1:9 and 1:99 (v/v) ratio with ethyl acetate to obtain the microgram quantities of OTC per millilitre of ethyl acetate. The absorbance of Solutions "C" were individually determined at 353nm for the construction of the calibration curve. The absorbance reading from Solutions A - D (Appendix 1) were used in assessing uv-vis spectrophotometer's limits of sensitivity. Specific care was taken to prevent loss of solvent from the solutions through volatilization: by using screw-capped bottles and minimal ambient temperature and air current.

Using similar dilution method, "Stock solution II" (SS.II) was prepared to contain 10mg/ml OTC in 0.1 N HCl solution. A set of working "Solutions X" was prepared from "SS.II" to contain OTC concentrations ranging from 0.025mg to 10mg per ml of 0.01 N HCl. Portions of these OTC in 0.01 N HCl were used: (i) to spot the TLC to determine the minimum spot visibility of OTC on the plate, (ii) assessment of TLC spot resolution for every spotted sample, and (iii) as internal standards in the OTC Recovery Evaluation.

3.2.6 Ultraviolet - Visible Spectroscopy

Instrument Optimization:

Make of Instrument: PYE UNICAM ultraviolet-visible double beam Spectrophotometer

Model: - Unicam SP 1800

Measurement Wavelength: 353nm

Set Absorbance reading range: 0--1 (with options to read higher at 0 - 2, or lower at 0 - 0.5) absorbance units.

Band Width: 0.6

Slit Width: 0.18

Sample cuvettes: Silica (1.0cm pathlength)

Absorbance Reading Measurements and the OTC Standard's Calibration Curve:

The pair of silica cuvettes were rinsed and thoroughly cleaned with soft tissue paper. They were filled with the blank sample ethyl acetate solution. One was placed in the reference/blank position and the second in the sample position. The "Zero" knob was adjusted to put the baseline reading at zero. The blank sample was decanted and replaced with each one of the prepared Working Solutions: $^{A}_{1}$-$^{A}_{10}$, $^{B}_{1}$-$^{B}_{10}$, $^{C}_{1}$-$^{C}_{13}$, and $^{D}_{1}$-$^{D}_{10}$, cleaning the cuvettes thoroughly within each sample. For all the group - samples (e.g. "Solutions A"), the measurements were done in successive increasing order of their known OTC contents at 353nm wavelength. The absorbance readings were obtained and recorded. Following this step, the absorbance measurement was made for each of the ethyl acetate extracts obtained from the various

biologic (tissue) samples for the chicks, and then, the layers.

Chemical Evaluation of Test Drug Terramycin Poultry Formula (TPF):

Twelve satchets of the drug preparation each containing 50 gram-weight of the OTC were purchased initially. Three satchets were randomly selected from the lot and analysed for their oxytetracycline antibiotic content using the chemical spectrophotometric method of Monastero et al (1951) and Mitscher (1978) with slight modification. Two subsamples (1 & 2) were obtained from each analysed satchet and quantitated against the "OTC. $2H_2O$" reference drug standard. First, 0.5g of the TPF was accurately weighed from each satchet and carefully dissolved in 50ml of 0.01 N alcoholic HCl. The pH was taken. Two twelve millilitre portions were taken from the 50ml TPF preparation per satchets I,II & III. They were correspondingly designated subsamples 1 & 2. These subsamples were each divided into (i) ten millilitre portion, and (ii) two millilitre portions. Three millilitre of distilled water was added to each of the two millilitre portion and then 5.0ml of 0.4 N Sodium hydroxide (NaOH) was added. These were boiled for 5 minutes and then cooled in ice for 2 minutes. These resultant six, 10ml preparations (from satchets I, II and III in pairs) were each further diluted to 20ml with distilled water. The pH after addition of 0.4 N NaOH was measured. Ten millilitre was taken from each of the 20ml volume. They were subsequently reacidified to about PH 1.7 with the addition of 15ml of 1.7 N HCl. This brought the total volume to 25.0ml. The UV-Vis absorbance readings were measured at 353nm for each of them, derived from the two millilitre portions (Table 5). To each of the 10ml aliquots (for satchets I, II & III in

pairs), 240ml of distilled water was added: bringing each's total volume to 250ml. The pH was taken for the six preparations as well as the absorbance at 353nm. The equivalent OTC quantities of: (iii) the base treated-reacidified (2ml) TPF portions and (i) the acidic (10ml) portion unalkalinized TPF preparation, were read-off from the regression analysed calibration curve.

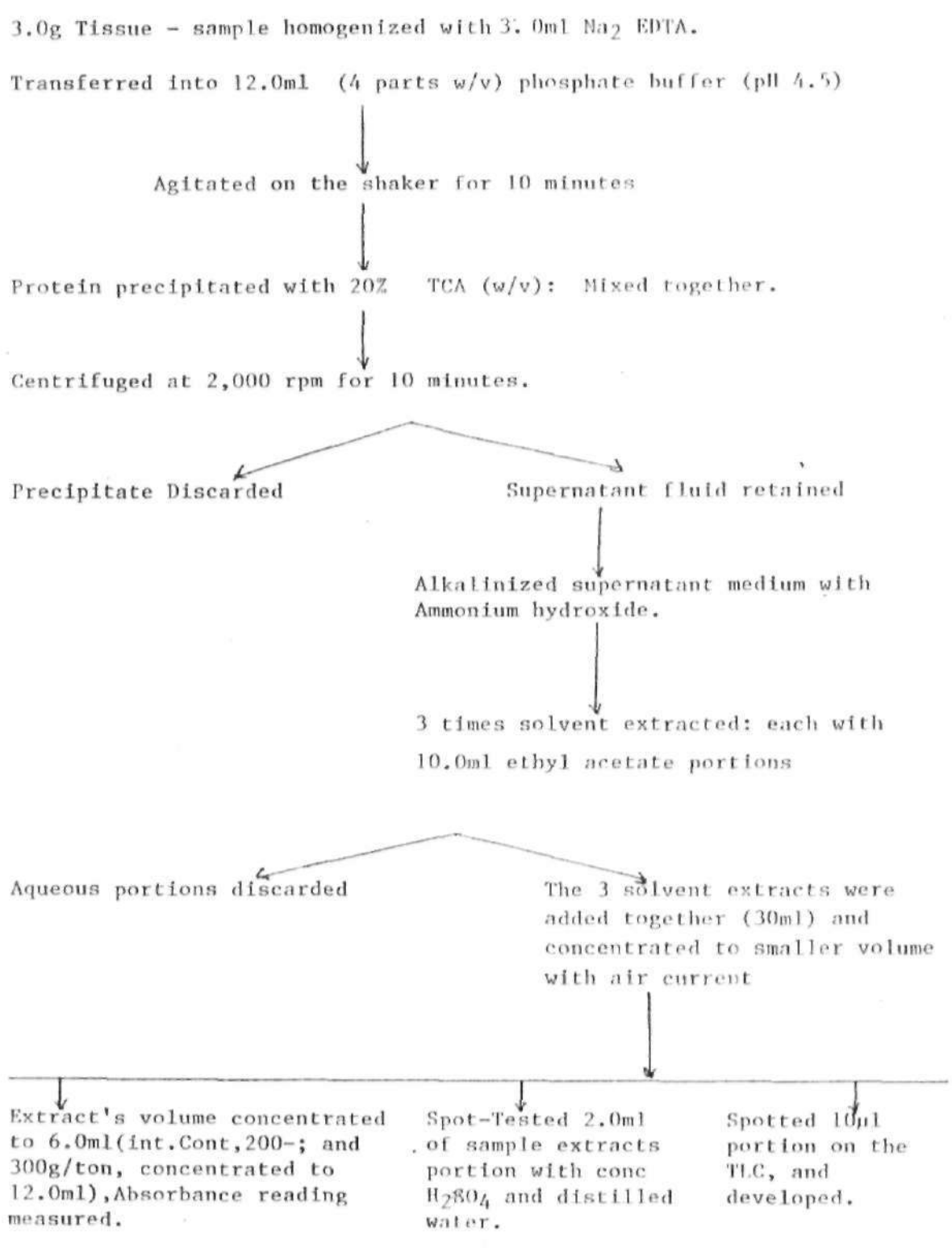
Extraction of OTC from the Biologic Matrix:

For the broiler-type tissues and blood, each known weight (eg. 3.0gm) of the specimen(s) was homogenized by hand chopping and grinding in a glass mortar with 3.0ml of 0.1M EDTA (di- Sodium salt). This was then completely transferred into a 250ml flask with 12ml of phosphate buffer (pH 4.5). These two liquids used for the homogenization were previously kept at 0°C.

For the egg samples, the yolk and albumin were homogenized together in a rotary-blender (Sterilmixer$^{(R)}$) for 30 sec. at 4,000 rpm (revolution per min). As done for the broiler-type tissues, 3.0g portion of the homogenate was added to the 3.0ml of 0.1M EDTA, and 12ml phosphate buffer. After homogenization, every sample prepared was then agitated on the shaker for 10min. Five millilitres of trichloroacetic acid (20% w/v) was added to the homogenate (in statu quo) to precipitate the protein. The mixture was then centrifuged at 2,000 rpm for 10min (Figure 8). The liquid portion was recovered and the precipitate discarded. Immediately prior to solvent extraction (which was the next step), 0.5ml of ammonium hydroxide was added to each homogenate, mixed together, covered and left for 5min to equilibrate

on the bench. It was tested positive to litmus. 10ml of ethyl acetate was added to each of two separate homogenates at a time. They were agitated on the shaker in pair for 15 min. The 10ml solvent extract was recovered using separating funnel.

FIGURE 8: EXTRACTION OF OXYTETRACYCLINE FROM THE BIOLOGIC MATRIX



The 10ml ethylacetate extraction was repeated two more times by gentle shaking and mixing for every sample. The three 10ml ethyl acetate extracts were combined. The solvent extract was kept and the aqueous portion discarded. The 30.0ml extract's volume obtained from each of the 3.0gm of the broiler-type tissues, and each got from those of the layers , were then concentrated to 6.0ml, i.e. 1/5th volume. Those of intestinal content at 200-, and 300 g/ton were also concentrated to 12.0ml using warm air current. Their absorbance readings were measured and multiplied by a factor of 2, to obtain equivalent OTC quantity for each (intestinal content), from the calibration curve in µg/g.

OTC recovery was evaluated from the six broiler-type tissues. Three pieces each weighing 1g, were obtained from the control birds for all the tissues (i.e. Blood, Fat, Intestinal content, Kidney, Liver and Muscle). They were labelled subsamples 1, 2, & 3 for each type of tissue. The six tissues in each subsamples 1, 2 & 3 were successively homogenised in glass morter, spiked with 5µg OTC standard (SS.II) and extracted with solvent as described above . The 30ml ethyl acetate extract was each concentrated to 5ml by warm air current and the UV-Vis absorbance readings measured. The equivalent OTC quantities in µg/g were calculated using five-fold conversion factor. The OTC residue levels are shown in Tables 2,3 & 5. Subsequently, the tissues' solvent extracts were spotted on the TLC, and evaluated for OTC content by colour spot tests.

Data Analysis

Beer - Lambert's Law or relationship was used for the absorbance measurements in respect of drug contents' quantitation using the UV-Vis spectroscopic data.

i.e. $A = \varepsilon . b . c$

Where A = The absorbance (or Optical Density) of substance of interest, in the solution at the appropriate wavelength (353nm).

ε = The molar Extinction coefficient (or molar absorbtivity).

b = Pathlength of the light through the solution.

c = Concentration of substance of interest (i.e. the absorbing species) in the solution of pathlength "b".

ε and b are constants (K). Value of ε is independent of b and c, and the pathlength "b" is represented by uniform cross-section (1.0cm) of the cuvette(s).

If $A = \varepsilon . b . c$

$\therefore A = K.c$

i.e. $A \propto c$ (at 1.0cm pathlength: equivalent to 1.0 cubic centimetre extract volume).

The 30.0ml extract's volume got from the 3.0gm of the broiler-type tissues, and those got from those of the layers, were each concentrated to 6.0ml i.e. 1/5th volume (those of intestinal content at 200-, and 300g/ton were concentrated to 12.0ml). Their absorbance readings were measured and multiplied by factor of 2, to obtain equivalent OTC quantity for each from the calibration curve in ug/gm. Extracts got from one-gram-piece tissues used for

extract ion in the recovery study were correspondingly evaluated to get the equivalent OTC quantities in µg/gm using five-fold conversion factor. The OTC residue levels are shown in Tables 2, 3 & 5.

3.2.7. Colour Spot Tests:

Obtaining the OTC standard's calibration curve was augmented by the use of colour spot testing on concentrations of markedly different OTC quantities but giving similar absorbance readings. It was observed that though the OTC concentration of 0.012 mg/ml in one sample for example, was significantly lower than another containing 3 mg/ml. Yet their absorbance readings were 0.480 and 0.435 respectively (Appendix 1). These absorbance readings were not quite different nor proportional for their convenient differentiation, nor for the reading of the two solutions' intermediate concentrations. Subjecting the two solutions to colour spot tests, could result in clear different colour reactions. This test gave colourless reaction to the former (0.012mg OTC/ml) while the later (3mg OTC/ml) was yellow homogenously. This led to the investigation of colour spot test described by British Veterinary Codex (B. Vet. C., 1953) for assessing the identity of oxytetracycline in a substance. Three categories (A, B & C) of OTC preparations were investigated. OTC contents of the tissue extracts from the experimental animals were also assessed. In all the assessed OTC preparations, whether amongst those in the tissues or those in the OTC standards, two millilitre portions were tested for the colour reactions. Two millilitre of Conc sulphuric acid was first added to produce the crimson colour, followed by 1ml of distilled water which should change the colour to yellow if oxytetracycline was present.

3.2.8 Thin Layer Chromatography:

Four 20 x 20cm Chromatoplates were prepared at a time, using slurry titurated in a glass mortar from 48.0gm of modified silica gel H_6F (254) and EDTA-buffer (pH 7). 16ml of the buffer was used for every 8.0g of silica gel. The buffer was prepared by mixing 5.0ml of 20% v/v polyethelene glycol (P E G-400) in glycerin, with 95.0ml of 0.1 M EDTA that has previously been adjusted to pH 7 with ammonium hydroxide (Ascione et al., 1967). The thin layer was gauged to be coated at 500μ (microns) thickness. After coating, the plates were left on the leveller to first air dry for 30 min. They were then transferred to an oven preheated to 105°C, and activated for 1 hour. After activation and cooling, the chromatoplates were spotted with 10μL of the ethyl acetate extracts of the tissues, obtained from the solvent extraction step above. OTC.$2H_2O$ standards were prepared from SS.II to give concentration of 10, 7.5, 5.0, 2.5, 1.0, 0.50, 0.25, 0.1, 0.05 and 0.025 mg/ml of 0.01 N HCl. These were spotted along with the tissues' solvent extracts, for the identification and measurement of their OTC contents. 5μL of these standards were used at 0.1, 1.0, and 10mg/ml for spotting each plate using Hamilton (R) precision Syringe at 1½ cm spacing-intervals provided by the transparent plastic spotting-guide device. The plates were left on the bench for 5 - 10 minutes to air-dry. Meanwhile, the development tank was prepared and equilibrated (Ascione et al.,1967) using ethyl

acetate and EDTA buffer (pH 7) at ratio 6: 1(v/v) and pouring the resultant 140ml mixture into prepared development tank. After marking the solvent fronts at 12cm from the starting points on the two plates, they were placed in the tank, closed normally, and watched the mobile phase's migration. On completion of development, the plates were airdried on the laboratory bench and later viewed under long wave ultraviolet light for the positive orange fluorescence of OTC, the extent of migration, and the spot size. In cases where there were positive OTC spot-separation, the area occupied by separated spots were traced on a transparent tracing-paper and measured on the graph-paper in centimetre square (cm^2) units. All measurements were recorded. Minimum spot visibility was evaluated using OTC standards absorbance readings. Five µL (microlitre) each of these preparations was spotted directly on the TLC and developed normally. The ten preparations from Solutions "X" (0.025 to 10.0mg OTC per ml of 0.01 N hydrochloric acid) were solvent extracted. The ethyl acetate extracts recovered were spotted after concentration, at 5µL quantities each, on the TLC and developed.

CHAPTER 4

RESULTS

4.1 Observations Made After Medication of Experimental Animals:

During the post mortem sampling of the animal tissues, a number of gross pathological changes were noted particularly in the liver of some of the sacrifised animals. These included: mottled appearance of the liver mass, petechial haemorrhages, paleness, focal necrotic points, some were severely hyperemic (dark reddish), while some had yellowish discolouration. These occurred more in the livers of birds fed higher drug concentrations (300-, and 200g/ton) for six weeks or longer (about 30% of them). Some birds that died during the course of the study and some of those sacrifised had inflammed caecum (about 10%). This indicated Eimeria tenella prevalence, even with the coccidiostat control. A few of the birds had ascariasis, and fewer still, had taeniasis.

All the birds fed with different drug concentrations in this study were observed to have increased weight (Table 1) which were not dose dependent. Mortality with various drug concentrations were 64, 57.1, 56.5, 52.6 and 44.4% for Groups F,D,B,E,C and A respectively. The highest weight-gain (93%) was obtained with 200g of OTC per ton of feed (200 ppm) after 12 weeks feeding duration. At both 6-, and 9-weeks feeding durations, the birds fed 100g/ton were observed to gain the highest weight of 80.1% and 87.9% respectively. Group F, with no drug supplementation in the feed, showed the least weight gain.

TABLE 1

PERCENTAGE WEIGHT GAIN OF BIRDS FED WITH VARIOUS CONCENTRATIONS OF OTC

GROUP	concentration of OTC in the feed(g/ton)	PERCENTAGE WEIGHT GAIN				GROUP AVERAGE WEIGHT GAIN
		3 WEEKS*	6 WEEKS*	9 WEEKS*	12WEEKS*	
A	300	49·6	79·0	84·8	92·9	76·62
B	200	23·9	72·7	73·5	93·0	65·77
C	150	40·5	69·5	87·5	85·0	70·62
D	100	25·3	80·1	67·9	86·4	69·92
E	50	25·9	78·3	76·7	72·6	63·39
F	0	14·3	28·0	45·2	63·7	37·80
INTERVAL'S AVERAGE WEIGHT GAIN:-		30·0	67·9	75·9	82·3	

* BIRDS WERE SACRIFISED AT THESE TIME PERIODS.

4.2. Levels of OTC Residues in Blood and Other Tissues: From Spectroscopy

For the quantitation of OTC contents of samples, a calibration curve was plotted (Appendix 4) using regression analysis (Appendix 3) and the absorbance readings in Appendix 2 ('t' test, P = 0.95). The spectrophotometric readings of OTC concentrations are shown in Table 2 and Appendix 5 for solvent extracts of the broiler-type tissues (i.e. Blood, Fat, Intestinal content, Kidney, Liver and Muscle); and also in Table 3 for the layers' blood and eggs. The drug quantities found in the tissues ranged between zero ug/g of blood at 3 weeks of feeding 50g of drug per ton of feed, to 24.40 ug/g of intestinal content at 6 weeks of feeding 200g of drug per ton of feed. Drug levels in all the tissues were least at 3 weeks, the highest levels occurred at 6 weeks. The quantities of Oxytetracycline in all tissues were not significantly different at 9 and 12 weeks study, but were slightly lower than the 6 weeks drug feeding study. No significant fat deposit was found in 3 weeks fed birds and as such,no drug value was obtained for that tissue at 3 weeks. Generally, drug levels were lowest in blood and highest in the intestinal contents. Also kidney levels of OTC were higher than levels in the liver. The thigh muscle had higher drug levels than the blood. The highest drug residue was found in the kidney at all drug feeding levels.

Following witharawal of drug, birds fed 50, 100-, or 150g/ton feeds were negative for OTC after 2 days, while those fed at 200, and 300g/ton were negative on the 4th and 5th day withdrawal periods respectively.

TABLE 2: *AVERAGE OXYTETRACYCLINE (OTC) CONCENTRATIONS (ug/g) IN THE BLOOD AND OTHER TISSUES OF CHICKS, AFTER ADMINISTRATION IN FEEDS AT VARIOUS CONCENTRATIONS AND TIME INTERVALS

OTC Concentrations in feeds(g/ton)	OXYTETRACYCLINE CONCENTRATIONS IN THE TISSUES (ug/g)											
	3WK	'6WK	'9WK	'12WK	3WK	'6WK	9WK	'12WK	'3WK	6WK	'9WK	'12WK
	B L O O D				F A T				INTESTINAL CONTENTS			
300	0.26	1.06	0.96	0.96	-	2.85	2.70	2.70	22.60	23.60	21.50	21.60
200	0.15	0.96	0.93	0.85	-	-	2.68	2.70	18.52	24.40	21.10	21.00
150	0.06	0.58	0.43	0.43	-	1.26	1.21	1.30	12.35	12.75	12.60	12.68
100	0.01	0.30	0.15	0.26	-	1.33	1.21	1.08	10.15	10.76	10.50	10.53
50	(a) NR	0.06	0.02	0.02	-	0.16	0.56	0.60	4.16	6.22	3.50	3.46
	K I D N E Y				L I V E R				M U S C L E			
300	3.68	4.00	3.92	3.95	1.15	2.65	2.10	2.12	0.48	1.70	1.33	1.30
200	3.36	3.60	3.38	3.38	1.20	2.43	1.92	1.92	0.38	1.65	1.16	1.20
150	2.46	2,63	2.52	2.46	0.85	1.98	1.65	1.68	0.08	1.36	1.20	1.20
100	1.33	1,63	1.38	1.38	0.40	1.20	1.18	1.20	0.06	0.83	0.56	0.62
50	0.55	1.36	0.76	0.60	0.15	0.63	0.63	0.30	0.06	0.62	0.60	0.60

* Evaluated from the regression analysis.
(a) NR: No spectrophotometer reading; zero drug quantity.
-- ie No sample for analysis: chicken too young.
NB: Og/ton drug feeding concentration (Group F) were used as the blank (≈0.00µg/g) for the other Groups (A-E :- 300g/ton to 50g/ton).

TABLE 3: QUANTITIES OF OXYTETRACYCLINE OTC RESIDUES FOUND IN THE LAYERS' BLOOD AND EGGS AT VARIOUS SACRIFICE INTERVALS AND DRUG FEEDING LEVELS; EVALUATED FROM THE REGRESSION ANALYSIS.

OTC LEVEL IN FEED (g/ton)	ABSORBANCE FOR EGGS						ABSORBANCE FOR BLOOD						EQUIVALENT OTC QUANTITY IN BLOOD (μg/g)	
	1st Week		2nd Week		3rd Week		1st Week		2nd Week		3rd Week		2nd Week	
	Premedication		Medication		Withdrawal		Premedication		Medication		Withdrawal		Medication	
	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
300	NE*	NR[a]	NR	NE	NR	NE	-	NR	0·01	-	NR	-	0·06	-
200	NE	NE	NR	NR	NR	NE	NR	-	0·009	0·008	NR	-	0·02	0·02
150	NE	NE	NE	NE	NE	NE	-	-	-	-	-	-		
100	NE	NR	NR	NE	NR	NE	—	NR	NR	-	NR	-		
50	NE	NE	NE	NE	NR	NE	-	-	-	-	NR	-		
OTC (Blank control)	0·00	0·00	0·00	NE	0·00	NE	0·00	0·00	0·00	-	0·00	-		

(i) ie First egg/blood sample
(ii) ie Second egg/blood sample

- ie No blood was sampled at that interval.

* NE ie No egg layed at that interval
(a) NR ie No Spectrophotometer reading; Zero drug quantity.

In respect of samples obtained from the layers' (Table 3), no drug residues were observed in the tissues at the drug feeding levels during premedication and drug withdrawal periods. During the one-week drug medication period, two birds, each placed respectively on 300-, and 200g OTC per ton of feed, had blood drug residues equivalent to 0.06 ug/g (in one sample) from the 300 g/ton fed bird, and 0.02 and 0.02 ug/g (in two samples) from the 200g/ton fed bird. No OTC residue was found in the eggs laid by the two birds at this same period and in all other birds', throughout the study periods.

The earliest laid egg was at the third day of drug withdrawal. The maximum number of eggs laid by any of the birds were two, during that period.

Results of the tissues recovery studies are shown in Table 4. Percentage recovery was highest from kidney and lowest from liver. Recovery study was not done for the layers' samples.

TABLE 4: TISSUES' OXYTETRACYCLINE (OTC) RECOVERY: EVALUATED FROM THE REGRESSION ANALYSIS.

	(d) Absorbance Readings of Tissue Extracts			(e) Equivalent OTC Quantity (µg/gm)			OTC Percentage recovery from tissues			Average percentage Recovery
SUB SAMPLE / TISSUES	1	2	3	1	2	3	1	2	3	%
BLOOD	0·150	0·150	0·142	3·6	3·6	3·4	72·0	72·0	68·0	70·7
FAT	0·185	0·165	0·173	4·5	4·0	4·2	90·0	80·0	84·0	84·7
INTESTINAL CONTENT	0·193	0·185	0·197	4·7	4·5	4·8	94·0	90·0	96·0	93·3
KIDNEY	0·185	0·209	0·197	4·5	5·1	4·8	90·0	102·0	96·0	96·0
LIVER	0·155	0·135	0·142	3·7	3·2	3·4	74·0	64·0	68·0	68·7
MUSCLE	0·165	0·173	0·165	4·0	4·2	4·0	80·0	84·0	80·0	81·3

(d) 1·0g tissue spiked with 5µg OTC, solvent extracted and concentrated to 5ml volume.

(e) Five-fold value of equivalent OTC quantity.

The TPF's (premix) chemical assay, showed that the potency was in close agreement with the manufacturer's declared quantity, and the standard by the United States Pharmacopeia (USP XX, 1980) for Oxytetracycline hydrochloride (Table 6).

4.3 Levels of OTC Residues in Blood and Other Tissues From Colour Spot Tests:

The observations made for the colour spot testing of the OTC are shown in Tables 6 and 7. OTC's presence was indicated by crimson/violet, brown or yellow colour. From the analysis of the tests, crimson colour that was considered positive for oxytetracycline identity using the B. Vet. C (1953) method, was found often with 0.5 mg/ml, contained in media preparations of 0.25ml and 0.5ml volumes. It was also positive for 0.5 mg OTC in 1.5ml or 0.5ml volumes (Table 7). When the test tube was not agitated and the concentrated sulphuric acid (H_2SO_4) was added gently along the side of the tube, the colours formed downwards as: Brown/violet, crimson interphasing layers.

On addition of 1ml of distilled water, the colour changed from crimson to yellow. In some other concentrations of the OTC preparations, the OTC colour changed to lighter shade(s) of the colour initially shown with addition of the acid (i.e. lighter shade(s) of either crimson, brown or yellow).

When the test tube was shaken, the colour of positive test cases were brownish to crimson. Such were the colour reactions observed with ten preparations of constant 1ml volume, having OTC quantities that ranged from 1.0mg to 10.0mg (Plate IV and V), (Table 6).



Plate IV: Colour Spot Test on ten preparations of Constant 1ml volumes having OTC concentrations from 1.0mg to 10.0mg. The blank (zero OTC concentration) is shown in the right (eleventh) test tube.



Plate V: Colour Spot Test shown in Plate IV. This was the Colour-change: from crimson to yellow, after adding 1.0ml distilled water.

Various concentrations of the OTC drug (i.e. >0.05 mg/ml) and volumes (>2.0ml, ≯ 10.0ml) were tested. These gave yellow colour reactions. The yellow colour manifested usually when the volume of the medium (containing the OTC) was more than 2.0ml (tested up to 10.0ml) and the OTC content was between 0.5 and 1.0 mg/ml. When 2.0ml was used as constant media volume, OTC in quantities of 0.05, 0.025 and 0.0125mg (i.e. 0.025, 0.0125 and 0.00625 mg/ml respectively) showed negative colourless results to the colour-test as it was for a blank 2.0ml of 0.01 N HCl diluent medium. Yellow colour manifested for 0.10g OTC in 2ml of the diluent HCl (i.e. 0.05mg/ml concentration). The 0.025mg/ml therefore appeared to be

TABLE 5 : EVALUATION OF OXYTETRACYCLINE (OTC) CONTENT OF TERRAMYCIN POULTRY FORMULA (TPF)

SAMPLE NUMBER	SUB SAMPLE	ACTION (f)	MEASURED PH	UV-VIS ABSCRBANCE	DIFFERENCE OF ABSORBANCE: (ii) - (iii)	OTC QUANTITY (g) (µg/gm)	AVERAGE OTC FROM SAMPLE	MANUFACTURER'S OTC QUANTITY (µg/gm)	OTC % FOUND
I	1	i	2·5	0·082	0.048	1·00	1·10	1.10	100·00
		ii	12.5	0·062					
		iii	1.7	0·034					
	2	i	2·5	0·080	0.054	1·20			
		ii	12·5	0·063					
		iii	1·7	0·026					
II	1	i	2·5	0·085	0.050	1·06	1·13	1.10	102·72
		ii	12·5	0·063					
		iii	1·6	0·035					
	2	i	2·5	0·083	0·055	1·20			
		ii	12·5	0·065					
		iii	1·6	0·028					
III	1	i	2·4	0·078	0·053	1·15	1·115	1·10	101·36
		ii	12·6	0·063					
		iii	1·7	0·025					
	2	i	2·4	0·084	0.051	1.08			
		ii	12·6	0·064					
		iii	1·7	0·033					

(f) CHEMICAL action on sample

i - 10ml portion acidic preparation of OTC premix
ii - 2ml portion acidic preparation of OTC premix alkalinised and heat treated.
iii - The caustic treated 2ml portion, reacidified.

(g) OTC quantity (µg/gm) from sample: evaluated from the regression analysed calibration curve.

TABLE 6: COLOUR SPOT TESTS ON OXYTETRACYLINE STANDARDS

6a: DIFFERENT OTC QUANTITIES IN DIFFERENT DILUENT VOLUMES

0·01N HCl Diluent(ml)	Total Qty of OTC(mg)	H_2SO_4 Colour Reaction	Dist H_2O Colour Reaction	0·01N HCl Diluent(ml)	Total Qty of OTC(mg)	H_2SO_4 Colour Reaction	Dist H_2O Colour Reaction
10·0	10	Yellowish	Light Yello.	0·25	0·125	Yellow/ crimson/ colourless interlayer	Yellowish
9·0	9	"	"				
8·0	8	"	"				
7·0	7	"	"	0·50	0·25	Brown/crimson	"
6·0	6	"	"	0·75	0·375	Yellowish	"
5·0	5	Brownish	Brownish Yellow	1·00	0·50	"	"
				2·00	1·00	"	"
4·0	4	"	"				
3·0	3	Crimson	Lighter Crimson	4·00	2·00	"	"
2·0	2	"	"				
1·0	1	"	"				
0.5	0.5	"	Darker Yello.				

7(b): DIFFERENT OTC QUANTITIES IN CONSTANT DILUENT VOLUME

1·0	1·0	Brownish	Brownish	2.0	10.0	Yellowish	Light Yello.
1·0	2·0	"	"	2.0	7·5	"	"
1·0	3·0	"	Yellowish	2.0	5.0	"	"
1·0	4·0	"	Brownish	2·0	2.5	"	"
1·0	5·0	Crimson	"	2·0	1·0	Brownish	"
1·0	6·0	"	Light crimson	2·0	0·5	Brownish Yellow	"
1.0	7.0	"	"	2·0	0·25	Light Yello.	"
1·0		"	"	2·0	0·10	"	"
1.0	9·0	"	"	2·0	0.05	Colourless	Colourless
1·0	10·0	"	"	2·0	0·025	"	"

7(c): CONSTANT OTC QUANTITY IN DIFFERENT DILUENT VOLUMES

10·5	0.5	Yellowish	Light Yellow	4·5	0.5	Yellowish	Light Yellow
9.5	0.5	"	"	3·5	0.5	Brownish	"
8.5	0·5	"	"	2·5	0·5	"	"
7·5	0·5	"	"	1·5	0.5	Crimson	Yellow
6.5	0.5	"	"	0·5	0·5	"	"
5.5	0·5	"	"				

TABLE 7: COLOUR SPOT TEST FOR OXYTETRACYCLINE RESIDUE IN TISSUES

TABLE 7(a): 3 WEEKS DRUG FEEDING DURATION

Feeding Levels with Positive Reaction				Feeding Levels that were Negative			
Tissue Extract (2·0ml)	Total Qty of OTC(mg)	H_2SO_4 Colour Reaction	Dist H_2O Colour Reaction	Tissue Extract (2·0 ml)	Total Qty of OTC (mg)	H_2SO_4 Colour Reaction	Dist. H_2O Colour Reaction
Liver (300 & 200 g/ton)	Unknown	Yellowish	Lighter yellow	Blood (all)	Unknown	Colourless	Colourless
Kidney (300 & 200 g/ton)	"	"	"	Muscle	"	"	"
				Liver (The rest)	"	"	"
Fat (300 & 200g/ton)	–	No Sample	No Sample				
Int. Content (150-150g/ton)	Unknown	Yellowish	Lighter Yellow	Kidney (The rest)	"	"	"
Int. Content (300 & 200g/ton)	"	Yellowish Brown	Yellowish	Fat (The rest)	–	No Sample	No Sample

BLE 7b: 6 WEEKS DRUG FEEDING DURATION

-do-	-do-	-do-	-do-		-do-	-do-	-do-	-do-
Liver (300 & 200g/ton)	Unknown	Yellowish	Light Yellow		Blood	Unknown	Colourless	Colourless
Kidney (300 & 200g/ton)	"	"	"		Muscle	"	"	"
Fat (300 & 200g/ton)	"	"	"		Liver	"	"	"
Int. Cont (50-150g/ton)	"	"	"		Kidney	"	"	"
Int. Cont (300 & 200g/ton	"	Yellow Brown	Yellowish		Fat	"	"	"

TABLE 7c: 9 WEEKS DRUG FEEDING DURATION

-do-	-do-	-do-	-do-		-do-	-do-	-do-	-do-
Liver (300 & 200g/ton)	Unknown	Yellowish	Light Yellow		Blood	Unknown	Colourless	Colourless
Kidney (300 & 200g/ton)	"	"	"		Muscle	"	"	"
Fat (300 & 200g/ton)	"	"	"		Liver	"	"	"
Int. Cont (50-150g/ton)	"	"	"		Kidney	"	"	"
Int. Cont (300 & 200g/ton)	"	Yell. Brown	Yellowish		Fat	"	"	"

TABLE 7: cont'd.

TABLE 7(d) 12 WEEKS DRUG FEEDING DURATION

-do-	-do-	-do-	-do-		-do-	-do-	-do-	-do-
Liver (300 & 200g/ton)	Unknown	Yellowish	LightYellow		Blood	Unknown	Colourless	Colourless
Kidney (300 & 200g/ton)	"	"	"		Muscle	"	"	"
Fat (300 & 200g/ton)	"	"	"		Liver	"	"	"
Int. Cont (50-150g/ton	"	"	"		Kidney	"	"	"
Int. Cont (300 & 200g/ton)	"	YelloBrown	Yellowish		Fat	"	"	"

the limit of the colour-test's sensitivity at that media volume of 2ml. It may vary however when the 0.05mg OTC (for example) was contained in 1.0 or 0.5ml of diluent media.

The tissues' solvent extracts for all the drug feeding levels at various sacrifice intervals were tested (Table 7). The colour test results were for tests done for OTC in the tissue extracts at all drug feeding levels and the indicated sacrifice intervals. Blood and muscle extracts at all feeding levels and sacrifice intervals gave negative, colourless results. Kidney, Liver and Fat extracts at 300-, and 200 g/ton feeding level showed yellowish colour reaction. All the extracts of the intestinal materials were yellowish to the colour test at 50 to 150 g/ton feeding level. But the test gave yellowish-brown colour for 200-, and 300 g/ton samples.

4.4 Levels of OTC Residues in Blood and Other Tissues: From Thin Layer Chromatography

The Chromatography of broiler-type tissue extracts did not show the orange fluorescence under UV light as would be expected for the presence of OTC spot/band in the sample extracts. In some extracts of the higher feeding levels (300-, and 200 g/ton) however, there were light bluish spots almost regularly appearing for extracts from liver, Kidney and intestinal content samples. The spots were not further identified though, but had spot and solvent fronts similar to that of OTC.

The OTC standards separately spotted at 5-, and 50 ug quantities gave positive, orange fluorescence under UV light, as they were applied along-side all the batch samples (Appendix 6). No spot separation was got with 0.5ug quantity of OTC sample (i.e. from 5 uL of 0.1mg/ml OTC concentration). The minimum spot visibility of the direct OTC standard was 5ug (i.e. 5uL of 1mg/ml concentration). The 5ug spotting quantity also gave the best spot resolution for the OTC standards. From the retardation (Rf) values in Table 7, the reproducibility of the Rf value was got from 40 (averages of two readings) data values to be 0.08 ('t' test, P 0.95). From the separated spots, an average of 0.1145 (cm) from 20 (averages of two readings) data values was obtained in respect of "square root of spot area" ($\sqrt{A}$) for 5ug spotting quantity. The value for 50ug quantity was 1.1429 also from the same number of readings. Of the 5uL quantities spotted from each component of Solutions A to D, all the spots gave positive fluorescent reaction for Solution A, the three highest concentrations were positive for Solutions B, while none was positive from the spottings of Solutions C and D (Appendix 6).

The solvent extracted, ten OTC standard preparations (0.025 to 10.0mg/ml) spotted on the TLC gave positive orange fluorescent spot separations for the highest two preparations (7.5-, and 10 mg/ml). Those of 5.0-, to 1.0mg/ml were light bluish. No spot separation was observed for the last five preparations (0.5-, to 0.025 mg/ml). The lower limit of the solvent's extracts' spot visibility from this work was observed to be 25ug (5uL of 5mg/ml concentration).

From the observed OTC standard's spots' resolution on the TLC, the calculated "square root" of the measured "spots areas" ($\sqrt{A}$) were directly proportional to the quantity (W) of the OTC spiked.

CHAPTER 5

DISCUSSIONS AND CONCLUSION

5.1 Management and Medication of Animals:

The use of bird, older than day-old chicks in drug residue research was reported by Amin et al (1977). They worked with chlortetracycline and Oxytetracycline, and evaluated the residue in 1,580 two-week old broiler chicks of both sexes. They reported another study where they studied the drug residues in 1,580 chicks whose ages that ranged from 2 to 8 weeks: housed in floor pens on wood shavings. Some other workers used day-old chicks, including Katz et al (1972). In respect of layers' studies, hens are known to lay eggs in a given cycle: which comprised of sequence and also the time intervals of interruption in the sequence. In this experimental situation therefore, the idea to get enough eggs dropped per given time interval prompted stocking both sexes of chicken. Reports showed that sexual behaviour is tied closely to the psychological behaviour of the animal (Swenson, 1970). Onset of breeding season had been hastened several days in ewes that had a ram added to the flock. The next oestrus period in many sows had been hastened by putting a boar in a pen adjacent to sows. The practice by local poultry breeders to herd hens and cocks together usually result in more frequent egg laying. Physiologically however, vasotocin (a hormone from the posterior pituitary) may start the contraction of

the uterus which leads to oviposition. Although it had been shown that posterior lobe of chicken contained the least amount of vasotocin coincidental to the time of laying (Najoko, 1962) due to its release into the blood stream. Douglas and Sturkie (1964) demonstrated the hormone (vasotocin) in the blood stream as manyfold higher blood titer before the egg was expelled, as compared to the level at preoviposition period.

Using OTC as a therapeutic agent, the manufacturer recommended dosages that ranged from 22 to 88 ppm for various management and herd health maintenance. The lower limit being included in the prevention dosages, while the upper limit was recommended for both prevention and control regimens. For OTC susceptible organisms, 200gm/ton for example was recommended for curative medication against Mycoplasma synoviae (Infectious synovitis), and 100gm/ton for the prevention (Siegmund 1973). The 200g/ton of feed would also suffice to effect rapid decline of mortality in field outbreak of Clostridial dermatomyositis (Cl. septicum) and Ulcerative enteritis (Cl. perfringens Type A) infection in fowls.

Although low-level antibiotic medication was used as early as 1950 (Goldberg, 1959 and Siegmund, 1973) in the range of 1 - 10 mg/kg (ppm), yet levels as high as 300 ppm were included for even prophylactic feeding. For therapeutic usages, levels up to 2,000 ppm had also been reportedly used (Goldberg, 1959).

There are however regulations now in some countries controlling the use of antibiotics (and other therapeutics) in food - animal production (Amuda-Giwa, 1984). This was seen in the light of drug - resistance due to subtherapeutic usages, and drug deposition in tissues on account of metabolism, high dosages and/or withdrawal periods. But the overall considerations for drug use in food - animal production after being primarily for enhanced animal protein supply, should be also for the drugs therapeutic efficacy and safety in the target animals. Alchogh not studied emphatically with drug-dose-quantity in this experiment, the hazard of liver lesions observed with the 300-, and 200 g/ton dosage levels could disfavour recommending such medication levels for continuous feeding of chicks from day-old to table weight. Besides, one of the highest mortality rates was recorded in the 200g/ton feeding level. OTC was reported to be hepatotoxic.

By evaluating the "group-average" gain-in-weight, the risk of drug toxicity/liver lesions, the mortality rate recorded, and the tendency of drug cummulation to residue level after usage exceeding 6 weeks, it could be noted that the 100g/ton drug feeding level would be optimal for prophylactic use, or as growth promoter.

5.2 Levels of Oxytetracycline Tissue Residues from Colour Tests, Chromatography and Spectroscopy:

A large-scale, modern food - animal production invariably incorporates drug medication of the livestock. Detection of such drugs to control those of residue importance would therefore be

a necessity. Screening method(s) are required, which should be rapid, cost-effective and applicable in non-laboratory environments. The colour-spot-test used for OTC detection in this work was considered for use, as OTC screening method in tissues of food-animals. Following the described procedure, 2.0ml of the solvent extract could be tested which would give the appropriate colour reaction changes when OTC is present at or above 0.025µg/gm. The positive samples could be further tested with sensitive instrumental method(s) for quantitation. The sensitivity of 25 ppm is however low for regulatory purposes, but could be enhanced by improved quantitative extraction and test volume adjustment. This colour spot test could also be a good guide for selecting the calibration range for a set of standard preparations, rather than assessing same by dilution and comparison of spectroscopic absorbance, that may be time consuming.

There were no orange fluorescence (positive colour for OTC's presence) on the TLC from the tissue extracts. This could be that the OTC drug epimerized which probably prevented the colour reaction. Tetracycline (a member of tetracycline group) is however known to undergo epimerization at pH 3 to 5 in aqueous solution to form 4 - epitetracycline. Other substances that influence such epimerization include phosphate, citrate, urea and some pharmaceutical ingredients (Mitscher, 1978). The data from the Spectroscopy were used for the drug quantitation.

The values obtained for the tissues at all drug feeding levels and various sacrifice intervals from the spectroscopy were shown in Tables 2 and 3. Feeding - level to drug - deposition relationship was observed at all sacrifice intervals. Katz et al (1973) observed also a tendency towards higher residues in tissues as drug feeding level was increased with OTC fed chickens. The report by Yoshida et al (1975), working with OTC in chickens at feeding levels that ranged from 0 to 4,000 ppm also showed that at over 500 ppm feeding levels, the tissues drug residues were directly proportional to the dietary medication levels. They found no OTC residue in the blood, muscle (thigh) and liver at 20ppm drug feeding level. From this work, the blood's drug level reached the peak value at 6 weeks' drug feeding duration; but decreased at 9 weeks, establishing a plateau up to 12 weeks. That tendency was observed for muscle and other tissues as well. The decline and levelling off of the drug residues suggest a lessening of potential residues when drug intake is assessed in relation to body weight. Katz et al (1972) observed in their study with Chlortetracycline (CTC) that the kidney had 8 - 10 times the drug concentrations found in muscle or liver. In the present study with OTC, residues in kidney although higher than those found in muscle or liver, the magnitude of difference observed was not as high as that obtained by Katz et al (1972). OTC residue found in Kidney by Katz et al (1973) were higher than those from

the liver, and were also 2 - 5 times the residue in muscle tissues. They found in muscle, liver and kidney, residues in the order of trace, no residue and 0.09µg/g respectively at 25g/ton; They found 0.20,0.29 and 0.78 µg/g respectively at 200g/ton drug feeding level. In the 3-, 6- and 9-week old/fed chickens, they found just detectable residue in the muscle tissues. Mercer et al (1978b) observed significant correlation between plasma levels of OTC and the levels in the liver, muscle, lung, kidney and stomach wall, when studying the drug's tissue kinetics in swine. They opined therefore that field monitoring for OTC could be done using blood plasma to screen for tissue residues.

The analysis of the intestinal contents that showed comparatively very high drug levels (4.16-24.4µg/gm) was not unexpected, being the primary portal of entry (GIT) of the drug-feed into the blood and the tissues.

The values detected varied with the sacrifice interval; and may also be related to the quantity of food ingested prior to the sacrifice of the birds. From the report by Katz et al (1973), feeding chickens with OTC at levels up to 400g/ton for 4 weeks did not give residue in the eggs. Whereas feeding for 6 weeks with levels of 150-, 200-, 300 and 400g/ton produced OTC residues in eggs at trace amounts, the levels they classified as positive presumptive. They opined therefore that several weeks (6) would be expected to pass before measurable residue would be found when 400g/ton level is fed.

5.3 Aspects of Regulations on Drug Residues in Human Foods:

The safety of animal drug residues in human food was generally not evaluated prior to 1958. From 1965 - 1971 however, methods were required for the analysis of all animal drugs, but were demanded only on a selected basis (Livingstone, 1985). Studies have shown also that animal(s) could not just be a filter for the biologically active drugs. Some drugs are converted in the body to much more toxic metabolites. Because of toxicological concern for all drug-related residues, for regulatory control therefore, tolerance limits for animal drugs are set based on total residues; i.e. parent drug and metabolites.

The official analytical method for antibiotic assay is still the microbiological method. Though sensitive, the test lacks specificity. In Canada for example, a sensitivity limit of 0.0125 IU/ml (ie between 0.012 to 0.014µg of procaine penicillin per mL of milk) is set for assaying penicillin content in the milk, using Sarcina lutea piate test. Ten percent of Canadians were estimated to be sensitive to penicillin, and all Canadians consume a dairy product each day (Mitchell, 1984). In Canada, there is zero tolerance for drugs in milk (Campbell, 1978). Some chemical, or chromatographic methods have been widely used however, a number

had also been recommended for adoption to compendial status (Mitscher, 1978). Drug extraction from the biologic matrix is also important. Analytical method for animal drug is considered acceptable for this purpose, if the averages of the observed recoveries from fortified samples are 80 - 110% of the added marker residue at levels equal to or greater than 0.1ppm (Livingstone,1985).

As a regulatory provision, the withdrawal times recommended for various brand-name Oxytetracycline preparations for chicken are as follow (USFDA BVMM 28, 1982):-

"Terramix - 10 medicated premix" ..... 1 day

"Terramycin injectable solution" ..... 5 days

"OTC & Robenz premix" ..... 5 days

Booth (1986b) also reported that if oxytetracycline is used at 50 - 200g/900kg of feed for chicken, zero day withdrawal time was recommended, and the tolerance for OTC residues in the kidney was set at 3µg/g, and 1µg/g for muscle, liver and fat. Even though this drug preparation (TPF) is sold directly to the laities and professionals alike in animal health and livestock production in Nigeria, it was observed that there was no instruction on the immediate pack about withdrawal time.

5.4 CONCLUSION:

Drug residue studies in biological tissues is undoubtedly a tidious task. That probably may be the reason for the scarcity of data on research works in this field. Even in countries where some studies have been carried out, very little was done on poultry. In general world-wide, the number of monitoring and surveillance tests carried out had been paltry compared to the number of food animals slaughtered for consumption.

In USA for example, just 0.2 million monitoring and surveillance tests are conducted each year. Whereas in 1979, over 120 million livestock and 3.5 billion poultry were slaughtered (Booth, 1986b). This difference would be higher if imported meat and poultry are included. This means that more analytical work has to be done to improve coverage. In Nigeria, even in officially designated slaughter slabs or meat plants, drug residue monitoring is non-existent (Diseases of Animal Act 1954, Nigerian Livestock and Meat Authority Act 1971, Food and Drugs Decree 1974, Animal Diseases (Control) Decree 1988). The Guardian Newspaper (Orere, 1984) carried an investigational report on the use of hormonal preparation (Sulgonan: a combination of serum gonadotrophin and chorionic gonadotrophin. Intervet International, Holland) on privately owned farms to increase fecundity of swines. Thats' a food for thought.

From the results of this research work however, it can be inferred that antibiotic medication of the local chicken

(Gallus domesticus L.) can be justified in normal agricultural practice. If percent weight-gain was 49.8% for example during 3 weeks of feeding trial (Table 1), that means that 49.8% of the final life weight at that trial was gained during the drug feeding interval. This could be compared to the unmedicated (Control) animals that gained 14.3% (average of two readings) at the same time interval. But the question of drug residue in animal-derived foods only then need be rightly and adequately addressed by the drug manufacturers, food animal producers/clinicians, drug regulatory authorities, and the consumers. The reality today however is that animal drugs are absolutely essential for the production of an inexpensive and plentiful food supply for local consumption and possibly for export. Poultry and poultry products constitute a very significant proportion of animal protein source for Nigerians.

A large number are also kept by the traditional farmers. Agricultural Extension efforts can guide antibiotic medication in the chick based on good agricultural practice, and to include the up-grading steps recommended for the local chicken by the AERLS. This should be a practicable management appraoch in improving the productivity of the chicken. Other research finding also attested to the quality of laboratory-processed local chicken's egg yolk to compare very well with its commercial (imported?) counterpart in the functional properties like: colour, viscosity, coagulation, emulsification, foam stability, etc which are often exploited for

producing industrially processed food (Bamidele et al., 1990). That might be an asset to local sourcing of industrial food raw materials in this country.

Among the eight principal elements of Primary Health Care agreed on at Alma-Ata in 1978 for achieving the goals of "Health for All by the Year 2000", the followings were however included:-

(i) Provision/Supply of Food and Proper Nutrition

(ii) Proper provision of essential drugs..., etc.

These go to recommend the need for effective control of veterinary use of drugs in food-producing animals in this country, by instituting field monitoring, laboratory analysis, and regulatory guidelines, to ensure that the usage will not pose a public health hazard.

BIBLIOGRAPHY

Addison, E.; Clark, R.G. (1963): The Determination of Epitetracycline and Tetracycline By Ion-Exchange Paper Chromatography and its Application fo Human Urine and Serum. J. of Phar., and Pharmacol. Vol. XV. PP. 268 - 272.

Agricultural Extension Research Liaison Services (1982): The Performance of Local Chickens. In: Extension Guide No. 79, Poultry Series No. 8. Ahmadu Bello University, Zaria, Nigeria. Pp. 7.

Allen, E.H. (1985): Review of Chromatographic Methods for Chloramphenicol Residues in Milk, Eggs and Tissues. JAOAC 68 (5). PP.990-999.

Alma Ata (1978): Health for all by the year 2,000. In: Addison, B.K. (1988, Dir. Food & Drug Admin. on behalf of Fed. Minister of Health). Text of the Keynote Address by the Honourable Minister of Health, Prof. O. Ransome-Kuti at the 10th Annual Conference of the Society of Public Analysts of Nigeria, Kaduna PP. 4.

Amin, M.; Kazemi, R.; Bondari, K.; Yazdani, C. (1977): Effects of Various levels of Clortetracycline and Oxytetracycline on broiler performance and tissue residue. Archiv fur Geflugelkunde 41 (5). PP. 221-4.

Amuda-Giwa, S.O. (1984): *Veterinary Drugs Control and Livestock Production.* The Book of Abstracts: Nigerian Veterinary Medical Association Annual Conference, Ilorin. PP. 35 - 36 Nov.,1984.

Amuda-Giwa, S.O. (1985): Staphylococcosis, Staphylococcal Food-borne in-toxication, and other food-borne infections and intoxications. Paper Presented for the Course VPH-603: Zoonoses: at the Dept. of Vet. Public Health & Preventive Medicine, Ahmadu Bello University, Zaria. PP. 50-51, 12th December.

Amuda-Giwa, S.O. (1987): Study of Total Aerobic Counts of Some Soft Drinks *and a Comparative Analysis of Palm Wine.* The Book of Abstracts and Programme: Nigerian Veterinary Medical Association 24th Annual Conference and Silver Jubilee Celebrations Lagos. PP.17 Nov. 1987.

Anadeon, A.; Martinez-Larranaga, M.R.; Diaz, M.J. (1985): Pharmacokinetics of Tetracycline in chickens after intravenous administration. Poultry Sci. 64 (12). PP. 2273-9.

Animal Diseases (Control) Decree (1988): Federal Republic of Nigeria Official Gazette No.13,Vol.75. The Federal Government Press, Lagos. PP. A477-A501.

Ascione, P.P.; Zagar,I.B.; Chrekian,G.P.(1967): Tetracycline I: Separation and Examination by Thin-Layer Chromatography. J. Pharm. Sci. 56 (II) PP. 1393-95.

Ashworth, R.B. (1985): Liquid chromatographic Assay of Tetracyclines in Tissues of Food-Producing Animals. J A O A C (68) 5. Pp. 1013 - 18.

Baggot, J.D. (1977): Principle of Drug Disposition in Domestic Animals. The Basis of Veterinary Clinical Pharmacology. W.B. Saunders Co. Philadelphia. PP. 1 - 238 .

Bamidele, E.A. (1990): The functional potentials of dried egg Yolk from indigenous breed of the domestic fowl. In: Book of Abstracts: Nigerian Institute of Food Science and Technology 14th Annual Conference. P. 15. Oct. 1990 Kaduna.

Bocker, R.; Warnke, L.; Estler, C.J. (1984): Blood and Organ Concentrations of Tetracycline and Doxycycline in Female Mice: Comparison to Males. Arzneim.-Forsch/Drug Res. 34 (1) N.r.4. PP. 445 - 8.

Booth,N.H. (1986a) Tetracyclines. In: Booth, N.H. and McDonald, L.E. (Eds.) 1982 Veterinary Pharmacology and Therapeutics. 5th Ed The Iowa University Ames, Iowa. P. 740.

Booth, N.H. (1986b): Drugs and Chemicals Residues in Edible Tissues of Animals. In: Booth, N.H.; McDonald, L.E. (Eds) (1982). Veterinary Pharmacology and Therapeutics 5th Ed. The Iowa State University Press, Ames, Iowa. P. 1065 - 1113.

Brander, G.C.; Pugh, D.M. (1971). Veterinary Applied Pharmacology and Therapeutics 2nd Ed. Bailliere Tindall, Lond. P. 3 - 485.

B. Vet. C (1953). Oxytetracycline. In: British Veterinary Codex. The Pharmaceutical Press, London. P. 250.

Campbell, D.J. (1978): Drug Residues in Animal Tissues and their Regulatory Significance - The Canadian Point of View. JAOAC 6 (5). P. 1194 - 7.

Clarke, E.G.C. (1974): Oxytetracycline. Isolation and Identification of Drugs in Pharmaceutical Body Fluids and Post-mortem Materials. The Pharmaceutical Press, London. P. 1 - 163.

Clarke, E.G.C.; Clarke, M.L. (1967): Garner's Veterinary Toxicology 3rd Ed. The Williams and Wilkins Co. Baltimore.

Davey, L.A.; Ferber, N.T.; Kaye, E. (1985): Comparison of the Serum Pharmacokinetics of a Long acting and a conventional Oxytetracycline injection Vet. Rec. 177 (17) P. 426-9.

Davis, R. C.; Wood, P.; Mendelsoln, M,L. (1977): In: Cancer Res 37 (12) P. 4539-45.

Diseases of Animal Act (1954): Laws of the Federation of Nigeria and Lagos Vol. II Cap 54 (1958) P. 1101-1105.

Douglas, A.C. (1963): Tetracyclines Deposition in Nails. Lancet, ii/1963, 283. In: Todd, R.G. (Ed.) (1967). Extra Pharmacopeia-MARTINDALE 25th Ed. The Pharmaceutical Press, Lond. P.995-1027.

Douglas and Sturkie (1964): Titer Levels of Vasotocin during oviposition. In: Swenson, M.J. (1970). Dukes Physiology of Domestic Animals 8th Ed. Comstock Publishing Associates, Cornell University Press, Ithaca PP. 1341.

Duggar, B.M. (1948). Aureomycin: A product of the continuing search for new antibiotics. Ann. N.Y. Acad. Sci. 51: 177-181.

Eagle, Harry, Fleishman., Ralph and Levy, Mina (1952): Development of Increased Bacteriological Resistance to Antibiotics. Continuous Spectrum of Resistance to Penirillin, Chloramphenicol and Streptomycin. Journal of Bacteriology 63 pp. 623

Ehrlich, P.(1854-1915): Immunotherapy. In: Wilson, G.S.; Miles, M.A. (Eds). (1948).Topley and Wilsons Principles of Bacteriology and Immunity. 3rd Ed. Edward Arnold & Co. Lond. P.238-9.

FAO (1966): Tola, A.; Laolu, A. (1983): Nutrition and Food Policy in Nigeria. Nat. Inst. Policy & Strategic Studies, Kuru, Nig.

FAO/WHO Expert Committee on Food Additives (1969): WHO Tech. Rep. Ser. 430.

FAO (1984): Safety Evaluation of Veterinary Drug Residues. In: Residues of Veterinary Drugs in Foods, Report of a Joint FAO/WHO Expert Consultation, No. 32. FAO, Rome. PP. 9-10. 29th Oct.- 5th Nov.

Federal Office of Statistics (1983): Statistical News: No.17. 28th June

Fellers, F. X.; Lindguist, R. (1964): Oxytetracycline Degradation. Fed. Proc. 23. PP. 575.

Food and Drugs Decree (1974): Federal Republic of Nigeria Official Gazette No. 55 Vol. 61. The Federal Government Press, Lagos PP. A191 - A207.

Franklin, T.J.; Snow, G.A. (1971): The Biochemistry of Antimicrobial Activity. Academic Press, New York. P. 99 ff.

Globus, R.K.; Bikle, D.D.; Morey-Holton,E. (1986): The temporal responses of bone to unloading. In: Endocrinology. 118 (2). PP. 733-42.

Goldberg, H.S. (1959): Antibiotics Thier Chemistry and Non-Medical Uses. D. Van Nostrand Co. Inc. Princeton N.J. PP. 1-594.

Griffiths, B.W. (1966): Separation and Analysis of Degradation Products of Tetracycline by Gel Filteration on Sephadex G-25. J. Pharm. Sci. 55(3). PP. 353-5.

Hamann, J.; Heeschen, W.; Tolle, A. (1979): Hilchwissenschaft 34: 357. In: Petz, M. (1984).

Harrison, J.E.; Hitchman, A. J.; Hitchman, A.; Hasany, S.A.; McNeil, K.G. (1980): Differences Between the effects of phosphate deficiency and Vitamin D Deficiency on bone metabolism. In: Metabolism 29(2). PP. 1225-33.

Idusogie (1971): In: Tola, A.; Laolu, A. (1983): Nutrition and Food Policy in Nigeria. NIPSS, Kuru, Nigeria.

IZmailov, N.A.; Shraiber, M.S. (1938): Thin Layer Chromatography. In: Farmatsiya (Sofia). No.3, 1.

Kane; J. H.; Finlay, A.C; Sobein, B.A. (1949): Science, 109, P.199.

Katz, S.E.; Fassbender, C.A.; Dorfman, D.; Dowling,(Jr.) J.J. (1972): Chlortetracycline in Broiler Tissue and Organs. JAOAC 55 (1). P. 134-8.

Katz., S.E.; Fassbender, C.A. (1973): Fluorimetric Determination of Oxytetracycline in Premixes. JAOAC 56 (1). P. 17-19.

Katz, S.E.; Fassbender, C.A.; Dowling,(Jr.) J.J. (1973): Oxytetracycline Residues in Tissue, Organs, and Eggs of Poultry Fed Supplemented Rations. JAOAC 56 (1). PP. 77 - 81.

Kerr, E.E. (1972): Bull. Assoc. Food Drug Off. 36. PP. 186.

Kirchner, J.G.; Miller, J.M. (1952) Equipment for TLC. In: Eng. Chem. 44, 318.

Kuzel, N.R.; Kavanagh, F.W. (1971): J. Pharm. Sci. 60 PP. 767 - 773.

Kwaga, J.K.P.; Adesiyun, A.A. (1984): Antibiograms of Staphylococcus aureus isolates from some ready-to-eat products. Journal of Food Protection Vol. 47 PP. 865-7.

Lawrie, R.A. (1975): Meat Science 2nd Ed. Pergamon Press Oxford PP. 324-6.

Livestock Feeds Ltd., Ikeja (1989): Poultry Prices Soar. In: Agoye, D. Vetpractice PP. 8 & 12, Sept. - Oct.

Livingston, R.C. (1985): Antibiotic Residues in Animal-Derived Food. JAOAC 68 (5). PP. 966-7.

Longenecker, J.B. (1963) In: Rao, K.K.P.N. (1976): Food Consumption and Planning Vol. 5. Pergamon Press New York. PP. 155.

Machery-Nagel & Co. (Ed. 74): Thin Layer,Gas,Paper,Column Chromatography. Machery-Nagel & Co. Germany. PP. 1 - 33.

McCoy, RE (1982): In: Rev Infect Dis. 4 Suppl: S 157 - 61

Mercer, H.D.; Heath, G.E.; Long, P.E.; Showalter, D.H.; Powers, T.E. (1978a). Drug residues in food animals I. Plasma and tissue Kinetics of Chloramphenicol in young Cross-bred swine. J. Vet. Pharmacol. Therap. 1. PP. 19 - 36.

Mercer, H.D.; Teske, R.H.; Long, P.E.; Showalter, D.H. (1978b). Drug residue in food animals II. Plasma and tissue Kinetics of Oxytetracycline in young cross-bred swine. J.Vet. Pharmacol. Therap. 1. 119-128.

Miller, B.L.; Wyatt, R.D. (1985): Effect of Dietary aflatoxin on the uptake and elimination of Chlortetracycline in broiler chicken Poult. Sci. 64(9) PP. 1637-43.

Mitchell, G.A. (1984): Benefits of Food Additives. Proceedings of 2nd International Consultation on Veterinary Products Registration Oslo Norway 18 - 22 June.

Mitscher, L.A. (1978): The Chemistry of the Tetracycline Antibiotics. Medicinal Research Series Vol.9. Mercel Dekker Inc. New York. PP. 1 - 139.

Monastero , F.; Means, J.A.; Grenfell, T.C.; Hedger, F.H. (1951): Terramycin: Chemical Methods of Assay and Identification J. Amer. Pharmct. Ass. Vol. XL, No.5, PP. 241 - 145.

Mottier, M.; Potterat, M. (1952): Thin Layer Chromatography. Ind. Eng. Chem. 43, 118(1952).

Najako (1962): Effect of Vasotocin on uterine contraction. In: Swenson, M.J. (1970) Dukes Physiology of Domestic Animals 8th Ed. Comstock Publishing Associates. Cornell University Press, Ithaca PP. 1341.

Nesheim, M.C.; Austic, R.E.; Card, L.E. (Eds) (1979): Poultry Production 12th Ed. Lea & Febiger Philadelphia. P. 1 - 20,

Nigeria Livestock and Meat Authority Decree (1971): Laws of the Federal Republic of Nigeria. Federal Ministry of Information, Printing Division, Lagos (1972). PP. A185 - A195.

Nillson - Ehle, I.; Yoshikawa, T.T.; Schotz, M.C.; Guze, L.B. (1976): Quantitation of Antibiotics Using High-Pressure liquid Chromatography: Tetracycline. Antimicrobial Agents and Chemotherapy. 9 (5). PP. 754 - 60.

Okon, E.E. (1975): Biology of the Domestic Fowl Ethiope Publishing House, Benin City, Nigeria P. 47 - 65.

Olurinola, O.F. (1988): Personal Communication. Department of Pharmaceutics (Head), Faculty of Pharmaceutical Sciences (Dean). Ahmadu Bello University, Zaria, March 1989 .

Orere, O. (1984): Experts Warn Against Eating Drug Induced Animals. The Guardian, Vol.2, No. 509, Nov.8.

Pascual, C.R. (1976): In: Rao, K.P.N. (1976): Food Consumption and Planning Vol.5. Pergamon Press New York. PP. 155 - 219.

Petz, M. (1984). Chemische Analyse von Tierarzneimittelruckstanden in Lebensmitteln:
1 Mitteilung: Allegemeine Methodik and gaschromatographische Verfahren
Chemical Residue Analysis of Veterinary drugs in Food: Part 1. General Methodology and Gas Chromatographic Procedure:
In: Z Lebensm Unters forsch/Ubersichtsbericht/Review)180: 267-79

Pollet, R.A.; Glatz, C.E.; Dyer, D.C. (1985): The Pharmacokinetic of Chlortetracycline orally administered to turkeys, influence of citric acid and Pasteurella multocida infection, J. Pharmacokinet. Biopharm. 13(3). PP. 243 - 64.

Powis, G. (1974): A study of the interaction of tetracycline with human serum lipoproteins and albumin. J. Pharm. Pharmacology 26. PP. 113 - 8.

Radeleff, R.D. (1970). Residues of Chemicals in Foods of Animal Origin. Veterinary Toxicology 2nd Ed. Lea & Febiger, Philadelphia PP. 302.

Radley, D.E.; Brown, C.C.D.; Cunningham, M.P.; Kimber, C.D.; Misisi, F.L.; Payne, R.C.; Furnell, R.E.; Stagg, S.M.; Young, A.S. (1980): East Coast Fever: Chemoprophylactic Immunization of cattle using Oxytetracycline and a combination of theleiria strains. In: F.A.O. Animal Production and Health Paper No.19. F.A.O. Rome, PP. 709 - 718.

Ragheb, H.S.; Cunnings, A.M.; Browning, B.M. (1973): Microbiological Assay of Oxytetracycline in Food: Manual Turbidimetric Assay and Plate Assay. JAOAC 56(1) PP. 23-30.

Runge, F.F. (1795-1867): Runge Pictures. In: Machery-Nagel & Co Ed. 74.

Sadee, W.; Beelen, D.C.M. (1980). Drug Level Monitoring: Analytical Techniques, Metabolism and Pharmacokinetics John Wiley & Sons New York. PP. 1 - 42.

Sanusi, A.; Aliu, Y.O. (1986): Field Observations on Adult Zebu Bulls Immunized Against Anaplasmosis by Infection and Treatment Plus Integrated Tick Control. In: Trop. Anim. Hlth. Prod. No.18. PP. 13 - 20.

Schifferli, D.; Gallazzi, R.L.; Nicolet, J.; Wanner, M. (1982): Pharmacokinetics of Oxytetracycline and Therapeutic Implications in veal calves J. Vet. Pharmacol. Therap. 5(4). PP. 247-257.

Schoental, R.(1965): Toxicology of Natural Products. Far East Med. J.l. PP. 291 - 296. In: Nwude, N. (1976): Toxicological Investigation of Lasiosiphon Kraussianus (Meisn). Unpublished Ph.D. Thesis. Ahmadu Bello University, Zaria, Nigeria.

Sharma, J.P.; Koritz, G.D.; Perkins, E.C.; Bevill, R.F. (1977a): High Pressure Liquid Chromatographic Determination of Tetracyclines in Urine. J. Pharm. Sci. 66(a). PP. 1319-22.

Sharma, J.P.; Perkins, I.C.; Bevill, R.F. (1977b): Reverse-Phase High-Performance Liquid Chromatographic Determination of Tetracyclines in Urine and Plasma. J.Chromatog. 134. PP. 441 - 50.

Siegel. B.B. (1959): Bull WHO. 21. PP. 703.

Siegmund, O.H. Ed. (1973): The Merck Veterinary Manual 4th Ed. Merck & Co., Inc. N.J. U.S.A. PP. 1-1600.

Skeeles, J.K.; Swafford, W.S.; Wages, D.P.; Hellwig, H.M.; Slavik, M.F.; Houghten, G.E.; Crawford, D.L. (1985): Studies on the use of a long-acting Oxytetracycline in Turkeys: Serum Levels and tissue residues following injection. Avian. Dis. 29 (1). PP. 145-9.

Stahl, E. (1964): New Apparatus for Preparative thin layer Chromatography In: Thin Layer Chromatography: Reprints from "Laboratory Practice" (Stahl,E. Proifle, J. (1964). Z Analyt. Chem 200,377 United Trade Press Ltd., London. PP. 3 - 7.

Stahl, E.; Schroter, G.; Kraft, G.; Renz, R. (1956) Pharmazie. 11, 633.

Steiger, M.; Reichstein, T.R. (1938): Liquid Chromatogram. Helv Chim Acta 21. P.546. In: Lederer,E.; Lederer,M. (1955). Chromatography: A Review of Principles and Applications Elsevier Publishing Co. Lond. PP. XVIII & 30-33.

Swenson, M.J. (1970): Dukes Physiology of Domestic Animals 8th Ed. Comstock Publishing Associates. Cornell University Press, Ithaca PP. 348.

Teske, R.J.; Rollins, L.P.; Candon, R.J.; Cater, G.G. (1973): Serum Oxytetracycline Concentrations after Intravenous and Intramuscular administration in horses. J.A.V.M.A. 162 PP. 119 - 20.

Todd, R.G. (Ed) (1967): Oxytetracycline. Extra Pharmacopoeia MARTINDALE. 25th. Ed. The Pharmaceutical Press, Lond. PP. 995-1027.

USFDA (1971, 1972). In: Mitscher, L.A. (1978). The Chemistry of Tetracycline Antibiotics.

USFDA. BVM (1982): Drug Use Guide: Poultry. Bureau of Veterinary Medicine MEMO. BVMM-29 H.H.S. Pub.No (FDA) 82-6030. U.S. Department of Health and Human Services. Public Health Service, Food and Drug Administration. PP. 4 - 6.

USP XX (1980): Oxytetracycline. United States Pharmacopoea XX. The U.S. Pharmacopoeial Convention Inc. Rockville, Md. PP. 577-8

Wallmam, I.S.; Hilton, H.B. (1962): Tetracyclines deposition in teeth. Lancet, ii/1962, 770. In: Todd, R.G. (Ed) (1967) Extra Pharmacopoeia-MARTINDALE. 25th Ed. The Parmaceutical Press Lond. 995 - 1027.

WHO (1974). Bioavalability of Drugs, Principles and Problems WHO Technical Report Series. No. 536. Geneva.

Windholz, M.; Budavari, S.; Stroumtsos, L.Y.; Fertig, M.N. Editors(1976). The Merk Index. An Encyclopedia of Chemicals and Drugs 9th Ed. Merck & Co., Inc. N.J. U.S.A. P.6790.

Wong, KM., Zika, J. Klein, L. (1983). In: Calcif Tissue Int.35 (4-5) PP. 562-5.

Xia,W.J.; Gyrd-Hansen, N.; Nielsin,P. (1983): Comparison of Pharmacokinetic Parameters for two oxytetracycline preparations in pigs. J. Vet., Pharmacol. Ther. 6 (2). PP. 112-9

Yoshida, M.; Hoshii, H.; Yonezawa, S.; Nogawa, H.; Yoshimura,H.; Ito, D. (1975): Residue and disappearance of dietary oxytetracycline in the blood, muscle, liver and bile of growing chicks. Japanese Poultry Science 12 (4). PP. 181 - 187.

Ziv,C.; Sulman,F.G.(1974): Analysis of Pharmacokinetic Properties of nine tetracycline analogues in dairy cows and ewes. Amer. J.Vet.Res.35. PP. 1197 - 1201.

APPENDIX 1: ABSORBANCE READINGS OF WORKING SOLUTIONS A–D

S/N	OTC Concentration(mg/ml	Absorbance
Solutions A		
1	1·0	0·065
2	2·0	0·125
3	3·0	0·435
4	4·0	1·920
5	5·0	1·930
6	6·0	1·940
7	7·0	1·940
8	8·0	1·950
9	9·0	1·960
10	10·0	1·960
Solutions B		
1	0.001	0.030
2	0.010	0.315
3	0.050	0.360
4	0.100	0.385
5	0.200	0.585
6	0.400	1.940
7	0.600	1.940
8	1.000	1.940
9	2.000	1.950
10	5.000	1.960

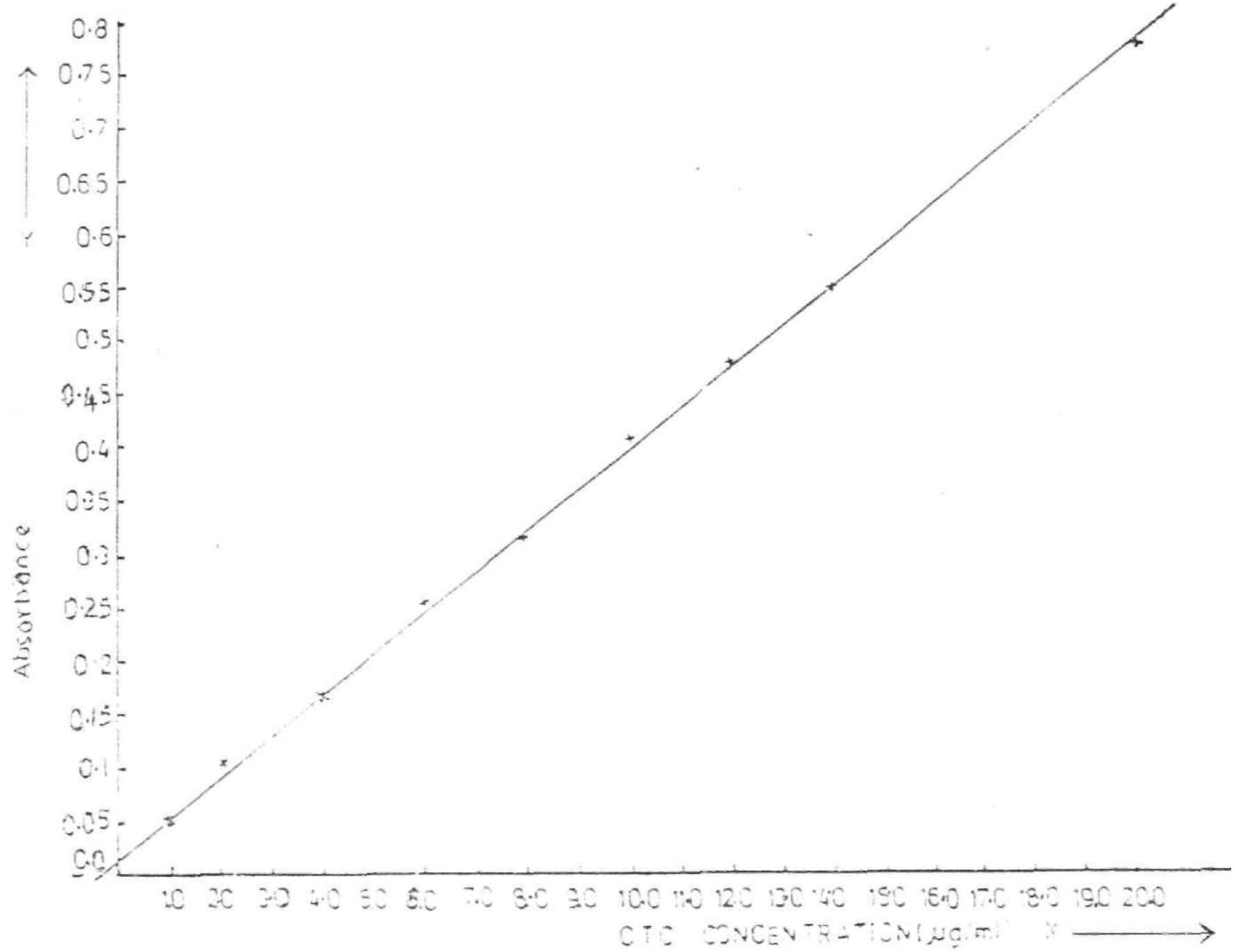
S/N	OTC Concentration(mg/ml)	Absorbance
Solutions C		
1.	0.00005	0.005
2	0.00010	0.012
3	0.00025	0.025
4	0.00050	0 044
5	0,00100	0.050
6	0.00200	0.105
7	0.00400	0.170
8	0.00600	0·255
9	0·00800	0·315
10	0·01000	0·410
11	0·01200	0.480
12	0·01400	0·550
13	0·02000	0·780
Solutions D		
1	10^{-8}	0.030
2	10^{-7}	0.070
3	10^{-6}	0.095
4	10^{-5}	0.140
5	10^{-4}	0.180
6	10^{-3}	0·225
7	10^{-2}	0·265
8	10^{-1}	0·360

APPENDIX 2: ABSORBANCE READINGS OF OXYTETRACYCLINE STANDARD SOLUTIONS: USED FOR PLOTTING THE CALIBRATION CURVE

S/N	OTC CONCENTRATION (mg/ml)	ABSORBANCE READING
1	0.00005	0.005
2	0.00010	0.012
3	0.00025	0.025
4	0.00050	0.044
5	0.00100	0.050
6	0.00200	0.105
7	0.00400	0.170
8	0.00600	0.255
9	0.00800	0.315
10	0.01000	0.410
11	0.01200	0.480
12	0.01400	0.550
13	0.02000	0.780

APPENDIX 3: LEAST SQUARE REGRESSION ANALYSIS OF UV-VISIBLE SPECTROPHOTOMETRY'S DATA OF OXYTETRACYCLINE STANDARDS.

S/N	x (μg/ml)	y	x^2	y^2	xy	$\bar{y}=\alpha+Bx$	Residual
1	0·05	0·005	0·0025	0·000025	0·00025	0·0102278	- 0·0052278
2	0·10	0·012	0·0100	0·000144	0·00120	0·0122136	- 0·0002136
3	0·25	0·025	0·0625	0·000625	0·00625	0·0181710	0·0068290
4	0·50	0·044	0·2500	0·001936	0·02200	0·0280999	0·0159001
5	1·00	0·050	1·0000	0·002500	0·05000	0·0479578	0·0020422
6	2·00	0·105	4·0000	0·011025	0·21000	0·0876735	0·0173265
7	4·00	0·170	16·0000	0·028900	0·68000	0·1671049	0·0028951
8	6·00	0·255	36·0000	0·065025	1·53000	0·2465363	0·0084637
9	8·00	0·315	64·0000	0·099225	2·52000	0·3259677	- 0·0109677
10	10·00	0·410	100·0000	0·168100	4·10000	0·4053991	0·0046009
11	12·00	0·480	144·0000	0·230400	5·76000	0·4848305	- 0·0048305
12	14·00	0·550	196·0000	0·302500	7·70000	0·5642619	- 0·0142619
13	20·00	0·780	400·0000	0·608400	15·60000	0·8025561	- 0·0225561
Total	77·90	3·201	961·3250	1·518805	38·17970	3·2010001	- 0·0000001



APPENDIX 4 OXYTETRACYCLINE CALIBRATION CURVE DERIVED BY LEAST SQUARE REGRESSION ANALYSIS.

APPENDIX 5: QUANTITIES OF OXYTETRACYCLINE (OTC) RESIDUES IN THE BROILER-TYPE TISSUES AT VARIOUS FEEDING DURATIONS: EVALUATED FROM THE REGRESSION ANALYSIS:

OTC LEVEL IN FEED (g/ton)	AVERAGE OF TWO SAMPLES' ABSORBANCE READINGS						EQUIVALENT OTC QUANTITY (µg/g)					
	BLOOD	FAT	(c) INT. CONT.	KIDNEY	LIVER	MUSCLE	BLOOD	FAT	(c) INT. CONT.	KIDNEY	LIVER	MUSCLE
	3 WEEKS FEEDING DURATION											
300	0.013	(b)	0.455	0.152	0.058	0.026	0.26		22.50	3.00	1.15	0.48
200	0.015	–	0.375	0.140	0.056	0.024	0.15	–	18.52	3.36	1.20	0.38
150	0.010	–	0.495	0.105	0.049	0.012	0.06	–	12.35	2.46	0.85	0.08
100	0.006	–	0.411	0.060	0.027	0.011	0.01	–	10.15	1.33	0.40	0.06
50	(a) NR	–	0.174	0.028	0.014	0.011	NR	–	4.16	0.55	0.15	0.06
	6 WEEKS FEEDING DURATION											
300	0.050	0.100	0.475	0.165	0.112	0.078	1.06	2.35	23.60	4.00	2.65	1.70
200	0.046	–	0.490	0.150	0.104	0.070	0.96	–	24.40	3.60	2.43	1.65
150	0.030	0.058	0.513	0.112	0.086	0.066	0.58	1.26	12.75	2.63	1.98	1.36
100	0.022	0.051	0.432	0.072	0.055	0.042	0.30	1.33	10.76	1.63	1.20	0.83
50	0.010	0.015	0.252	0.068	0.032	0.034	0.06	0.16	6.22	1.36	0.63	0.62

APPENDIX 5 Contd.

OTC LEVEL IN FEED (g/ton)	AVERAGE OF TWO SAMPLES ABSORBANCE READINGS						EQUIVALENT OTC QUANTITY (µg/gm)					
	BLOOD	FAT	INT. CONT. (c)	KIDNEY	LIVER	MUSCLE	BLOOD	FAT (b)	INT. CONT. (c)	KIDNEY	LIVER	MUSCLE
9 WEEKS FEEDING DURATION												
300	0·046	0·116	0·435	0·163	0·090	0·062	0·96	2·70	21·50	3·92	2·10	1·33
200	0·044	0·114	0·427	0·141	0·088	0·054	0·93	2·68	21·10	3·38	1·92	1·14
150	0·025	0·056	0·506	0·109	0·078	0·055	0·43	1·21	12·60	2·52	1·65	1·20
100	0·015	0·055	0·425	0·064	0·054	0·030	0·15	1·21	10·50	1·38	1·18	0·56
50	0·008	0·030	0·146	0·036	0·032	0·029	0·02	0·56	3·50	0·76	0·63	0·60
12 WEEKS FEEDING DURATION												
300	0·046	0·116	0·436	0·166	0·092	0·060	0·96	2·70	21·60	3·95	2·12	1·30
200	0·042	0·116	0·426	0·141	0·088	0·055	0·85	2·70	21·00	3·39	1·92	1·20
150	0·026	0·058	0·508	0·105	0·079	0·055	0·43	1·30	12·68	2·46	1·68	1·20
100	0·017	0·051	0·426	0·064	0·055	0·032	0·26	1·08	10·53	1·38	1·20	0·62
50	0·008	0·032	0·145	0·030	0·021	0·030	0·02	0·60	3·46	0·60	0·30	0·60

Keys

(a) NR ie No Spectrophotometer reading. Zero drug quantity.

(b) - ie No tangible fat deposit found at the period of sacrifice.

(c) ie For intestinal content samples, the readings for feeding levels 300, and 200g/ton at all sacrifice intervals represent the absorbance of the solvent diluted tissue extracts (12.0 instead of 6.0ml). The OTC quantity is the doubled equivalent OTC value.

APPENDIX 6: CHROMATOGRAPHY OF BROILER TYPE TISSUES AND OXYTETRACYCLINE STANDARDS. 3 WEEKS FEEDING DURATION

OTC LEVEL IN FEED (g/ton)	SAMPLE	(h) Sample size	Spot Separation (i)	UV Reaction (j)	Solvent front	Spot's Distance	Spot's Area	R_f value	VA	$\log_{10} W$
300	Blood	10	-	-	12.0	-	-	-	-	-
	Muscle	10	-	-	12.0	-	-	-	-	-
	Kidney	10	-	-	12.0	-	-	-	-	-
	Liver	10	-	-	12.0	-	-	-	-	-
	Fat	10	-	-	12.0	-	-	-	-	-
	Int. cont	10	-	-	12.0	-	-	-	-	-
	OTC Std I	5	-	+	12.0	-	0.00	-	0.00	4.699
	" " II	5	+	+	12.0	0.96	0.015	0.08	0.122	3.699
	" " III	5	+	+	12.0	0.96	1.400	0.08	1.184	2.699
200	Blood	10	-	-	11.8	-	-	-	-	-
	Muscle	10	-	-	11.8	-	-	-	-	-
	Kidney	10	-	-	11.8	-	-	-	-	-
	Liver	10	-	-	11.8	-	-	-	-	-
	Fat	10	-	-	11.8	-	-	-	-	-
	Int. Cont	10	-	-	11.8	-	-	-	-	-
	OTC Std I	5	-	+	11.8	-	0.00	-	0.00	4.699
	" " II	5	+	+	11.8	1.0	0.014	0.08	0.116	3.699
	" " III	5	+	+	11.8	1.1	1.360	0.08	1.166	2.699
150	Blood	10	-	-	12.5	-	-	-	-	
	Muscle	10	-	-	12.5	-	-	-	-	
	Kidney	10	-	-	12.5	-	-	-	-	
	Liver	10	-	-	12.5	-	-	-	-	
	Fat	10	-	-	12.5	-	-	-	-	
	Int. Cont	10	-	-	12.5	-	-	-	-	
	OTC Std I	5	-	+	12.5	-	0.00	-	0.00	4.699
	" " II	5	+	+	12.5	1.0	0.013	0.08	0.114	3.699
	" " III	5	+	+	12.5	1.1	1.320	0.08	1.149	2.699
100	Blood	10	-	-	12.0	-	-	-	-	-
	Muscle	10	-	-	12.0	-	-	-	-	-
	Kidney	10	-	-	12.0	-	-	-	-	-
	Liver	10	-	-	12.0	-	-	-	-	-
	Fat	10	-	-	12.0	-	-	-	-	-
	Int. Cont	10	-	-	12.0	-	-	-	-	-
	OTC Std I	5	-	+	12.0	-	0.00	-	0.00	4.699
	" " II	5	+	+	12.0	0.96	0.014	0.08	0.116	3.699
	" " III	5	+	+	12.0	0.97	1.36	0.08	1.166	2.699
50	Blood	10	-	-	12.0	-	-	-	-	-
	Muscle	10	-	-	12.0	-	-	-	-	-
	Kidney	10	-	-	12.0	-	-	-	-	-
	Liver	10	-	-	12.0	-	-	-	-	-
	Fat	10	-	-	12.0	-	-	-	-	-
	Int. Cont	10	-	-	12.0	-	-	-	-	-
	OTC Std I	5	-	+	12.0	-	0.00	-	0.00	4.699
	" " II	5	+	+	12.0	0.96	0.012	0.08	0.109	3.699
	" " III	5	+	+	12.0	0.96	1.20	0.08	1.09	2.699

OTC LEVEL IN FEED (g/ton)	SAMPLE	6 WEEKS FEEDING DURATION -do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
300	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0·00	–	0·00	4·699
	" " II	5	+	+	12·0	0·96	0·012	0·08	0·114	3·699
	" " III	5	+	+	12·0	0·96	1·32	0·08	1·149	2·699
200	Blood	10	–	–	13·2	–	–	–	–	–
	Muscle	10	–	–	13·2	–	–	–	–	–
	Kidney	10	–	–	13·2	–	–	–	–	–
	Liver	10	–	–	13·2	–	–	–	–	–
	Fat	10	–	–	13·2	–	–	–	–	–
	Int. Cont	10	–	–	13·2	–	–	–	–	–
	OTC. Std I	5	–	+	13·2	–	0·00	–	0·00	4·699
	" " II	5	+	+	13·2	1·1	0·014	0·08	1·118	3·699
	" " III	5	+	+	13·2	1·1	1·26	0·08	1·166	2·699
150	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC-Std I	5	–	+	12·0	–	0·00	–	0·00	4·699
	" " II	5	+	+	12·0	0·96	0·012	0·08	0·109	3·699
	" " III	5	+	+	12·0	0·96	1·20	0·08	1·09	2·699
100	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont.	10	–	–	12·0	–	–	–	–	–
	OTC-Std I	5	–	+	12·0	–	0·00	–	0·00	4·699
	" " II	5	+	+	12·0	0·96	0·012	0·08	0·109	3·699
	" " III	5	+	+	12·0	0·96	1·240	0·08	1·113	2·699
50	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC-Std I	5	–	+	12·0	–	0·00	–	0·00	4·699
	" " II	5	+	+	12·0	0·96	0·012	0·08	0·103	3·699
	" " III	5	+	+	12·0	0·96	1·20	0·08	1·09	2·699

APPENDIX 6: Cont'd. 9 WEEKS FEEDING DURATION

-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
300	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. cont	10	–	–	12·0	–	–	–	–	–
	OTC std I	5	–	+	12·0	–	0.00	–	0·00	4·699
	" " II	5	+	+	12·0	0.96	0·013	0.08	0·114	3·699
	" " III	5	+	+	12·0	0·96	1·32	0·08	1·149	2·699
200	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0·00	–	0.00	4·699
	" " II	5	+	+	12·0	0.96	0·013	0·08	0·114	3·699
	" " III	5	+	+	12·0	0.96	1·32	0·08	1·149	2·699
150	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0.00	–	0.00	4·699
	" " II	5	+	+	12·0	0·96	0.013	0.08	0.114	3·699
	" " III	5	+	+	12·0	0.96	1·28	0·08	1·131	2·699
100	Blood	10	–	–	12.0	–	–	–	–	
	Muscle	10	–	–	12·0	–	–	–	–	
	Kidney	10	–	–	12·0	–	–	–	–	
	Liver	10	–	–	12·0	–	–	–	–	
	Fat	10	–	–	12·0	–	–	–	–	
	Int. Cont	10	–	–	12·0	–	–	–	–	
	OTC Std I	5	–	+	12·0	–	0·00	–	0·00	4·699
	" " II	5	+	+	12·0	0·96	0·012	0·08	0·109	3·699
	" " III	5	+	+	12·0	0·96	1·24	0·08	1·113	2·699
50	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0.00	–	0.00	4·699
	" " II	5	+	+	12·0	0·96	0·012	0·08	0·109	3·699
	" " III	5	+	+	12·0	0·96	1·20	0·08	1·09	2·699

APPENDIX 6: Cont'd 12 WEEKS FEEDING DURATION

-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
300	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0.00	–	0.00	4.699
	" " II	5	+	+	12·0	1.0	0.014	0.08	0.118	3.699
	" " III	5	+	+	12·0	1.0	1.40	0.08	1.183	2.699
200	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0.00	–	0.00	4.699
	" " II	5	+	+	12·0	0·96	0·014	0.08	0.118	3.699
	" " III	5	+	+	12·0	0.96	1·36	0.08	1·166	2·699
150	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int, Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0.00	–	0.00	4.699
	" " II	5	+	+	12·0	0.96	0·014	0·08	0.118	3.699
	" " III	5	+	+	12·0	0·96	1·36	0·08	1·166	2·699
100	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0.00	–	0.00	4.699
	" " II	5	+	+	12·0	1.0	0.014	0.08	0.118	3.699
	" " III	5	+	+	12·0	1.0	1.40	0.08	1.183	2.699
50	Blood	10	–	–	12·0	–	–	–	–	–
	Muscle	10	–	–	12·0	–	–	–	–	–
	Kidney	10	–	–	12·0	–	–	–	–	–
	Liver	10	–	–	12·0	–	–	–	–	–
	Fat	10	–	–	12·0	–	–	–	–	–
	Int. Cont	10	–	–	12·0	–	–	–	–	–
	OTC Std I	5	–	+	12·0	–	0·00	–	0.00	4.699
	" " II	5	+	+	12·0	0·96	0·014	0·08	0.118	3.699
	" " III	5	+	+	12·0	0·96	1·36	0·08	1·166	2·699

Keys:- (h) ie Sample size W (mg): 10µL of tissue extract or 5µL of OTC std spotted. (OTC std: I - 0.1mg/ml, II - 1.0mg/ml, III - 10 mg/ml)

(i) ie (+) = Orange fluorescence or (-) = No orange fluorescence.

(j) ie All the tabulated data were averages of two samples readings. The solvent front, and the Spot's Area (A) were measured in centimetres (cm).