TOXICITY AND RESIDUE STUDY OF FLORFENICOL IN CHICKEN

T H E S I S

Submitted
in partial fulfillment of the requirements for the Degree of

M A S T E R OF V E T E R I N A R Y S C I E N C E

IN

VETERINARY PHARMACOLOGY AND TOXICOLOGY

BY

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Enrolment No : V/06/0234

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(INDIA)
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PGR-ANNEXURE-XIII

DECLARATION BY THE STUDENT

I hereby declare that the experimental Research work and interpretation of the thesis entitled, "**TOXICITY AND RESIDUE STUDY OF FLORFENICOL IN CHICKEN**" or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date : Signature

(Miss. ELIZABETH KIMTEI)

Enrolment No: V/06/0234

Counter signed by
Chairman, Advisory Committee
with date

PGR-ANNEXURE-XIV

DECLARATION OF ADVISORY COMMITTEE

Ku. ELIZABETH KIMTEI has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled, **"TOXICITY AND RESIDUE STUDY OF FLORFENICOL IN CHICKEN"** submitted by her is the result of research work is sufficient to warrant its presentation to the examination in the subject of Veterinary Pharmacology and Toxicology for the award of **MASTER OF VETERINARY SCIENCE** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part there of has not been previously submitted by her for a degree of any other University.

Place: Mumbai-12.
Date:

(Dr. M. M.GATNE)
Advisor/Guide
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PGR-ANNEXURE-XV

CERTIFICATE

This is to certify that the thesis entitled, **"TOXICITY AND RESIDUE STUDY OF FLORFENICOL IN CHICKEN"**, submitted by **MISS ELIZABETH KIMTEI** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **MASTER IN VETERINARY SCIENCE** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

Name and Signature of External Examiner	Signature with Seal Head of Department	Dr. M. M. GATNE Advisor/Guide Professor of Pharmacology and Toxicology, BVC

Advisory committee

Name	Designation	Signature
1. Dr. D.V. KESKAR	Professor and Head of Medicine and Pharmacology and Toxicology	
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'Coming together is a beginning; keeping together is a progress; working together is a success.'

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Date:

Place: Mumbai-12 *Dr. Elizabeth Kimtei*

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LIST OF ABBREVIATIONS

Abbreviation	Full form
b.i.d	Bis in die/twice daily
C_{max}	Maximum plasma concentration
EMEA	European Agency for the Evaluation of medicinal products
Fig.	Figure
g	Gram
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
i.e.	That is
Kg	kilogram
L	Litre
LC_{50}	Lethal concentration-50
LD_{50}	Lethal dose-50
LOD	Limit Of Detection
LOQ	Limit Of Quantification
μg	Microgram
mg	Milligram
MIC	Minimum Inhibitory Concentration
ml	Millilitre
mm	Millimeter
m-RNA	Messenger-ribonucleic acid
MRT	Mean Residence Time
ng	Nano-gram
nm	Nano-meter
NOEL	No Observed Effect Level
ppm	Parts Per Million
rpm	Revolutions Per Minute
RT	Retention Time
S.E	Standard Error
$t_{1/2}$	Elimination half-life
t-RNA	Transfer RNA

1 INTRODUCTION

Indian poultry industry has made a tremendous and remarkable progress, evolving from a small-scale backyard venture to the status of commercial, full fledged, self-sufficient and most progressive agro-based industry with the annual growth rate of 6-8 % per annum. The total broiler production of India is 2000 million/year, total poultry meat production is 1.92 million tonnes/year, number of layers is 215 million/year (Evans, 2007), the total egg production is 45 billion eggs, per capita availability of chicken meat is 1.76 kg and that of egg is 42 eggs. Indian poultry industry ranks 4th in egg production and 5th in broiler production (Bootwala, 2005), with contribution of 2% to GDP (Gross Domestic Product) and provides employment to 1.5 million people. Production of the quality assured eggs and the broiler meat has opened the global market opportunity for better economics of poultry farming.

The growth of poultry sector is sometimes hampered due to many diseases viz. bacterial, viral, fungal and protozoal. These diseases are caused by bacteria (Fowl cholera, Fowl typhoid etc.), virus (Fowl pox, Bird flu, New castle etc.), fungus (Aspergillosis) and protozoa (Coccidiosis). Viral diseases are taken care by vaccination, however to treat bacterial diseases, various antimicrobials are commonly used. Bywater (2005) stated that in poultry industry these antimicrobials are used for treatment, prophylaxis and growth promotion (their use as growth promotants however is not common in India). The success of antimicrobial therapy depends upon the maintenance of antimicrobial concentration at the site of infection resulting in bacteriostatic or bactericidal effect. Hence, it is desirable to know the distribution of antimicrobial agents at these sites (Radostits *et al.,* 2000).

The most common Pharmacokinetic parameter considered to assess the extent of distribution of drug is its apparent volume of distribution. However, volume of distribution may be deceptive many a times, especially when selective accumulation of drug occurs in particular tissue. The definitive information on the distribution pattern can be obtained only by measuring concentration of that drug in various organs and tissues (Prescott and Baggot, 1993). Unfortunately, scientists find it difficult and extremely expensive to determine concentration of

drug at target sites in large animals. Here lies the advantage of using poultry birds for a more realistic and practical approach to the problem observed in large animal study. Hence, by using poultry birds, measurement of the exact concentration of the antibacterial agent reaching the target site can be assessed which serves a true indicator of the therapeutic efficacy of the drug in infections involving the target tissue. Similarly, measuring the concentration in egg provides the assessment of use of an antimicrobial in controlling vertically transmitted diseases caused by sensitive organisms. On one side the estimation of drug levels in tissues dictates the therapeutic value of antimicrobial whereas on other hand it serves useful to predict the residue levels and persistence in edible animal tissues.

The use of drugs in food-producing animals is an accepted and well-established practice worldwide. The advantages of enhanced food production arising from pharmacologic intervention must be weighed against the attendant disadvantages of residue formation in edible tissues. The most important basis for regulatory authorization of drug use in animals is the establishment of drug safety. This requires adequate toxicological evaluation of residues in tissues and organs likely to be eaten by humans.

Antibiotic therapy is most commonly performed under veterinary supervision or against the authority of a veterinarian's prescription. Unacceptable antibacterial tissue residues do not occur in the human food supply, if the specified withdrawal time of the drug is rigidly observed. Antibiotics are administered to many animals for prolonged periods, during times of peak production or near to slaughter giving rise to the potential for residue formation in edible tissues and eggs. Emergency slaughter of antibiotic-treated animals is undoubtedly a significant residue hazard, and in such cases, tissue monitoring for drug residues is of utmost importance (Barragry, 1994).

The presence of xenobiotics, especially antibiotic residues in the foodstuffs of animal origin is one of the most important indexes for their safety. Protection of public health against possible harmful effects of veterinary drug residues is a very important problem. As per USA estimate, approximately 80% of all food-producing animals receive medication for part or most of their lives

which may result in the presence of residues in meat and offal (Pavlov *et al.,* 2008).

Antibiotic residues in food constitute a variety of health hazards to humans. These hazards depend on the frequency and degree of exposure. The most serious objections to the presence of antibiotics and their metabolites in food intended for human consumption arise as a consequence of human health hazards. Food of animal origin having residues of antibiotic produces the effect on consumer presumably as that of the equivalent dose of antibiotic given directly.The consumption of antibiotic residues by humans could produce harmful effects from direct toxicity or from allergic reactions to the antibiotic. Antibiotic residues could also lead to the emergence of resistant strains of organisms in humans. The quantities that could be ingested by such means however scarcely approach the therapeutic range and do not reach the levels ordinarily required to produce toxicity, which are usually atleast 10 times the therapeutic dose (Barragry, 1994). However, chronic exposure to the residues in animal products may lead to several health complications in consumer.

To eliminate health risks to consumers as well as a negative impact to the environment and the technology of food production, the control of foods of animal origin must become more effective. Therefore, the availability of simple and reliable screening systems for the detection of antibiotics is an essential tool to ensure food safety (Hussein *et al.,* 2005).

Antibiotics are used by the poultry industry and veterinarians to enhance growth and feed efficiency and reduce disease. Antibiotic usage has facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. Antibiotic usage has also enhanced the health and well-being of poultry by reducing the incidence of disease. Although these uses benefit all involved, unfortunately, the consumer perceptions in developed countries are that edible poultry tissues are contaminated with harmful concentrations of drug residues. Owing to the considerations involved in use of drugs in food animals, veterinarians are left with the limited options for choosing appropriate antibiotic. The option is further narrowed down in poultry due to the different physiology and specific formulation requirement.

Amphenicol is a group of synthetic antimicrobials popularly used in veterinary medicine but among Amphenicol, Chloramphenicol, the pioneer member of the group has been reported to cause reversible dose related bone marrow suppression in human by a mechanism involving inhibition of mitochondrial protein synthesis. It also causes irreversible non dose related aplastic anaemia in human (Yunis, 1981) that has led to ban on Chloramphenicol use in United States. Thiamphenicol has not been linked to aplastic anaemia as seen with Chloramphenicol. However, antibacterial potency of Thiamphenicol is less than that of Chloramphenicol and it could be inactivated by bacterial Chloramphenicol acetyl transferase which has limited its use.

Florfenicol is a fluorinated analogue of Thiamphenicol used in veterinary medicine. Florfenicol is a broad-spectrum, primarily bacteriostatic antibiotic with a range of activity similar to that of Chloramphenicol, including many gram-negative and gram-positive organisms; however, it does not carry the risk of inducing human aplastic anaemia that is associated with Chloramphenicol (Sams,1994).The antibacterial activity of Florfenicol does not get affected by Chloramphenicol acetyl transferase since 3'- hydroxyl group , which is the site of acetylation in Thiamphenicol and Chloramphenicol is replaced by fluorine atom in Florfenicol (Neu & Fu,1980) which has possibly made the antibiotic more resistant to bacterial inactivation (Adams, 2001).

Florfenicol is especially active against many Chloramphenicol-resistant strains of organisms such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Shigella dysenteriae*, *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris* and *Haemophillus somnus* (Neu and Fu, 1980; Syriopoulou *et al.,*1981 and Varma *et al.,* 1986). The potency and broad spectrum activity of Florfenicol should make it a good antibiotic to replace Chloramphenicol, which has been banned from use in food-producing animals.

Florfenicol was first introduced in the markets as injectable solution for treatment of respiratory diseases in cattle and now it is introduced in some countries as oral solution for treatment of several poultry diseases. The antimicrobial spectrum of Florfenicol indicates that it may become valuable

antibiotic in the treatment of infectious diseases in poultry. Florfenicol is developed exclusively for veterinary use and it is not used in human beings. It is also therefore appropriate to assess risk to the poultry birds through toxicity studies in chicken considering the possibility of accidental overdosing due to human errors. The present study is hence proposed with following objectives:

1. To study the concentration of Florfenicol (10% and 20% w/v) in different tissues of different organs viz., liver, kidney, lungs and muscle.
2. To study the duration for which Florfenicol residues are detectable in these tissues.
3. To study the concentration of Florfenicol (10% and 20% w/v) in egg albumin and egg yolk of laying chicken.
4. To study the duration for which Florfenicol residues appear in eggs of laying chicken.
5. To determine acute oral toxicity of Florfenicol in chicken

2 REVIEW OF LITERATURE

Florfenicol is used exclusively in Veterinary Medicine. However, owing to the fact that human population could be exposed to Florfenicol by way of residues, various toxicity and residue analysis studies are performed for establishing the ultimate safety of human population.

Keeping in mind the present research work the literature pertaining to Florfenicol is reviewed under following topics

1. Pharmacological features of Florfenicol
2. Residue studies (tissue and egg)
3. Toxicity
4. Methods of assay of residues
5. Toxicity testing for chicken

2.1 Pharmacological features

2.1.1 Historical development of Amphenicols

The discovery of Amphenicol was reported in 1947 by Ehrlich and co workers following intensive study of the organism isolated from about 6000 soil samples by Burkholder, a botanist at Yale University. A new species of Actinomycetes named *Actinomycetes venezuelae* after the origin from soil sample were found to produce a highly active antibiotic to which was given trade name Chloromycetin. In 1949, determination of relatively simple structure of the crystalline material made Chloramphenicol as first synthetic antibiotic on commercial scale (Brander *et al.,* 1982). However, Chloramphenicol has been reported to cause reversible dose related bone marrow suppression and irreversible non dose related aplastic anaemia in human (Yunis, 1981) that led to ban on Chloramphenicol use in United States and subsequently in other countries in food animals. Structural modification in Chloramphenicol further resulted in synthesis of new antimicrobial of Amphenicol group i.e. Thiamphenicol

which is not linked to aplastic anaemia as seen with Chloramphenicol. However, antibacterial potency of Thiamphenicol is less than that of Chloramphenicol and it could be inactivated by bacterial Chloramphenicol acetyl transferase which has limited its use (Fuglesang and Bergan, 1982).

Florfenicol is a veterinary fluorinated analogue of Thiamphenicol approved in 1996 in the United States for Bovine Respiratory Disease pathogens and recently launched for the use in poultry.

2.1.2 Chemical structure of Florfenicol (Anon, 2004)

Florfenicol, is D-(threo)-1-p-methylsulfonyl phenyl-2-dichloroacetamido-3-fluoro-1- propanol

2.1.2.1 Molecular formula: $C_{12}H_{14}Cl_2FNO_4S$

2.1.2.2 Molecular weight: 358.21.

2.1.3 Physicochemical properties of Florfenicol (Anon, 2004)

2.1.3.1 Melting point: 153 to 154 °C

2.1.3.2 Solubility: Soluble in water (23±1^{0}C) at pH 7 = 1.32±0.05 mg/ml.

2.1.3.3 Appearance: White to off white

2.1.3.4 UV absorption: Maximum molar absorbability occurs at 224 nm in aqueous solution containing 1%methanol.

2.1.3.5 Dissociation constant: Florfenicol molecule contains no functional groups that ionize between pH 2 and pH 12

2.1.3.6 Stability of florfenicol in Drinking Water: (Hayes *et al.,* 2003).

Florfenicol was found to be stable (above 90%) after 24 hours of its dilution in drinking water.

2.1.4 Structure activity relationship of Amphenicol

The basic structural component of Amphenicol is propanediol nucleus. Various substitutions on the propanediol nucleus of Chloramphenicol resulted in development of other Amphenicols such as Thiamphenicol & Florfenicol.

Basically Chloramphenicol has a three functional groups on propanediol nucleus that determine its biological activity. These groups are para nitro group (p-$NO_{2)}$, the dichloroacetyl moiety and the primary alcoholic group at carbon 3 of the propanediol chain. An intact propanediol moiety is required for full biological activity (Brock ,1961). Alteration in the propanediol portion of the molecule at carbons 1, 2 and 3 (Chloramphenicol–ribosome interaction site) generally leads to loss in biological activity (Triton, 1979). However, this is not a rigid requirement since replacement of the alcoholic group at carbon 3 has been done with retention of biological activity **(**Della Bella, 1981). Thus, fluorinated derivative of Chloramphenicol and Thiamphenicol have been prepared in which the primary alcoholic group on carbon 3 has been replaced by fluorine i.e. Florfenicol (Fig. 3). These derivatives not only retained the biological activity, but were also found to be active against Chloramphenicol resistant micro organisms.

The electro negativity of p- NO_2 group is essential for the proper conformation of Chloramphenicol (Fig. 1) but it is possible to replace the p-NO_2 with other electronegative groups without any drastic effect on conformation or biological activity. An example is replacement of p-NO_2 group by methyl sulphonyl (H_3C So_2 group) in Thiamphenicol (Fig. 2) (Della Bella, 1981).

The methylsulfonyl group of Thiamphenicol allows conformational changes in the molecule as in Chloramphenicol & therefore an intact drug ribosomal interaction occurs and Thiamphenicol inhibits bacterial ribosomal protein synthesis by similar mechanism and share an identical binding site on the 50S ribosomal subunit (Contreas *et al.,* 1974). A number of other analogues have been synthesized in which the $_p$- NO_2 group has been substituted. In general, all analogues in which the substitute group is electronegative retained antimicrobial activity though at reduced level. Except for Thiamphenicol, however none has undergone sufficient evaluation to merit clinical use (Della Bella, 1981).

The dichloroacetyl side chain is also important for biological activity but may be replaced by other acetyl side chains. Removal of side chain altogether results in loss of activity (Della Bella, 1981).

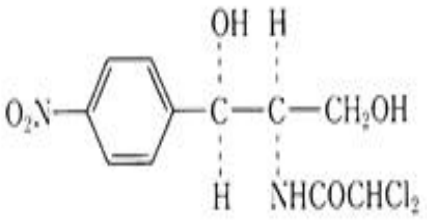

Fig 1 Chloramphenicol

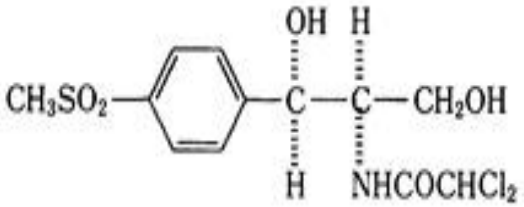

Fig. 2 Thiamphenicol

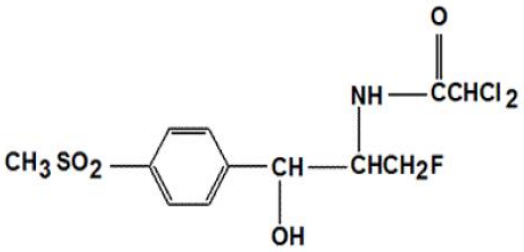

Fig. 3 Florfenicol

2.1.5 Antibacterial activity of florfenicol

Florfenicol is a broad-spectrum, primarily bacteriostatic antibiotic with a range of activity similar to that of Chloramphenicol, including many gram-negative and gram-positive organisms. The conventional way of assessing activity of antibiotic involves estimating MIC_{50} and MIC_{90} against either the isolates available as standard bacterial cultures or those which have been isolated from clinical cases. The reviewed literature reflects such attempts in some animal species.

Florfenicol has been demonstrated to be active *in vitro* and *in vivo* against *Pasteurella haemolytica, Pasteurella multocida* and *Haemophilus somnus* (Lobell *et al.,* 1994). Florfenicol's activity was demonstrated against *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi* and *Shigella dysenteriae* at MIC 2 to 10 fold higher than that for *Pasteurella and Haemophilus*. (Syriopoulou *et al.,* 1981 and Marshall *et al.,* 1996). It also has activity against some Chloramphenicol resistant strains of bacteria (Martel, 1994) possibly because it is less affected by the major enzymes produced in plasmid-mediated bacterial resistance against Chloramphenicol and Thiamphenicol (Cannon *et al.,* 1990). Florfenicol has MIC_{50} of 4 µg/ml and MIC_{90} of 8 µg/ml against the *E. coli* strains in turkey (Sarah and Jeffrey, 2000) whereas, Yang *et al.* (2002) reported that MIC of Florfenicol against *E.coli* (O_{78}) is 6 µg/ml.

In addition Pu *et al*. (2002) found that the MIC of Florfenicol against *Mycoplasma gallisepticum* as 1.25 µg/ml indicating that the drug may be of value for controlling *Mycoplasma* infection in broilers and turkeys.

Neu and Fu (1980) studied the comparative activity of Chloramphenicol, Thiamphenicol and three analogues (Table 1).

Jacks *et al.* (2003) reported *in-vitro* activity of Florfenicol against *Rhodococcus equi.* (MIC_{90} >8 µg/ml) and other common equine pathogen like *β haemolytic Streptococci* (MIC_{90} ≤1 µg/ml), *Staphylococci spp* (MIC_{90} 4 µg/ml), *Pasteurella spp* (MIC_{90} ≤1 µg/ml), *S. enterica* (MIC_{90} >8 µg/ml), *E. coli* (MIC_{90} 8 µg/ml) and *Klebsiellae spp* (MIC_{90} 8 µg/ml).

Table 1: **Comparative MIC values of Chloramphenicol, Thiamphenicol and its fluorinated analogs against enteric organisms.** (Neu and Fu,1980)

Organism (No. of isolates)	Antibiotics	Range (mcg/ml)	MIC_{50} (mcg/ml)	MIC_{90} (mcg/ml)
E.coli (51)	Chloramphenicol	3.1≥200	6.3	>200
	Thiamphenicol	1.6≥200	100	>200
	SCH24893	6.3-100	12.5	25
	SCH25393	6.3≥200	12.5	25
	SCH25298	3.1≥200	12.5	25
Klebsiella (35)	Chloramphenicol	0.4≥200	3.1	>200
	Thiamphenicol	6.3≥200	3.1	>200
	SCH24893	1.6≥200	12.5	50
	SCH25393	3.1≥200	12.5	50
	SCH25298	0.8≥200	6.3	100
Enterobacter (10)	Chloramphenicol	3.1≥200	6.3	200
	Thiamphenicol	50≥200	100	200
	SCH24893	3.1≥200	12.5	200
	SCH25393	6.3≥200	12.5	200
	SCH25298	3.1≥200	12.5	200
Citrobacter (16)	Chloramphenicol	3.1≥200	6.3	100
	Thiamphenicol	12.5≥200	100	>200
	SCH24893	3.1-50	25	50
	SCH25393	6.3≥200	6.3	12.5
	SCH25298	6.3-100	12.5	25
Proteus mirabilis (13)	Chloramphenicol	12.5-25	25	25
	Thiamphenicol	12.5≥200	200	> 200
	SCH24893	12.5-25	12.5	25
	SCH25393	3.1-12.5	6.3	12.5
	SCH25298	6.3-12.5	6.3	12.5
Serratia (16)	Chloramphenicol	>200		
	Thiamphenicol	>200		
	SCH24893	>200		
	SCH25393	>200		
	SCH25298	>200		
Shigella (32)	Chloramphenicol	0.8 ≥200	3.1	>200
	Thiamphenicol	0.8 ≥200	3.1	>200
	SCH24893	1.6-12.5	12.5	12.5
	SCH25393	0.8-6.3	3.1	6.3
	SCH25298	0.8-6.3	6.3	6.3
Salmonella (32)	Chloramphenicol	3.1 ≥200	6.3	100
	Thiamphenicol	25 ≥200	50	>200
	SCH24893	12.5-25	25	25
	SCH25393	3.1-6.3	6.3	6.3
	SCH25298	3.1-12.5	6.3	6.3

Table 1: Contd…

Organism (No. of isolates)	Antibiotics	Range (mcg/ml)	MIC_{50} (mcg/ml)	MIC_{90} (mcg/ml)
Proteus Indole positive (32)	Chloramphenicol	6.3≥200	12.5	100
	Thiamphenicol	6.3>200	50	>200
	SCH24893 25	6.3-100	12.5	25
	SCH25393	3.1-25	3.1	6.3
	SCH25298	3.1-25	3.1	12.5
Acinetobacter (13)	Chloramphenicol	3.1 ≥200	50	>200
	Thiamphenicol	100 ≥200	100	>200
	SCH24893	0.8-50	12.5	12.5
	SCH25393	3.1-200	>200	>200
	SCH25298	3.1 ≥200	>200	>200
P.aeruginosa (10)	Chloramphenicol	12.5 ≥200	>200	>200
	Thiamphenicol	50 ≥200	>200	>200
	SCH24893	12.5 ≥200	>200	>200
	SCH25393	25 ≥200	>200	>200
	SCH25298	12.5 ≥200	>200	>200
Bacteroides (51)	Chloramphenicol	3.1-50	6.3	25
	Thiamphenicol	1.6-50	6.3	25
	SCH24893	<0.4-3.1	1.6	3.
	SCH25393	3.1-12.5	6.3	12.5
	SCH25298	0.8-6.3	3.1	3.1
S. aureus (15)	Chloramphenicol	0.8-50	6.3	50
	Thiamphenicol	1.6 ≥200	25	>200
	SCH24893	<0.4-3.1	1.6	3.1
	SCH25393	0.8-6.3	3.1	6.3
	SCH25298	0.4-6.3	3.1	6.3
Enterococci (15)	Chloramphenicol	6.3-50	6	50
	Thiamphenicol	6.3≥100	6.3	>200
	SCH24893	6.3-12.5	1.6	3.1
	SCH25393	3.1-12.5	6.3	12.5
	SCH25298	3.1	3.1	3.1
Haemophillus influenzae (15)	Chloramphenicol	<0.4-1.6	1.6	1.6
	Thiamphenicol	0.8-25	1.6	25
	SCH24893	0.8	0.8	0.8
	SCH25393	3.1-63	3.1	3.1
	SCH25298	<0.40.8	0.8	0.8

Antimicrobial susceptibility of Florfenicol against bacterial agents isolated from cattle and pigs with respiratory disease were investigated (Shin *et al.,*2005 and Priebe and Schwarz, 2003). All *Actinobacillus pleuropneumoniae, Pasteurella multocida, Mannheimia haemolytica* and 98.6% of the *Bordetella bronchiseptica* isolates were susceptible to Florfenicol. It also showed high *in-vitro* antimicrobial activities ($MIC_{90} \leq 1$ µg/ml) against all strains tested with an MIC ranging from 0.12 to 4 µg/ml.

Ueda *and* Suenaga (1995) investigated that the MIC of Florfenicol against *A. pleuropneumoniae* isolated from porcine pneumonic lungs and it ranged from 0.20 to 1.56 µg/ml.

In vitro antibacterial activity of Florfenicol and its combination with Doxycycline were studied (Gongzheng *et al.* 2004).The results revealed growth of *E. coli* and *Staphylococcus aureus* was inhibited at 1 or 2 MIC and killing was evident at 4 & 8 MICs. Duration of post antibiotic effect induced by Florfenicol against *E. coli* (1.45-2.07 hours) was longer than that against *S. aureus* (0.48-1.18 hours). Fractional inhibitory concentration of Florfenicol plus Doxycycline (1:2) against *E. coli*, *S. pullorum* and *S. suis* was 1.5. The time-killing curve showed synergism at 4 MIC of Florfenicol or higher.

2.1.6 Mechanism of Action

The bacterial ribosome consists of a 50S subunit and 30S subunit. The other element in peptide synthesis are messenger RNA (mRNA) which forms the template for protein synthesis and transfer RNA (t RNA) which brings the individual amino acid to the ribosome. The ribosome has three binding site for tRNA, the A, P, and E site. mRNA, which is transcribed from DNA becomes attached to the 30S subunit of the ribosome. The 50S subunit then binds to the 30S subunit to form 70S subunit which moves along the mRNA so that successive codons of the messenger pass along the ribosome from the A position to P position (Rang *et al*., 2003).

Florfenicol is a bacteriostatic antibiotic that inhibits bacterial protein synthesis. Florfenicol binds to 50S ribosomal subunits of susceptible bacteria

and prevent the binding of the amino acid containing end of the aminoacyl tRNA to the acceptor site on 50S ribosomal subunit by inhibiting peptidyl transferase and thereby preventing the transfer of amino acid to growing peptide chains and subsequent protein formation. The bacterial receptor that is the site of action of Florfenicol, is considered to be the same as that of Chloramphenicol and Thiamphenicol (Sams, 1994 and Cannon *et al.,* 1990). Site of inhibition of protein synthesis by Amphenicol is presented in Fig. 4.

The structure of Florfenicol allows it to be less susceptible to deactivation by bacteria (as compared to Thiamphenicol and Chloramphenicol) with plasmid-transmissible resistance that involves acetylation of the C-3 hydroxyl group (Sams, 1994 and Cannon *et al.,* 1990).

2.1.7 Clinical Indications and efficacy of Florfenicol

Clinical efficacy of different formulations in various species has been studied in natural or induced bacterial infection. El Banna *et al.* (2007) reported that Florfenicol administration for five successive days @ 30 mg/kg body weight twice daily was highly efficacious in control of disease induced by *E coli.* There was decrease in mortality percent, post mortem lesions and bacterial reisolation from birds treated with 30 mg/kg body weight twice daily for five days as compared to 20 mg/kg body weight twice daily for five days.

Marien *et al.* (2007) assessed the efficacy of drinking-water administration of Enrofloxacin for three and five days, Amoxicillin for five days and Florfenicol for five days for the treatment against *Ornithobacterium rhinotracheale* and *E. coli O2:K1* dual infection in 3 week old turkeys following avian metapneumovirus (APV) priming oculonasally. A three days interval between viral and bacterial inoculation and approximately 8 hours between the two bacterial inoculations was followed. Compared with the untreated group, clinical signs as well as *E. coli* multiplication in the respiratory tract were significantly reduced by both Enrofloxacin and Florfenicol treatment. However, Enrofloxacin treatment showed significantly better reductions than the Florfenicol treatment.

Fig. 4 Site of inhibition of protein synthesis by Amphenicol

Long acting formulation of Florfenicol is effective in treating naturally occurring Bovine Respiratory Disease when administered intramuscularly @ 20 mg/kg body weight after every 48 hours and it was found to be superior to treatment with Oxytetracycline (Varma *et al.,* 1991).

When Florfenicol was administered by intramammary infusion (750 mg/cow) to cattle with sub-clinical mastitis, there was poor efficacy (Wilson *et al*.,1996).

On the other hand, Booker *et al.* (1997) reported that Florfenicol administered @ 20 mg /kg body weight was efficacious in treatment of undifferentiated fever in feedlot calves whereas, Jim *et al.* (1999) reported that Florfenicol is superior to Tilmicosin for the treatment of undifferentiated fever.

De Craene *et al.* (1997) stated that Florfenicol has a potential use in the treatment of bacterial meningitis due *to Haemophilus somnus* in calves as levels of Florfenicol in CSF remained above the MIC for over a period of 20 hours at a dose of 20 mg/kg body weight intramuscularly in cattle.

Several studies have been conducted to support the use of Florfenicol for treating Bovine Respiratory Disease. Florfenicol has been found to be effective for treating undifferentiated Bovine Respiratory Disease in cattle with doses of 20 mg/kg when administered intramuscularly after every 48 hours in the neck (Hoar *et al*., 1998).

In another study, Florfenicol has been found to be effective in calves for treating experimentally induced infection and naturally occurring Infectious Bovine Keratoconjunctivitis when administered as a single dose of 40 mg/kg body weight subcutaneously and two doses 48 hours interval at 20 mg/kg body weight intramuscularly (Dueger *et al.,* 1999; Angelos *et al.,* 2000).

Aslan *et al.* (2002) studied the clinical efficacy of Florfenicol in respiratory tract disease of calves and found it to be effective in terms of bacteriological and clinical use @ 20 mg/kg twice within 48 hours via intramuscular injection.

Rooney *et al.* (2005) and Skogerboe *et al.* (2005) and compared the efficacy of Tulathromycin @ 2.5 mg/kg body weight subcutaneously to Florfenicol @ 40 mg/kg body weight or Tilmicosin @ 10 mg/kg body weight in calves for the treatment of cattle with undifferentiated Bovine Respiratory Disease. Success rates of curing calves when treated with Tulathromycin were significantly higher than those calves treated with Florfenicol and Tilmicosin.

Palacios Arriaga *et al.* (2000) demonstrated that medication with the feed containing 40 ppm of Florfenicol for 12 consecutive days blocked efficiently the signs and lesions caused by *Actinobacillus pleuropneumoniae* and increased daily body weight gain in pig.

Efficacy of orally administered Oxolinic acid and Florfenicol in the treatment of experimentally induced *vibriosis* in cod *Gadus morhua* were investigated by Samuelsen *et al.* (2004). The dosages used were 10 or 20 mg/kg/day for both antibacterials and administered at days 1, 2, 4, 6, 8 and 10 following initiation of treatment for Oxolinic acid and daily for 10 consecutive days for Florfenicol. They reported cumulative mortality of 87.5% in untreated group whereas it was 34% and 28% in Oxolinic acid and 31% and 20% in Florfenicol treated group @ 10 & 20 mg/kg /day.

Soto-Rodriguez *et al.* (2006) evaluated survival and bacterial population in experimental infection with luminescent *Vibrio campbelli* in shrimp larvae exposed for 24 hours to Enrofloxacin and Florfenicol. At concentration higher than 20 µg/ml, both antimicrobials were found to be toxic to the larvae with an LC_{50} of 46.47 and 32.42 µg/ml for Enrofloxacin and Florfenicol respectively. Both the agents were found to be effective in reduction of bacterial load.

2.1.8 Dosage regimen of Florfenicol

As the plasma concentration of Florfenicol above 2 µg/ml remained up to 11 hours, Shen *et al.* (2003) suggested an oral dose of Florfenicol 30 mg/kg body weight twice daily in broiler chicken.

El- Banna *et al.* (2007) have recommended a dose of 30 mg/kg body weight of Florfenicol orally twice daily for five days for treating systemic infection with *E coli* in broiler chicken. Afifi and Abo el-Sooud (1997) also suggested that Florfenicol should be given twice a day at a dose @ of 30 mg/kg body weight to maintain its therapeutic concentration in plasma of broiler chicken.

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Soback *et al.* (1995) stated that intravenous administration of Florfenicol @ 20 mg/kg body weight twice daily is suitable for treatment of pathogen having MIC of 1 µg/ml or less and intramammary infusion of Florfenicol is sufficient for systemic treatment of pathogen possessing MIC ≤ 0.5 µg/ml or b.i.d for MIC of ≤ 2 µg/ml.

Varma *et al.* (1991) reported that intramuscular administration of Florfenicol after every 48 hours as a long acting formulation is effective in treating naturally occurring Bovine Respiratory Disease.

Alcorn *et al.* (2004) recommended that Florfenicol should be administered subcutaneously to elk every 24 hours at a dose level of 40 mg/kg.

2.1.10 Absorption of Florfenicol

Florfenicol is absorbed quickly after its administration by various routes. After studying the kinetics of Florfenicol in broiler chicken, Shen *et al.* (2003) reported that it was absorbed faster after intramuscular administration with an absorption half life 10±9 and 12±8 minutes whereas it was 21±13 and 32±15 minutes after oral administration @15 & 30 mg/kg body weight respectively.

El Banna, (1998) reported $t_{1/2ab}$ in healthy and diseased duck as 13.18±0.17 minutes and 14.8±0.54 min. respectively, after single intramuscular administration of Florfenicol @ 30 mg/kg body weight.

Pharmacokinetics of Florfenicol, Thiamphenicol and Chloramphenicol after single intravenous and oral administration @ 30 mg/kg body weight in turkeys was studied by Switała *et al.* (2007). They reported that after oral administration, the calculated mean C_{max} values for Florfenicol, Thiamphenicol and Chloramphenicol was 12.25±2.62, 8.99±2.78 and 4.69± 1.86 µg /ml which were reached at 2±1.22, 4.57±2.23 and 2.54±1.40 hours respectively. The MRT values of Florfenicol, Thiamphenicol and Chloramphenicol after intravenous injection were 3.37±0.63, 2.43±0.29 and 2.12±0.21 hours respectively.

2.1.11 Distribution of Florfenicol

Florfenicol has a wide distribution in most tissues of the body. Serum and tissue concentration (lung, liver, kidney, spleen, heart and breast muscle) of florfenicol in *Pasteurella* infected Muscovy duck were studied by El Banna,1998. The drug was administered @ 30 mg/kg body weight intramuscularly twice daily for 5 consecutive days. He reported that Florfenicol concentration in kidney, liver and bile was higher than concurrent serum concentration indicating that penetration of Florfenicol into these tissues was good. Florfenicol was detected in the kidney, bile and liver of diseased duck only after 7 day of cessation of treatment. He further reported that lower serum concentration of drug could be attributed to a more rapid intravascular distribution of drug in diseased than in healthy duck.

El Banna *et al.* (2007) further studied tissue residue depletion of Florfenicol in healthy and *E. coli* infected broiler chicken when administered orally @ 30 mg/kg body weight twice daily for five consecutive days. They reported that highest tissue concentration of drug after stopping of treatment were found in *E. coli* infected chicken than healthy birds. Bile was having highest tissue concentration followed by liver and kidney. Lowest concentration was determined in fat and brain of healthy as well as infected birds, which indicates that Florfenicol has low affinity for fat containing tissues and it can cross blood brain barrier (BBB) to a limited extent.

Afifi and Abo El-Sooud (1997) reported that the highest tissue concentration of Florfenicol in broiler birds were found in kidney, bile, lungs,

muscle, intestine, heart, liver, spleen and plasma after multiple intramuscular doses @ 30 mg/kg body weight for five successive days. They reported that penetration of Florfenicol into these tissues were good.

2.1.12 Metabolism of Florfenicol

In a radiometric study in chicken with ^{14}C Florfenicol, the drug was administered by oral gavage twice daily 12 hours apart at 20 mg/kg body weight/dose for three days. Florfenicol amine was the major metabolite measured in edible tissues of chicken, Florfenicol the only microbiologically active compound being only detected in skin+ fat in low concentrations (4%) and in kidney (1%). The other metabolites i.e. Florfenicol oxamic acid, Florfenicol alcohol and monochloroflorfenicol represents 3.45, 11.30 and 13.05% of total radioactivity at day 1st in skin + fat, liver and muscle respectively (Anon, 1999a).

Martinez-larranaga *et al.* (2003) reported that florfenicol amine represented 34.5% of the Florfenicol plasma concentration when Florfenicol was administered orally to broiler chicken @ 20 mg/kg body weight.

2.1.13 Excretion of Florfenicol

After repeated oral administration of ^{14}C Florfenicol twice daily 12 hours apart at 20 mg/kg body weight/dose for three days in chicken, approximately 93.7% and 98.2% of total reactivity administered were excreted within one day and seven days respectively after the last dose. The parent compound represented the major fraction of the radioactivity (42%), followed by florfenicol amine (25%), florfenicol oxamic acid (5%) and florfenicol alcohol (10%) and the remaining part of radioactivity being represented by a small percentage of three unknown compounds in excreta at seven days while no monochloroflorfenicol was detected in excreta (Anon, 1999a)

2.1.14 Adverse Effects of Florfenicol

In animals, there is no report of dose dependent bone marrow suppression but in humans aplastic anaemia cannot be protected due to the difference in structure of Florfenicol and Chloramphenicol as this does not protect against suppression of mitochondrial protein synthesis in bone marrow and subsequent reversible anaemia (Sams, 1994)

When toxic overdose of Florfenicol (200mg/kg body weight) were administered to calves, there was marked anorexia, decreased body weight, ketosis and elevated liver enzyme. In dogs administered high doses of Florfenicol for prolonged period, there was CNS vacuolation, haematopoietic toxicity and renal tubule dilatation (Papich and Riviere, 2000).

Mild diarrhoea was reported by McKeller and Varma (1996) in all three horses and three ponies administered a single dose of Florfenicol @ 22 mg /kg body weight by either the oral or parenteral route.

2.1.15 Development of resistance

Keyes *et al.* (2000) detected resistance to Florfenicol in clinical isolates of avian *E. coli* by molecular typing demonstrating that the Florfenicol resistance gene, '*flo'*, was independently acquired and was plasmid encoded.

Kim and Aoki (1993) stated that Florfenicol was only registered for commercial use in aquaculture in Asia in 1989, and Florfenicol resistance did not emerge in aquaculture pathogens until 1992.

2.2 Residue studies

2.2.1 Tissue residue study

Tissue residue studies of Florfenicol have been carried out in various species such as chicken (Afifi and Abo el-Sooud,1997; Anon,1999a; Chen *et al.*,2005 and El-Banna *et al.*, 2007); duck (El-Banna, 1998); cattle (Adams *et al.*,1987); swine (Anon,1999b; Limin *et al.,* 2005; Jiancheng *et al.*, 2006 and Suxia *et al.*, 2006a); sheep (Lane *et al.,* 2008); fish (Hormazabal *et al.,* 1993; Nagata and Oka, 1996; Guo *et al.,* 2006; Suxia *et al.,* 2006a and Zhang *et al.,* 2007); catfish (Gaikowski *et al.,* 2003; Wrzesinski *et al.,* 2003 and Yong-tao *et al.,* 2007); eel (Fang *et al.,* 2005) and shrimp (Pfenning *et al.,* 2000; Tao *et al.,* 2005 and Suxia *et al.,* 2006a).

When Florfenicol was administered @ 30 mg/kg body weight for five consecutive days by oral or intramuscular route in broiler chicken, it persisted in the tissue for 72 hours as reported by Afifi and Abo el-Sooud (1997), whereas according to El-Banna (1998), Florfenicol when administered @ 30 mg/kg body weight intramuscularly for five days, was detected in the kidney, bile and liver of *Pasteurella* infected muscovy duck only on day 7 after discontinuation of treatment. In a similar study conducted by El-Banna *et al.* (2007) in healthy and *E.coli* infected broiler birds, Florfenicol was detected up to 6^{th} day in all the tissues except in liver where it was detectable even on the 7^{th} day after discontinuation of medication. According to Jiancheng *et al.* (2006), when swines were administered Florfenicol @ 20 mg/kg body weight by intramuscular route at 24 hours interval, the sum of Florfenicol and florfenicol amine concentrations in all tissues analyzed was below the accepted MRL at 8 days post treatment while Lane *et al.* (2008) reported that by day 40, all the tissue samples were below the tolerance level when sheep were administered Florfenicol @ 40 mg/kg body weight subcutaneously for three days.

Irrespective of species and dose of administration, highest concentration of Florfenicol was seen in liver as reported by Anon (1999a) and Lane *et al.* (2008); whereas according to Adams *et al.* (1987); Afifi and Abo el-Sooud, (1997); El-Banna, (1998) and Jiancheng *et al.* (2006), the highest concentration of

Florfenicol was observed in kidney indicating that liver and kidney are the target organs.

Florfenicol is the only relevant microbiologically active residue and its metabolites include Florfenicol amine, Florfenicol oxamic acid, Florfenicol alcohol and monochloroflorfenicol of which Florfenicol amine is considered as the marker residue (Anon, 1999a). The established MRLs of Florfenicol in chicken for different tissues are mentioned in Table 2.

In a study performed by Afifi and Abo el-Sooud (1997) in broiler chickens, Florfenicol was administered @ 30mg/kg body weight via intramuscular and oral routes for five consecutive days. The birds were slaughtered at 1, 3, 6, 12, 24, 48, 72 and 96 hours after administration and the tissue samples (liver, lung, kidney, muscle, heart, spleen, intestine, bone marrow and fat) were collected and determined microbiologically using *Bacillus subtilis* (ATCC 6633) for estimation of Florfenicol concentration. It was observed that high tissue concentrations of the drug were found in the kidney (4.10 µg/g) and lung (2.80 µg/g) at one hour post administration of drug which indicate that Florfenicol may be an excellent drug for treating urinary and respiratory tract infections caused by susceptible organisms. The limit of quantification of Florfenicol in tissue was 0.01 µg/ml with a mean correlation coefficient (r^2) of 0.998. Recovery of Florfenicol from spiked tissues and fluid samples ranged from 85-100%. No Florfenicol residues were found in tissues after 72 hours.

El-Banna (1998), performed multiple dose study on diseased group of *Pasteurella* infected Muscovy ducks administering Florfenicol @ 30 mg/kg body weight intramuscularly twice daily for 5 consecutive days. Three birds were slaughtered at 1 hour, then at 1, 3, 5 and 7 day after the last dose. Tissue samples (lung,liver,kidney,spleen,heart and breast muscle) were collected and estimated microbiologically for Florfenicol concentration using *Bacillus subtilis* (ATCC, 6633). Highest concentration was observed in kidney (4.63 µg/g) followed by liver (3.95 µg/g), lung (2.79 µg/g) and muscle (2.30 µg/g) at one hour after the last dose administration. The detectable limit of Florfenicol was 0.050 µg/ml. The drug was detected upto the 5^{th} day after last dose administration in all

Table 2: MRLs of Florfenicol in tissues of chicken (Anon, 1999a)

Pharmacologically active substance(s)	Marker residue	Target tissues	MRLs	Other provisions
Florfenicol	Sum of Florfenicol and its metabolites measured as Florfenicol amine	Muscle Kidney Liver	100 µg/Kg 750 µg/Kg 2500µg/Kg	Not for use in birds from which eggs are produced for human consumption

the samples while in kidney, bile and liver, Florfenicol was detectable upto the 7th day post-administration.

Using the same dose, duration of dosing and method, El-Banna *et al.* (2007) found similar lower detection limit as above in healthy and *E.coli* infected broiler chickens. Three chickens each were slaughtered at 2 hours and at day 1, 2, 4, 6 and 7 and tissue samples were collected and estimated for Florfenicol concentration. High concentration of the drug were observed in liver (7.77 µg/g) and kidney (7.19 µg/g). Florfenicol was detectable on all the tested tissues on the 6th day after discontinuation of medication in healthy and infected birds. At day 7 after stopping of drug administration, all tissues were considered drug free except liver of infected birds which had a concentration of 0.11 µg/g.

In another study, broiler chickens were administered Florfenicol @ 17 to 30 mg/kg body weight/day via drinking water for three days. It was observed that at 0.5 day after the end of the treatment, the concentrations of Florfenicol amine were lower than the limit of quantification (LOQ) for muscle (< 50 µg/kg) and skin+fat (<109 µg/kg), whereas in liver and kidney 2862 and 1161 µg/kg of Florfenicol amine were measured respectively. One day after the end of the treatment the concentration in liver and kidney were found to be 2038 and 679 µg/kg respectively and after three days it was 1215 and 484 µg/kg respectively. Seven days after the end of treatment, the concentrations of Florfenicol amine were below the LOQ for liver (<461 µg/kg) and could be still measured in kidney (136 µg/kg) (Anon, 1999a).

In a study conducted by Chen *et al.* (2005) for the analysis of Chloramphenicol, Thiamphenicol and Florfenicol in chickens, the limit of detection (LOD) were 0.01 µg /kg and the LOQ were 0.10 µg /kg. The linear plots were obtained between 0.050 and 1.00 µg /L and overall recoveries were between 69.0% and 92.8% with relative standard deviations between 6.3% and 12.9%.

In a radiometric study, chicken received 20 mg/kg body weight of ^{14}C Florfenicol orally twice daily 12 hours apart for three days. Twenty-four hours after the end of oral administration, the levels of radioactivity in edible tissues were 146 µg/kg in muscle, 475 µg/kg in skin+fat, 11148 µg/kg in liver and 3125

μg/kg in kidney. Then it declined to reach approximately 50 μg/kg in muscle and in the skin+fat five days after the end of treatment, whereas in liver and kidney significant amount of residues of 2403 and 844 μg/kg were found respectively. Recovery percentage observed were 70% for muscle, 60 to 76.3% for skin+fat, 65 to 80% for liver and about 80% for kidney (Anon, 1999a).

Adams *et al.* (1987) determined tissue concentrations and pharmacokinetics of Florfenicol in male veal calves administered @11 mg/kg body weight intravenously and orally every 12 hours for 7 doses. After the 7^{th} dose administration, tissue concentrations were determined using High Performance Liquid Chromatography (HPLC) and it was observed that high concentrations of Florfenicol were measured in the urine, kidney and bile whereas low concentrations were estimated in the brain, CSF and aqueous humour. The result indicates that Florfenicol may be an excellent drug for treating urinary tract infections caused by susceptible organisms. Concentrations in all other tissues and fluid were found to be similar to the concurrent serum concentrations indicating that the penetration of Florfenicol into these tissues was good.

Tissue residues of Florfenicol were investigated (Lane *et al.,* 2008) in sheep administered @40 mg/kg body weight subcutaneously daily for 3 days using different injection sites for each daily dose. The LOQ for liver, muscle, kidney and fat was 0.32; ppm; 0.05ppm; 0.10ppm and 0.04ppm respectively. Quantifiable concentrations of Florfenicol amine were detected in all tissues except fat, at all time points.

A study was performed to determine Florfenicol and its metabolite Florfenicol amine in swine tissue (Jiancheng *et al.*, 2006) in which the pigs were administered Florfenicol @ 20 mg/kg body weight intramuscularly at 24 hours interval. They found that standard curves were linear from 100 to 20000 ng/ml (r^2=0.9997 for Florfenicol and r^2=0.9999 for Florfenicol amine). The limit of detection in muscles were 10 and 15 ng/g in liver and kidney respectively for both Florfenicol and Florfenicol amine and the limit of quantification was found to be 20 ng/g in muscle and 30 ng/g in liver and kidney respectively. The interday mean recovery of Florfenicol was between 76.1 and 87.9% and an intraday mean

recovery between 75.2 and 85.5%. While, in Florfenicol amine, the interday mean recovery was between 74.1 and 88.8% and an intra-assay mean recovery between 72.4 and 83.8%.

A study was conducted in 6 pigs receiving Florfenicol orally via drinking water @12.99 mg/kg body weight for five days. It was observed that after one day of treatment, the concentrations of Florfenicol amine in edible tissues were 527 µg/kg in muscle, 884 g/kg in skin+fat, 9860 µg/kg in liver and 3270 µg/kg in kidney. After 9 days the concentration declined to 218, 2410 and 385 µg/kg in skin+fat, liver and kidney respectively whereas it was found that in muscle the concentration was lower than the LOQ (<150 µg/kg). Twelve days post dosing, 237 µg/kg were measured in skin+fat, 2410 µg in liver and 385 µg/kg in kidney. Twenty one days after the administration, significant amounts of Florfenicol amine could still be measured in fat (122 µg/kg) and in kidney (123 µg/kg) whereas in liver the concentration was below the LOQ (<1000 µg/kg**)** (Anon, 1999b).

Residue study of Florfenicol in pork showed that the correlation coefficient was greater than 0.999 with an LOD of 2.0 µg/kg. The average recoveries were 88.4%, 84.0% and 71.2% for the concentration 1.0, 10 and 100 µg/kg respectively (Limin *et al.,* 2005).

2.2.2 Egg residue study

2.2.2.1 Formation and composition of egg

Antibacterials also appear in eggs after administration to laying hens. They enter the egg during the process of egg formation. The main components of poultry egg are egg shell, white and yolk (Fig.5). Egg yolk and albumen are formed at different times in different parts of reproductive tract of hen (Fig.6). The follicles later become the yolks and grow on the ovary. After ovulation the free ovum is picked up by the infundibulum of the oviduct and then transported towards the albumen secreting region or magnum. Via the isthmus, where membranes are formed, the egg then enters the shell gland or the uterus. There,

Fig 5 Composition of egg

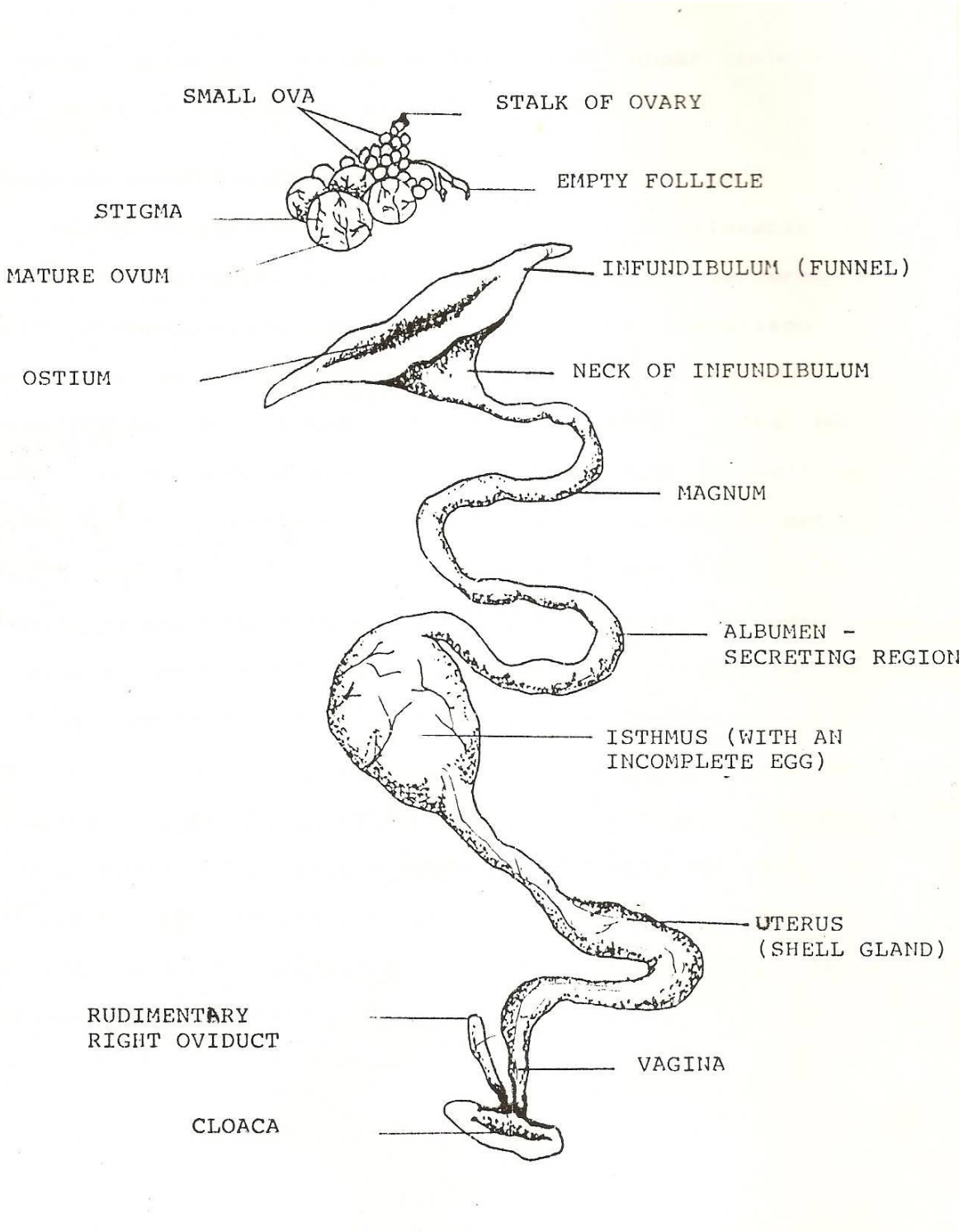


Fig. 6 The reproductive tract of hen

fluid and minerals gets added during the plumping process to the egg white and the shell is formed. Then the ready-to-lay egg is transported via the vagina and through the cloacae, and finally the egg is laid. (Kan, 2003). The time frame of the whole process is outlined in Table 3 (Ensminger, 1992).

Use of Florfenicol in layers has been banned in regulatory countries but in non-regulatory countries however extralabel use continues. The references pertaining to levels of Florfenicol in eggs of chicken were not traceable. However, such studies have been carried out with Thiamphenicol, Chloramphenicol and other antibacterials.

According to Giorgi *et al.* (2000), when layers were administered Thiamphenicol @ 40 mg/kg body weight as a single or multiple (five successive days) oral doses, the drug persisted longer in yolk than in albumin and were present in yolk for ten days after administering a single dose and for eight days after multiple doses. After multiple doses, the values of Thiamphenicol concentrations were under the limit of detection at ninth day after the end of the treatment. In another study conducted by Sisodia and Dunlop (1972), when Chloramphenicol was administered @ 40 ppm through drinking water for five days, concentration in the yolk reached its peak of 0.33 ppm on the sixth day and gradually fell to 0.14 ppm on the tenth day whereas following withdrawal of the drug at 120 hours, the concentration in albumin slowly declined and fell sharply to undetectable level on day nine. Similarly Acet *et al.* (1989) estimated Chloramphenicol concentration in eggs and found that no residues were detected in eggs 30 days after withdrawal of the drug at 400 mg/kg body weight for five days.

Data of levels of some antibiotic in egg white and yolk in chicken are summarized in Table 4.

Giorgi *et al.* (2000) investigated that when Thiamphenicol is administered @ 40 mg/kg as a single and multiple (5 successive days) oral doses to twenty laying hens, the retention time (RT) was 6.30 minutes and LOQ was 10 µg/kg. Extraction recoveries for yolk and albumin were 75.2±2.0 and 80.4±1.1% respectively. Residues (>10 µg/Kg) persisted longer in yolk than in albumin and

Table 3: Function of oviduct

Table 4 Antibiotics in eggs

Table 4 antibiotics in eggs page2

were present in yolk for ten days after administering a single dose and for eight days after multiple doses. After multiple doses, the values of Thiamphenicol concentrations were under the LOD at ninth day after the end of the treatment.

Chloramphenicol residues in eggs were determined microbiologically by Sisodia and Dunlop (1972) wherein layer birds were administered 40 ppm of Chloramphenicol through drinking water for five days. It was observed that day, eight hours following withdrawal of medicated water and gradually fell to 0.14 ppm on the tenth day. On the other hand, in albumin, the drug concentration was <0.1 ppm on day one, goes up to 0.12 ppm on 2nd day, reached up to 0.17 ppm on concentration of Chloramphenicol in yolk reached a peak of 0.33 ppm on the sixth day four. Following withdrawal of the drug at 120 hours, the concentration in albumin slowly declined and fell sharply to undetectable level on day nine. The average recovery of yolk and albumin was approximately 20 and 31 grams respectively. It was concluded that following termination of medication the persistence of Chloramphenicol was longer in yolk than in albumin supporting the hypothesis suggesting a longer lag period and persistence for residues in yolk than in albumin.

In a study conducted by Acet *et al.* (1989), four groups each of 30 hens were given Chloramphenicol at 0, 20 or 40 mg/kg of feed for two months or 400 mg/kg for five days. It was observed that there was no effects on egg production, egg weight, feed intake or the feed efficiency ratio. HPLC did not detect any egg residues with the 20 mg/kg dosage. Residues of 5-20 ng/g were found with the 40 mg/kg dosage. Eggs of those given 400 mg/kg showed increasing residue levels (58-197 ng/kg) during the five days of administration, the residues then decreased and no residues were detected in eggs 30 days after withdrawal of the drug.

Akhtar *et al.* (1995) found that although recoveries of Chloramphenicol were essentially the same for both albumen and yolk, the standard deviation was narrow for albumen as compared to yolk. There was no statistical difference in recoveries of spiked Chloramphenicol from whole liquid or freeze-dried eggs. The method was validated with eggs of chickens given Chloramphenicol in drinking

water at 1-200 mg/ml for 10 consecutive days followed by a withdrawal period of 10 days. There was no loss observed during freeze-drying. It is concluded that the method has the potential for being used routinely for monitoring Chloramphenicol in eggs and eggs products.

2.3 Toxicity

Florfenicol lacks the para-nitro group that could contribute to the induction of aplastic anemia associated with Chloramphenicol use in humans. Therefore, if residues were to occur in animals treated with Florfenicol, no dangerous public health risk would ensue. However, it is possible that Florfenicol can still produce dose-related form of reversible bone marrow suppression with prolonged use or high doses, although such reactions have not been reported from routine use of Florfenicol in animals.

Various toxicity studies of Florfenicol have been conducted such as acute toxicity study, teratogenicity and carcinogenicity studies in rats and mice (Anon, 2002b); chronotoxicity in mice (Picco *et al.,* 2001); bone marrow hypoplasia in Thomson's gazelle (Tuttle *et al.,* 2006); toxicity study in *Ictalurus punctatus* (Gaikowski *et al.,* 2003); *Tetraselmis chuii* and *Artemia parthenogenitica* (Ferreira *et al.,* 2007).

Picco *et al.* (2001) studied circadian rhythmicity in the toxicity of Florfenicol overdose in 63 months old homozygote male mice. Animals were kept under a regimen of 12 hours light, 12 hours darkness (12:12 LD). The LD_{50} dose was determined in a preliminary experiment and was administered to groups of 10 mice at six different clock times (hours) after light onset (HALO): 0, 4, 8, 12, 16, and 20 HALO. There was significant ($P < .04$) circadian rhythm in the toxic effect (mortality) of Florfenicol. Mortality was greatest when the drug was injected 4 hours after the commencement of the activity span (16 HALO) and least when injected 4 hours after the start of the diurnal rest span (4 HALO). Mortality was 2.5 times greater when drug injection was given at 16 HALO than at 4 HALO.

After oral administration, Florfenicol was not acutely toxic to mice and rats and no LD_{50} could be established (above 2000 mg/kg body weight). After Intraperitoneal administration, the LD_{50} was close to 2000 mg/kg body weight in rats while in repeated dose toxicity study conducted in mice (13 weeks), rats (7, 14, 28 days and 13, 52 weeks) and dogs (14, 28 days and 13, 52 weeks), it was observed that there were changes in haematologic parameters and atrophy of testes in rats while in dogs there was increase in liver weights. The dog was the most sensitive species and the NOEL was 1 mg/kg body weight/day (Anon, 2002a).

In a multi-generation study carried out in rats, the oral doses of Florfenicol had adverse effects on the male reproductive system with an NOEL of 1 mg/kg body weight. Several teratogenic studies were performed in mice (0, 1, 3, 60 mg/kg body weight/day) and in rats (0, 4, 12, 40 mg/kg body weight/day). High doses induced maternal effects and delayed ossification. The NOELs for maternotoxicity were 3 mg/kg body weight/day for mice and 4 mg/kg body weight/day for rats. Florfenicol induced no fetal malformation at any dose level and showed no potential for embryo or fetotoxicity. On the other hand, in a two carcinogenicity studies carried out in mice (0, 20, 100, 200 mg/kg body weight/day) and in rats (0, 3, 12, 48 mg/kg body weight/day) for 104 weeks, mortality was observed to be high (about 60%). In mice the two toxic effects were observed on the male reproductive system exposed to 200 mg/kg body weight and on the liver producing benign hepatocellular tumors. In rats, the toxic effects were observed on the testes showing atrophy of the tubular epithelium, spermatogenic cells in the epididymis and increased incidence of non-malignant interstitial cell tumors (Anon, 2002a).

Tuttle *et al.* (2006) reported Bone marrow hypoplasia secondary to Florfenicol toxicity in a Thomson's gazelle (*Gazella thomsonii*). The animal was given an accidental antibiotic dose of 9600 mg (400 mg/kg) of Florfenicol subcutaneously and observed that the animal showed panleukopenia and anaemia. After several days the animal was progressively depressed and developed polydipsia and severe diarrhoea and resulting in ultimate death of the animal.

Gaikowski *et al*. (2003) studied safety of Aquaflor (Florfenicol, 50% type a medicated article), administered in feed to channel catfish, *Ictalurus punctatus* at doses of 0 (control), 10, 30 and 50 mg/kg body weight/day for twenty consecutive days.Parameters evaluated includes daily mortality, behavioral (appetite, distribution, flight/fright response), and water chemistry observations, initial and terminal weight measurements, and gross and microscopic pathology. There were no mortalities or clinically observable changes noted at any of the dose levels tested. Aquaflor-related changes were limited to the food consumption and histopathology data. Although Aquaflor-related decreased feed consumption was noted in the 30 and 50 mg/kg body weight/day/groups, there were no differences in fish growth among the treatment groups. Aquaflor-related histopathology findings were limited to a histomorphologically evident dose-dependent decrease in hematopoietic/lymphopoietic tissue in the anterior kidneys, posterior kidneys and spleens of channel catfish.

Acute toxicity of Florfenicol to microalgae *Tetraselmis chuii* and to the crustacean *Artemia parthenogenetica* was evaluated by Ferreira *et al.* (2007). They found that florfenicol inhibit the growth of *T.chuii* cultures with 96 hours LC_{50} values of 6.06 mg/L. Florfenicol did not cause mortality of *A. parthenogenetica* indicating that the drug was considerably more toxic to *T. chuii* than to *A.parthenogenetica.*

Li-wen *et al.* (2005) performed 96 hours acute toxicity study and histological toxicology of Florfenicol in abalone *Haliotis diversicolor* reeve. The LC_{50} was 162.67±17.41 mg/L. Toxicity symptoms observed includes mucus secretion, adhesion significantly weakened, offal mission expansion, unresponsiveness to outside stimulation and paralysis. Digestive gland and kidney were damaged. It was also found that basophils cell membrane were gradually falling from the disintegration of the connective tissue between the collagen fibers fracture along with wide dispersion necrosis, cavity renal epithelial cell shrink, separation of basal membrane, chamber became smaller, loss of microvilli, renal cell fusion and disappearance of nuclear disintegration.

2.4 Methods of assay of residues

One of the most important requirements for development of new animal drugs is to have an assay method that is reliable indicator of the possible drug residues in treated animals. Methods of analysis are essential not only for the determination of residues of veterinary drugs in food, but also for the ultimate safety evaluation of these drugs. Various methods have been developed by various scientists for estimation of florfenicol residues in tissues and certain antibiotics in chicken eggs (Barragry, 1994).

There are various methods of analysis of Florfenicol concentration in tissues, milk, feed and fishery products using Microbiological assay method (Afifi and Abo El-Sooud., 1997; El-Banna, 1998; El-Banna *et al.*, 2007), High Performance Liquid Chromatography (Adams *et al.*, 1987; Hormazabal *et al.*, 1993; Fang *et al.*, 2005; Hayes, 2003; Guo *et al.*, 2006; Yong-tao *et al.*, 2007 and Zhang *et al.*, 2007), HPLC-ESI-MS-MS (Chen *et al.*, 2005), HPLC with photodiode array (PDA) detection (Jiancheng *et al.*, 2006), HPLC with UV detection method (Lane *et al.,* 2008 and Wrzesinski *et al.*, 2003), LC/MS (Van De Riet *et al.,* 2003); HPLC-tendem Mass Spectrometry (Tao *et al.,* 2005), Gas Chromatographic (GC) (Suxia *et al.*, 2006a and Suxia *et al.*, 2006b), Gas Chromatography/Mass Spectrometry (GC/MS) (Nagata and Oka, 1996 and Limin *et al.,* 2005), GC with electron capture detection (ECD) (Pfenning *et al.*, 2000), Micellar electrokinetic chromatographic method (MEKC) (Pezza *et al.*, 2006) and radiometric study using ^{14}C isotope (Anon,1999b).

Thiamphenicol residue in egg have also been assessed by Giorgi *et al.* (2000) by using HPLC and Chloramphenicol residue in egg by Sisodia and Dunlop (1972) wherein the concentration of the drug was determined microbiologically. In one study performed by Hee-Jung *et al.* (2007), Fluoroquinolone residue in chicken egg was estimated by microbiological assay using *E.coli* as the test organism and confirmation was done using Liquid chromatography. They concluded that bioassay can be used as a routine screening methods for the presence of Fluoroquinolones in chicken eggs, which can be confirmed and quantified using Liquid Chromatography.

The three major methods of estimation of residues in tissues and eggs are Microbiological assay, High Performance Liquid Chromatography and Gas Chromatography technique. The limitation of using microbial assay method is that quantitative estimation of Florfenicol and its metabolites separately is not possible. However, the method is simple and needs no separate extraction procedures for obtaining Florfenicol in pure form. The advantage of using HPLC and GC is that the method can be used to assay the parent compound as well as its metabolite, economical and time saving. However, HPLC is found to be superior to GC and has greater applicability. Although the versatility and sensitivity are similar to GC, HPLC has and edge over GC in that its scope is not limited by sample volatility or thermal stability (Sandu, 2006).

2.5 Toxicity testing for chicken

The Avian acute oral toxicity test is divided into two steps i.e. a limit dose study in five birds, wherein the birds were administered 2000 mg/kg body weight of the test formulation (Florfenicol 20%) in first step. The toxicity symptoms and mortality observed in the first step guide to choose from among the options available to proceed with Step2. The method has been designed in a way to minimize the numbers of birds used (Anon, 2002b).

3 MATERIALS AND METHODS

The present work was divided into three phases of the study

1. Residues of Florfenicol in tissues of broiler birds.
2. Residues of Florfenicol in eggs of layer birds.
3. Toxicity testing of Florfenicol in chicken.

3.1 Residues of Florfenicol in tissues of broiler birds

3.1.1 Experimental Birds

Tissue residues of Florfenicol were estimated in chicken ready for slaughter. These were procured from local market and their weight ranged from 1.04 to 1.19 kg. The birds were maintained on standard balanced ration (Appendix I) and water was provided *ad-libitum*. The birds were healthy, active and free from any clinical sign of disease or disease condition at the time of experiment.

3.1.2 Drugs used for the study

The drugs used were two formulations of Florfenicol viz, 10% and 20% oral solution. The standard Florfenicol (100 % purity) was used for preparing the standard graph of Florfenicol in tissue of chicken**.**

3.1.3 Design of experiment

To determine the time period up to which the levels of Florfenicol are detected in the different body tissues, a pilot study was carried out in 10 broiler birds. Florfenicol (20%, w/v) was administered to all the birds, at the dose rate of 30 mg/kg body weight, individually, using oral gavage tube No. 3 (Fig.7) Two birds each were sacrificed at 6, 8, 10, 12 and 24 hours interval, post-administration. The final study was to be performed by collecting the organ

Fig NO 7 oral gavage

samples for estimating Florfenicol concentration at the period up to which Florfenicol was detectable in these tissues. Florfenicol could not be detected in samples collected at 10, 12 and 24 hours post-treatment whereas it was detected at 6 and 8 hours samples.

Final study was carried out in twelve birds. The birds were weighed using balance having sensitivity of 10 mg and weighed between 1.04 to 1.19 kg. These birds were then divided in two groups viz., Group A and Group B, consisting of six birds each. Florfenicol 10% w/v @30 mg / kg was administered to Group A by oral gavage using tubing No. 3, individually. Similar procedure was followed for Group B with individual administration of Florfenicol 20% w/v formulation.

Each bird received Florfenicol @ 30 mg /kg body weight orally. The weight of each bird was recorded and volume of Florfenicol (10% or 20% w/v) administered was as per Table 5.

3.1.4 Collection and processing of tissue

As results of the pilot study indicated non-detection of Florfenicol at 10 hours collection times, the tissues in the final study were collected at 8 hours post administration for estimation of Florfenicol concentration. The muscle and organ samples i.e. lung, liver and kidney were processed as per the method of Bielecka (1981). The tissue samples weighing about 2 g were cut into small pieces and triturated along with 4 ml phosphate buffer (Appendix II). The samples were transferred to the homogenizer (Universal Motor, Mumbai-53, RPM: 8000) and were homogenized for two minutes to obtain uniform suspension. Suspension thus obtained was kept standing at room temperature for better extraction of Florfenicol. The extracts were heated in water-bath at 80^0 C for five minutes to decrease the bacterial activity considerably and were cooled to room temperature. The samples were centrifuged at 4500 rpm for ten minutes. The supernatant was then assayed microbiologically for estimation of Florfenicol concentrations.

Table 5: Weights of experimental chicken and volume of formulation (Florfenicol 10% & 20% w/v) administered for tissue residue study.

Bird No.	Weight (Kg)	Volume administered (ml)
Group A (10% Florfenicol)		
1.	1.040	0.31
2.	1.150	0.34
3.	1.180	0.35
4.	1.130	0.34
5.	1.110	0.33
6.	1.150	0.34
Group B (20% Florfenicol)		
1.	1.190	0.18
2.	1.120	0.17
3.	1.150	0.18
4.	1.110	0.17
5.	1.150	0.18
6.	1.120	0.17

3.1.5 Estimation of Florfenicol concentration from tissue

Concentration of Florfenicol was estimated by microbiological assay technique using large glass assay plates and *Bacillus subtilis* ATCC 6633 as the test organism. The whole procedure of the assay was as follows

3.1.6 Microbial assay of Florfenicol

Glassware used during assay was washed thoroughly subjected to dry heat in hot air oven at 160^0 C for one hour. The large glass assay plate (25 X 25 cm) manufactured by M/s Aarchal Corporation, Pune was used for assay. In the present study, to apply samples in seeded plates, wells were punched in media using stainless steel punch having inner diameter of 8 mm. For performing microbiological assay Mueller Hinton Agar (M/s Hi Media Laboratories, Mumbai) was used. The composition was elaborated in Appendix III.

The organism used for microbiological assay of Florfenicol was *Bacillus subtilis* ATCC 6633. The culture of *Bacillus subtilis* ATCC 6633 was obtained from the FDA (Food and drug administration), Mumbai.

The test organism was sub cultured on Dextrose agar (M/s Hi Media Laboratories, Mumbai) slants. The slant was incubated at 37^0 C for 24 hours to get sufficient growth of the organism. The organism was sub cultured every three days to maintain its viability. The growth on agar slant was washed using 3 ml sterile normal saline. The density of microbial suspension was adjusted as per the method of Kirshbaum and Arret (1967). The microbial suspension was diluted with sterile normal saline to give 25 % transmittance at 580 nm, against normal saline as the blank. This microbial suspension was dispensed in aliquots of 1.5 ml and stored at 4^0 C until used. In the standardization experiment, 1.0 ml of culture was found suitable for 180 ml medium to get clear zone of inhibition.

The sterilized assay plate stand was levelled with adjustable screws using a spirit level for getting uniform thickness of molten medium. The sterilized large glass assay plate was placed on the stand. The molten medium was allowed to cool up to temperature of 45-50^0 C. About 1.0 ml of microbial suspension was added into the bottle containing 180 ml of the medium and was gently shaken in

clockwise and anticlockwise direction to distribute the microorganisms uniformly in the medium. Seeded medium was poured on the surface of the glass assay plate rapidly to form a uniform layer of the medium. The medium was allowed to solidify for 15-20 minutes.

Standard concentrations of Florfenicol were prepared in distilled water. Initially the standard Florfenicol powder having purity of 100 % was diluted in distilled water to give a concentration of 100 μg/ml. Subsequently serial dilutions were done to achieve concentrations of 20, 10, 5, 2.5, 1, 0.5 and 0.25 µg/ml, using distilled water. The concentrations were freshly prepared each time for the assay.

In the present study, to place the samples on the surface of the medium on large glass assay plates, wells having inner diameter of 0.8 mm were prepared using stainless steel punch and each well accommodating 100-µl tests or standard sample. All the test samples were assayed in triplicates and care was taken that the samples of standard Florfenicol in different dilutions were invariably put while each assay plate processed.

After placing the samples in respective wells, the plate was covered with the lid and kept at room temperature for 30 minutes under sterile conditions for better diffusion of antibiotic in wells and then incubated at 37^0 C for 12 hours. The zones of inhibition were measured by inverting the plate and their averages were recorded (Fig. 8).

The zones of inhibition were measured by Hi Antibiotic Zone scale C manufactured by M/s Hi Media Laboratories, Mumbai. The zone sizes were measured and a standard curve of concentration (µg/ml) versus zone of inhibition (diameter in mm) was plotted using semi-logarithmic graph paper by taking zone of inhibition on Y axis against the log of antibiotic concentrations (µg/ml) of each standard on X axis.

Fig 8 Microbial plate assay

The best-fit line (Fig. 9) joining these points had r^2 value of 0.83. The lowest detectable concentration was 0.25 µg/ml. The concentrations of Florfenicol in the test samples (lung, liver, kidney and muscles) were determined from standard curve of Florfenicol with reference to the zones of inhibition obtained after putting the test samples.

3.2 Residues of Florfenicol in eggs of layer bird

3.2.1 Experimental Birds

Egg residue of Florfenicol was studied in 56 weeks old White Leghorn chicken (Strain BV-300) weighed between 1.20 to 1.70 kg. The layers were maintained in Cage system at Layer poultry farm and fed on commercial balanced layer ration and water was provided *ad-libitum*. They were healthy, active and free from any sign of disease or disease condition at the time of experiment. Weekly egg production record of five weeks prior to the start of the treatment on percentage basis is given in Appendix IV.

3.2.2 Drug used for the study

The drug used was Florfenicol 10% and 20% oral solution. The standard Florfenicol (100 % purity) was used for preparing the standard graph of Florfenicol in egg yolk and albumin of layer chicken.

3.2.3 Design of experiment

Birds (20) included in the study were divided into two groups of ten birds each viz. Group A and Group B. Birds from Group A received Florfenicol 10% @ 30 mg/kg body weight orally for three consecutive days while birds from group B received Florfenicol 20% @ 30 mg/kg body weight orally for three consecutive

Fig 9 Graph best fit line

days. The weight of each bird and volume of Florfenicol (10% or 20% w/v) administered was recorded (Table 6).

3.2.4 Collection and processing of eggs

Daily egg collection was done from medicated birds, a day prior to Florfenicol administration and up to seven days post-treatment thereafter. Egg collection and drug administration to the birds were carried out at fixed time daily i.e. at 11.00 a. m. and 11.30 a. m. respectively. All the eggs laid by the birds of the respective groups were collected daily, for estimation of Florfenicol levels in albumin and yolk.

Egg yolk and egg albumin were processed within 24 hours for assaying Florfenicol concentration. Before collection of albumin and yolk from the egg, the egg surfaces were properly cleaned with a spirit swab and made sterile. Just prior to the assay, the eggshell was carefully broken with sterile forceps and egg albumin was carefully collected in pre-sterilized glass test-tube. After collection of albumin the egg yolk was taken out from the egg and kept on the Whatman filter paper No. 1, to remove the remnants of albumin sticking to the vitelline membrane. Thereafter the vitelline membrane was pierced and yolk was collected in the sterile glass tube.

3.3.5. Estimation of Florfenicol concentration from yolk and albumin

Concentration of Florfenicol was estimated by microbiological assay technique using large glass assay plates and *Bacillus subtilis* ATCC 6633 as the test organism. The whole procedure of the assay was followed as described in Section 3.1.6

Table 6: Weights of experimental chicken and volume of formulation (Florfenicol 10% & 20% w/v) administered for egg residue study.

Bird No.	Weight (kg)	Volume administered (ml)
Group A (10% Florfenicol)		
1.	1.220	0.37
2.	1.250	0.37
3.	1.790	0.54
4.	1.230	0.37
5.	1.460	0.44
6.	1.600	0.48
7.	1.350	0.40
8.	1.320	0.40
9.	1.440	0.43
10.	1.450	0.44
Group B (20% Florfenicol)		
1.	1.490	0.22
2.	1.600	0.24
3.	1.520	0.22
4.	1.450	0.21
5.	1.490	0.22
6.	1.380	0.21
7.	1.310	0.20
8.	1.450	0.21
9.	1.330	0.20
10.	1.660	0.25

3.3 Toxicity testing of Florfenicol in chicken

3.3.1 Experimental Birds

Toxicity of Florfenicol was studied in broiler chicken in mature plumage. The birds were procured from local market and weighing between 900-1400 grams. The birds were maintained in cage system and fed commercial balanced ration. Water was provided *ad-libitum*. They were healthy, active and free from any sign of disease or disease condition at the time of experiment.

3.3.2 Drug used for the study

The drug used was Florfenicol 20% w/v oral solution. Florfenicol 10% test formulation is not used for toxicity study.

3.3.3 Housing and Management of Birds

Before arrival of the birds, the room and all the cages were cleaned thoroughly and disinfected with 5% phenol solution. The birds were maintained in ambient temperature and humidity and adequate ventilation was provided. Throughout the period of investigation, the birds were reared under standard managemental condition. Individual caging is preferred to identify birds regurgitating the dose and to prevent fighting. Each cage was supplied with separate feed and water troughs along with tray underneath the cage for collection of excreta.

3.3.4 Design of experiment

The toxicity of Florfenicol (20% w/v florfenicol formulation) was assessed by carrying out two steps i.e. a limit dose study in five birds in first step and in second step, four birds were administered the doses of Florfenicol liquid as per the Stage 1 of the Appropriate D-optimal design referring Draft OECD guidelines 223.

3.3.4.1 STEP 1

Florfenicol is an antibiotic and was expected to have very high lethal dose. As there were no data available, it was decided to select 2000 mg/kg body weight (i.e. limit dose) as the starting dose for estimating acute toxicity by Avian Acute Oral Toxicity Test (Anon, 2002b), with limit dose test.

Florfenicol liquid was administered as a single oral dose by gavage to each bird. Syringes of 5 ml and 10ml capacity were used. It was administered at 2000 mg/kg body weight into the proventriculus. The total volume administered to each of the chicken was as per Table 7.

Feed restriction was followed for twelve hours prior to Florfenicol liquid administration. After administration birds were monitored individually for any clinical symptoms continuously for first 30 minutes of administration and at hourly intervals for next 24 hours and daily thereafter for a total of 14 days. Post mortem and gross pathological examination was performed immediately in dead birds and the lesions were recorded.

3.1.4.2. STEP 2

The next step was carried out again in four new birds as per the Stage 1 of the Appropriate D-optimal design (OECD guidelines).

Stage 1 of the Appropriate D-optimal Design was followed, as per Draft OECD guidelines. In the 1st stage the doses were equally spaced around the initial estimate of the LD_{50} and the ratio of the highest to the lowest dose was set to 50. Each dose of the test substance was given to a single bird. Thus in this step four birds for four different doses were selected. The procedure for oral administration of Florfenicol liquid followed in this step was similar to that of Step 1, using a tuberculin syringe and syringes of 5ml and 10ml capacity.

Calculations regarding the dose selection are elaborated in Appendix V. The total volume administered to each bird was as per Table 8.

Table 7: Volume of drug administered to the chicken –Step 1.

Sr. No.	Initial body Weight (kg)	Dose rate (mg/kg)	Total dose administered (mg)	Total volume administered (ml)
1	1.3	2000	2600	13
2	1.3	2000	2600	13
3	1.4	2000	2800	14
4	1.3	2000	2600	13
5	1.2	2000	2400	12

Table 8: Volume of drug administered to the chicken- Step 2.

Sr. No.	Initial body Weight (kg)	Dose rate (mg/kg)	Total dose administered (mg)	Total volume administered (ml)
1	1.1	251.69	281.90	1.44
2	1.1	793.74	873.11	4.37
3	1.0	2920.96	2920.96	14.61
4	0.9	3330.00	3023.03	15.12

4. RESULTS AND DISCUSSION

4.1 Residues of Florfenicol in birds

In the present study, residues of Florfenicol were estimated in tissues (liver, lung, kidney and muscle) of broiler chickens and eggs (albumin and yolk) of layer birds. All the birds were healthy and no clinical signs of diseases were seen at the time of experiment. The birds were administered the dose of 30 mg/kg body weight orally to individual birds using oral gavage. The dose of 30 mg/kg body weight used in the present study was selected from the available literature (Afifi & Abo el-Sooud, 1997; Anon, 1998; El-Banna, 1998; Shen *et al.,* 2002; Shen *et al.,* 2003 and El-Banna *et al.*, 2007). In chicken it's customary to administer drug either through feed or water. Florfenicol, due to its higher water solubility and efficacy against *E.coli*, is administered through water. Water medication to a flock always carries a possibility of non-uniform consumption and hence that of non-uniform medication in birds (Giguire *et al.,* 2006). In order to avoid this possibility it was preferred to administer drug individually to each bird. The exact dosing and administration was assured due to the use of oral gavage method in the present study.

4.1.1 General observations

The formulations administered was syringable, so the drug could be administered within five seconds and the chickens tolerated formulations very well at the dose of 30 mg/kg body weight and there were no signs indicative of any adverse effects were observed.

4.1.2 Microbiological assay of Florfenicol

The concentrations of Florfenicol in tissue and egg were assayed microbiologically using large glass plate by punching wells on the surface of the seeded medium in such a way that each well accommodated 100 µl of the sample.

The bioassay method can be used as a routine screening method for the presence of antibiotic in tissues and eggs (Hee-Jung *et al.,* 2007).

The test organism used for the assay was *Bacillus subtilis* (ATCC 6633). El- Banna (1998) and Afifi and Abo el-Sooud (1997) had used the same organism for Florfenicol assay in serum of Muscovy duck and plasma of the broiler chicken respectively.

The chemical methods of analysis take into account both biologically active as well as inactive component of an antibiotic. Microbial assay always accounts the biologically active component. It fails to measure the activity attributable to the metabolite (if any). However, Sams (1994) reported that the presence of metabolites which are microbiologically active may not necessarily interfere with determination of therapeutic dosage regimen.

Time within which the material should be processed for assay varies with the stability of antibiotic. Florfenicol is stable at room temperature as well as in drinking water. Stability of Florfenicol remains above 90% after 24 hrs of its dilution (Hayes *et al.,* 2003). Considering this, the samples were stored at -20^{o}C (Shen *et al.,* 2003) and processed for microbiological assay within a period of five days after collection.

4.1.3 Sensitivity of the assay method

In the present study, lowest limit of detection of Florfenicol by the microbial assay method was 0.25 µg/ml. The sensitivity of the assay method was somewhat less than the other workers. Afifi and Abo el-Sooud (1997) reported that the lower limit of quantification of Florfenicol in plasma, bile and tissue was 0.01µg/ml using *Bacillus subtilis* (ATCC, 6633) as test organism. Whereas according to El-Banna (1998) and El-Banna *et al.* (2007) the lower detectable limit of Florfenicol was 0.050 µg/ml using the same test organism in tissue. However, MIC of Florfenicol against the important pathogens of poultry is much higher than the sensitivity of the assay in the present work. The MIC_{50} of

Florfenicol against *E.coli* is 4 µg/ml (Sarah and Jeffrey, 2000) and against *Salmonella* is 6.3 µg/ml (Neu and Fu, 1980). The r^2 value of the present study was 0.83 indicating good correlation between the concentration and zone size.

4.1.4 Florfenicol concentration

4.1.4.1 Florfenicol concentration in tissue

The concentration of Florfenicol in different tissues of birds after single dose administration @ 30 mg/kg are presented in Table 9. The muscle and organ samples i.e. lung, liver and kidney were processed as per the method followed by Bielecka (1981). Each ml of test sample represented the antibiotic activity contained in 0.5 g of tissue (as PBS was added in the ratio 1:2). The mean Florfenicol concentrations per gram of liver, kidney, lung and muscle of individual bird are presented in Appendix VI considering the conversion factor and these were 0.58±0.058, 0.62±0.095, 0.56±0.070 and 0.51±0.050 µg/g from the birds administered with 10% Florfenicol and after administration of Florfenicol 20%, levels of Florfenicol were 0.49±0.056, 0.54±0.049, 0.47±0.046 and 0.45±0.054 µg/g in liver, kidney, lung and muscle, respectively indicating distribution of Florfenicol at these sites and Florfenicol hence can be used to combat the infections caused by susceptible organisms at these sites.

Estimating concentrations of drug in tissues like muscle, liver, lung, kidney etc. gives idea about the residues that are likely to persist in these tissues after treating the birds. On one hand these data are useful to know the tissue targets at which the antibiotic exerts its antibacterial activity whereas on the other hand it also warns about the period up to which the consumption of the treated birds should be avoided.

Pilot studies revealed that Florfenicol was detectable in the tissues up to 8 hours post-administration of a single oral dose (30 mg/kg). Hence the liver, muscles, lungs and kidney tissues of the birds in Group A and B were collected at 8 hours after administration of 10 and 20% Florfenicol formulations respectively.

Table 9: Florfenicol concentration (µg/g) in the organ samples of medicated experimental broiler birds, eight hours after single oral administration of Florfenicol 10% & 20% w/v at 30 mg / kg body weight (Mean ±S.E.).

Organ	Mean Concentration of Florfenicol (µg/g) (n=6)	
	GROUP A (Florfenicol 10% w/v@ 30 mg/kg)	GROUP B (Florfenicol 20% w/v@ 30 mg/kg)
Liver	0.58 ± 0.058	0.49 ± 0.056^{NS}
Kidney	0.62 ± 0.095	0.54 ± 0.049 ^{NS}
Lung	0.56 ± 0.070	0.47 ± 0.046 ^{NS}
Muscle	0.51 ± 0.050	0.45 ± 0.054 ^{NS}

NS: Non significant

The route of administration as well as method of administration influences the duration for which the drug is detected in tissues.

When Florfenicol was administered @ 30 mg/kg body weight for five consecutive days by oral or intramuscular route in broiler chicken, it persisted in the tissue for 72 hours after the last treatment as reported by Afifi and Abo el-Sooud (1997) whereas according to El-Banna (1998), Florfenicol when administered @ 30 mg/kg body weight intramuscularly for five days, was detected in the kidney, bile and liver of *Pasteurella* infected Muscovy duck only on day 7 after treatment. In a similar study conducted by El-Banna *et al.* (2007) in healthy and *E.coli* infected broiler birds, Florfenicol was detected upto 6^{th} day in all the tissues except in liver where it was detectable even on the 7^{th} day after discontinuation of medication. In a study conducted by Adams *et al.* (1987), Florfenicol was administered @ 11 mg/kg body weight intravenously and orally to male veal calves every 12 hours for 7 doses. After the 7^{th} dose administration, two calves were sacrificed at 4, 8 and 12 hours. It was observed that the concentration of Florfenicol were detectable upto 12 hours of the study and highest concentration were observed at 4 hours of the study. According to Jiancheng *et al.* (2006), when swines were administered Florfenicol @ 20mg/kg body weight at 24 hours interval, the sum of Florfenicol and Florfenicol amine concentrations in all tissues analyzed was below the accepted MRL at 8 days post treatment while Lane *et al.* (2007), reported that by day 40, all the tissue samples were below the tolerance level when sheep were administered Florfenicol @ 40 mg/kg body weight subcutaneously for three days. However, in the present study levels of Florfenicol were detectable in the different organs till eight hours post-treatment. The difference could be attributed to the fact that only single dose was administered to birds and also possibly to the formulation used in the present study.

The various tissues analysed in the present study include muscle, liver, lung and kidney. Muscle and liver are the edible tissues in poultry and so these tissues were analysed considering possible exposure to human being through consumption of these tissues. Florfenicol shows high efficacy against *E.coli* induced Air Sacculitis and hence it was thought to estimate concentration in lungs. *Salmonella* is another important pathogen in which the predilection site is liver. In spite of kidney not being an edible tissue, they usually remain with the chicken

carcass and can be potential risk for the consumers (Pavlov *et al.,* 2008). Considering all these reasons, the various organs were chosen for analysis in the present study.

The highest concentration of Florfenicol was observed in kidney in the present study which were in accordance with the findings of Adams *et al.* (1987); Afifi and Abo el-Sooud (1997); El-Banna, (1998) and Jiancheng *et al.* (2006) (0.62±0.095 µg/g and 0.54±0.049 µg/g for 10% and 20% Florfenicol test formulation respectively). Whereas in contrast, Anon, (1999a) and Lane *et al.* (2008) reported that concentration of Florfenicol was found to be highest in liver.

The statistical comparison of the levels of Florfenicol in different tissues after administration of 10% and 20% formulation revealed non-significant difference in Student's 't' test indicating that both 10% and 20% formulations were handled uniformly by the body during distribution. Numerical consideration however, indicated that the mean values of Florfenicol concentration in Group B were slightly lower than Group A, the reason for which could not be traced out.

The MRLs of Florfenicol in muscle, kidney and liver is 100, 750 and 2500 µg/kg respectively (Anon, 1999a). In the present work, the concentration of Florfenicol was 620 µg/kg in kidney and 580 µg/kg in liver which are much below the specified MRLs whereas in muscle, the concentration of Florfenicol (510 µg/kg) was higher than the established MRL.

4.1.4.2 Florfenicol concentration in eggs

The levels of Florfenicol at various days were estimated in egg yolk and albumen separately (Table10) taking into consideration selective food habits of human beings. When antibiotic is administered to chicken, the practice of continuous administration through feed and water is followed under field condition for consecutive 3-5 days. In light of this, Florfenicol was administered for three consecutive days.

Table 10: Florfenicol concentration (µg/ ml) in the eggs of medicated experimental layer birds after oral administration of Florfenicol 10% & 20% w/v at 30 mg / kg body weight (Mean ±S.E.)

Time (Days)	Group A (n=6)		Group B (n=6)	
	Egg albumin	Egg yolk	Egg albumin	Egg yolk
0	ND	ND	ND	ND
1	ND	ND	ND	ND
2	0.54±0.071	ND	0.83±0.15	ND
3	0.70±0.095	ND	0.71±0.13	ND
4	0.57±0.11	ND	0.72±0.14	ND
5	ND	ND	ND	ND
6	ND	ND	ND	ND
7	ND	ND	ND	ND

ND: Not detected

The weekly egg production data of the birds included in the study assures that birds were maintained under optimal managemental condition. In each of the Group A and B, ten birds were included with the view to get atleast 6 eggs/day/group. Table 11 represents mean data after processing 6 eggs/group. The eggs were processed within 24 hours of collection.

Scanning of literature indicated that immediately after egg collection, albumen and yolk are separated and preserved separately (Sisodia and Dunlop, 1972). This is probably for avoiding the possible diffusion of drug from one compartment to the other during storage. However, in the present study eggs were stored as a whole without separating egg albumen and yolk prior to storage. The levels of Florfenicol were detected in egg albumin from day 2-4 of the treatment in both the groups. The mean concentration of Florfenicol in egg albumin from the birds of Group A were 0.54, 0.70 and 0.57 µg/g whereas in Group B were 0.83, 0.71 and 0.72 µg/g on day 2^{nd}, 3^{rd} and 4^{th} respectively. The peak level of Florfenicol (0.70 µg/g) was observed on 3^{rd} day in Group A whereas on 2^{nd} day (0.83 µg/g) in Group B.

Florfenicol was not detected in egg yolk samples at any time. The difference in Florfenicol concentration in albumin and yolk can be explained accounting the fact that the process of egg formation requires 24 hours. Egg yolk is formed gradually and it has a turnover time of 6-8 days whereas albumin formation is an immediate process occurring in magnum about 20 hours before laying of eggs.

Goria *et al.* (1997); Furusawa (1999, 2001) and Kan (2003) attempted to explain the partitioning of drug in yolk and eggs and stated that fat soluble compound gradually occur in yolk. The other factors controlling distribution of drug in yolk and white were stated as pKa value of the drug, pH of egg yolk and white and protein binding of the drug (Kan, 2003). However, despite high lipid solubility Sulphonamides showed high levels in egg white in a study conducted by Kan, 2003. Florfenicol also has high lipid solubility allowing its distribution to various tissues but it fails to appear in yolk. The correlation between higher lipid solubility and concentration in egg was also not observed in case of Tetracyclines group of antibiotic.

Table 11: Florfenicol concentration (µg/ g and µg/kg) in the egg albumin of medicated experimental layer birds after oral administration of Florfenicol 10% & 20% w/v at 30 mg / kg body weight for consecutive three days (Mean ±S.E.).

Time (Days)	Florfenicol concentration in albumin (µg/g)		Florfenicol concentration in albumin (µg/kg)	
GROUP	**A**	**B**	**A**	**B**
0	ND	ND	ND	ND
1	ND	ND	ND	ND
2	0.54	0.83	540	830
3	0.70	0.71	700	710
4	0.57	0.72	570	720
5	ND	ND	ND	ND
6	ND	ND	ND	ND
7	ND	ND	ND	ND

(1 ml albumin = Approx. 1 gm when weighed actually)

Florfenicol is not recommended for use in layers in United States and the data of Florfenicol on eggs were not traceable. However, studies on its closely related antibiotic such as Thiamphenicol and Chloramphenicol were conducted by Giorgi *et al.* (2000) and Sisodia and Dunlop (1972) respectively. Levels of Thiamphenicol in egg yolk were much lower than levels in albumin. However, the persistence of Thiamphenicol in yolk was for longer period i.e. for ten days after single oral dose administration and for eight days after terminating multiple (five) doses. The maximum level following multiple oral dose of 40 mg/kg/day for five consecutive days was 356.64±86.23 µg/kg on day 8th after initiating the treatment.

On the other hand in another study conducted by Sisodia and Dunlop (1972), the levels of Chloramphenicol residues in yolk was higher than in albumin wherein concentration of Chloramphenicol in yolk reached a peak of 0.33 ppm on the sixth day, eight hours following withdrawal of medicated water and gradually fell to 0.14 ppm on the tenth day. In albumin, the drug concentration was <0.1 ppm on day one, went up to 0.12 ppm on 2nd day, reached up to 0.17 ppm on day four. Following withdrawal of the drug at 120 hours, the concentration in albumin slowly declined and fell sharply to undetectable level on day nine.

Acet *et al.* (1989) found appearance of Chloramphenicol in eggs in dose dependent manner. At 20 mg/kg, there were no residues in eggs whereas the levels were 5-20 ng/g at 40mg/kg dose and 58-197 ng/kg at 400 mg/kg dose.

Kan (2003) stated that residues of drug in yolk generally require exposure for about 8-10 days to reach a constant level. In the present study, Florfenicol was administered only for three days considering the therapeutic schedule suggested for Florfenicol and this could probably be one of the reasons for non-detection of Florfenicol in yolk.

4.2 Toxicity testing of Florfenicol in chicken

The Acute toxicity studies were conducted in chicken as per OECD guidelines and these aimed at estimating LD_{50}. The LD_{50} was calculated as 1597 mg/kg body weight.

All the birds were healthy and free from all clinical signs of disease or disease conditions at the time of experiment. The drug used for this study was Florfenicol 20% w/v oral solution. Florfenicol 10% w/v oral solution was not administered to the birds because the volume of drug to be administered to the birds was beyond the capacity of the birds. The dose of drug to be administered to the birds should not exceed 10ml/kg body weight (Anon, 2002b). As the volume of drug to be administered in 10%formulation calculated was above 20 ml which was beyond the capacity of the birds, 10% w/v oral solution was not administered.

The study included 2 Steps. In the first step after administration of Florfenicol @ 2000mg/kg, all birds showed the symptoms of dullness and transient depression. They huddled at the corner of cage. Observation after first and second hour revealed the same symptoms. Birds were off-feed. After twelve hours, the birds were extremely depressed, sluggish and anorexic. Birds were lying down with twisted neck (Fig.10). Symptoms of gasping were also noted. There was greenish watery diarrhoea in all birds (Fig.11).

Mortalities were observed in three birds out of five, in one bird on 2nd day and in two birds on 3 rd day (Table 12). All the three birds were subjected to post-mortem and gross pathological lesions were recorded. Externally, the beak was open and slight discharge was seen from nostrils. Internally, the liver showed pale areas of necrosis (Fig.12), gall bladder was engorged with bile (Fig.13), intestines were haemorrhagic and congested (Fig.14), congestion in lungs and kidney, haemorrhages in proventriculus and in heart ventricles were empty and blood clot in auricles.

Fig. 10 Twisting of neck after administration of limit dose (2000mg/kg)

Fig.11 Greenish watery diarrhoea after administration of limit dose (2000mg/kg) in chicken.

Table 12: Record of mortalities in chicken administered 2000 mg/kg Florfenicol in Step 1

Dose (mg/kg)	Number of birds used	Mortality after dosing						
		Hour(s)		Day(s)				
		½ - 4	-	1	2	3	4-7	8-14
2000 mg/kg of Florfenicol liquid	5	Nil	Nil	Nil	One bird died	Two birds died	Nil	Nil

Fig. 12 Pale areas of necrosis on the liver

Fig. 13 Gall bladder engorged with bile.

Fig. 14 Hemorrhage and congestion on intestines

Considering the flexibility that could be previledged while proceeding to Step 2 as per this guideline, it was decided to estimate only LD_{50} without estimating the slope of Dose Response curve. Four birds were included and individual bird exposed to different doses.

Birds which received dose 3 and dose 4 appeared dull and depressed. They were unable to move, reluctant to eat and there was greenish watery diarrhoea. Both birds died on 2nd day after administration of Florfenicol as per Table 13. These birds were subjected to postmortem examination and gross external and visceral examination was carried out and necropsy findings were similar to Step1. Birds receiving Dose 1 and Dose 2 were normal and did not exhibit any clinical signs.

This test Guideline is designed to estimate the acute oral toxic dose of substances in birds. This method provides a procedure for estimating precise LD_{50} which is required for hazard assessment of the compound.

The studies in laboratory animals after repeat dose administration are relevant for risk assessment to the human beings considering the possibility of chronic exposure of human population to Florfenicol residues through consumption of meat. However, the target animal for therapeutic purpose i.e. chicken is never likely to get exposed to the drug chronically and overdosing due to human error is the only risk seems to be associated with Florfenicol in poultry. Considering this, acute toxicity studies were carried out in poultry.

The advantages of using this method lies in the fact that it provides a sequential testing procedure that optimizes the placement of doses and matches the precision of the endpoint with the precision required for hazard assessment and labeling. Moreover, the method has been designed in a way to minimize the numbers of birds used (Anon, 2002b).

Table 13: Record of chicken mortalities in Step 2 of Acute toxicity study as per OECD guidelines

Dose (mg/kg)	Number of birds used	Mortality after dosing						
		Hour(s)		Day(s)				
		½ - 4	-	1	2	3	4-7	8-14
Dose 1	1	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Dose 2	1	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Dose 3	1	Nil	Nil	Nil	Nil	**M**	---	---
Dose 4	1	Nil	Nil	Nil	Nil	**M**	---	---

Note: M indicates Mortality

The estimated LD_{50} value is 1597 mg/kg body weight i. e. its lethal dose lies between 0.5-5 g/kg body weight Florfenicol liquid, therefore, can be classified as belonging to the Class of Slightly toxic compound for chicken (Garg,2002). The therapeutic dose of Florfenicol is 30 mg/kg in poultry. Thus LD_{50} is almost 50 times the therapeutic dose assuring remote possibility even of acute toxicity in poultry.

5 SUMMARY AND CONCLUSIONS

Florfenicol is a fluorinated analogue of Thiamphenicol used in Veterinary medicine. It was first introduced in the markets as injectable solution for treatment of respiratory diseases in cattle and now it is introduced in some countries as oral solution for treatment of several poultry diseases. The antimicrobial properties of Florfenicol indicate that it may become valuable antibiotic in the treatment of infectious diseases in poultry. Taking into consideration its possible use in poultry, it was decided to generate data on Florfenicol residue levels in tissue and egg and to study its acute toxicity in chicken.

For residue studies in broilers, Florfenicol 10% and 20% were administered 30 mg/kg, orally. The concentrations of Florfenicol in liver, kidney, lungs and muscle of medicated birds were estimated by microbiological assay. Florfenicol levels estimated were 0.58 ± 0.058, 0.62 ± 0.095, 0.56 ± 0.070 and 0.51 ± 0.050 µg/g from the birds administered with 10% Florfenicol and after administration of Florfenicol 20%, levels of Florfenicol were 0.49 ± 0.056, 0.54 ± 0.049, 0.47 ± 0.046 and 0.45 ± 0.054 µg/g in liver, kidney, lung and muscle respectively.

To estimate levels of Florfenicol in egg, Florfenicol 10% and 20% were administered at the dose of 30 mg/kg, orally for consecutive three days to layer chicken. The concentrations of Florfenicol in egg yolk and albumin from the eggs of medicated birds were estimated by microbiological assay. The levels of Florfenicol were detected in the egg albumin from the day second up to day fourth of the treatment in both the groups. Mean concentrations of Florfenicol in egg albumin collected from birds of Group A were 0.54, 0.70 and 0.57 µg/ml and 0.83, 0.71 and 0.72 µg/ml in Group B on 2^{nd}, 3^{rd} and 4^{th} day respectively. Throughout the study, Florfenicol was not detected in egg yolk.

Acute oral toxicity study was performed with Florfenicol liquid (20%) in chicken as per the Draft guidelines of OECD 223 (October 2002). The study was performed in two steps Step 1 involved exposure of five broiler birds to a limit dose of 2000mg/kg. Results of the first Step led to selection of doses of 251.69,

793.74, 2920.96 and 3330.00 mg/kg to each one of the four birds included in Step2 and after analysis of the mortality data, LD_{50} was estimated as 1597 mg/kg.

Based on the above findings, it was concluded that:

- After oral administration of Florfenicol to broiler chicken, at a dose of 30 mg/kg body weight, biologically active levels of Florfenicol were detectable in the liver, kidney, lung and muscle, of the birds sacrificed, till eight hours after the single dose treatment, when analyzed by microbiological assay having detection limit of 0.25 µg/ml. Florfenicol was not detected in these tissues at 10, 12 and 24 hours post-treatment. Hence, a withdrawal period of one day of the drug is recommended before slaughter of the birds for the formulation tested.

- After oral administration of Florfenicol to layer chicken, at a dose of 30 mg/kg body weight for three consecutive days, biologically active levels of Florfenicol were detectable in the egg albumin from 2^{nd} to 4^{th} day of the treatment period when analysed by the microbiological assay having detection limit of 0.25 µg/ml. Florfenicol was not detectable in the eggs of chicken at 48 hours following completion of the treatment. If used extralabely the eggs should be consumed only after 24 hours after the last treatment.

- The therapeutic dose of Florfenicol is 30 mg/kg body weight in poultry. Thus LD_{50} value is almost 50 times the therapeutic dose assuring remote possibility even of acute toxicity in poultry. Since the lethal dose of Florfenicol lies between 0.5-5 g/kg body weight. Therefore, it can be classified as belonging to the Class of Slightly toxic compound for chicken.

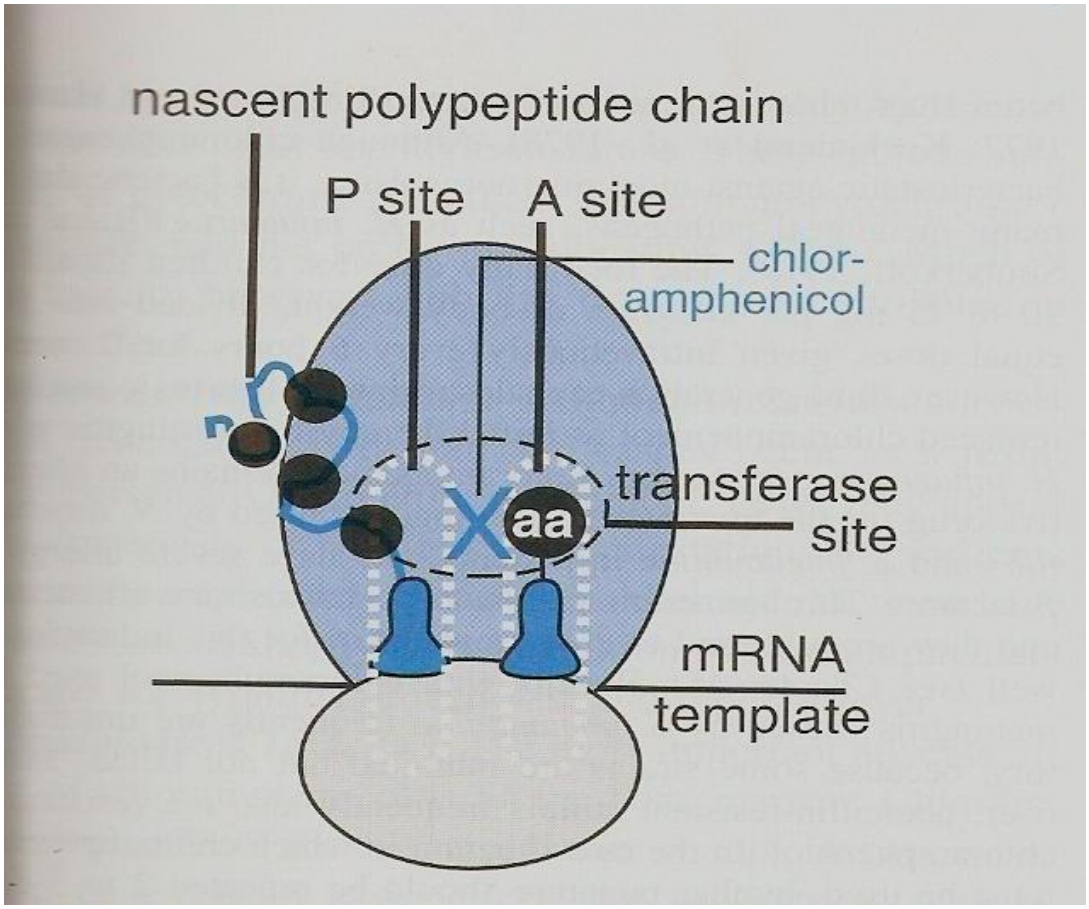


Fig. 4 Site of inhibition of protein synthesis by Amphenicol

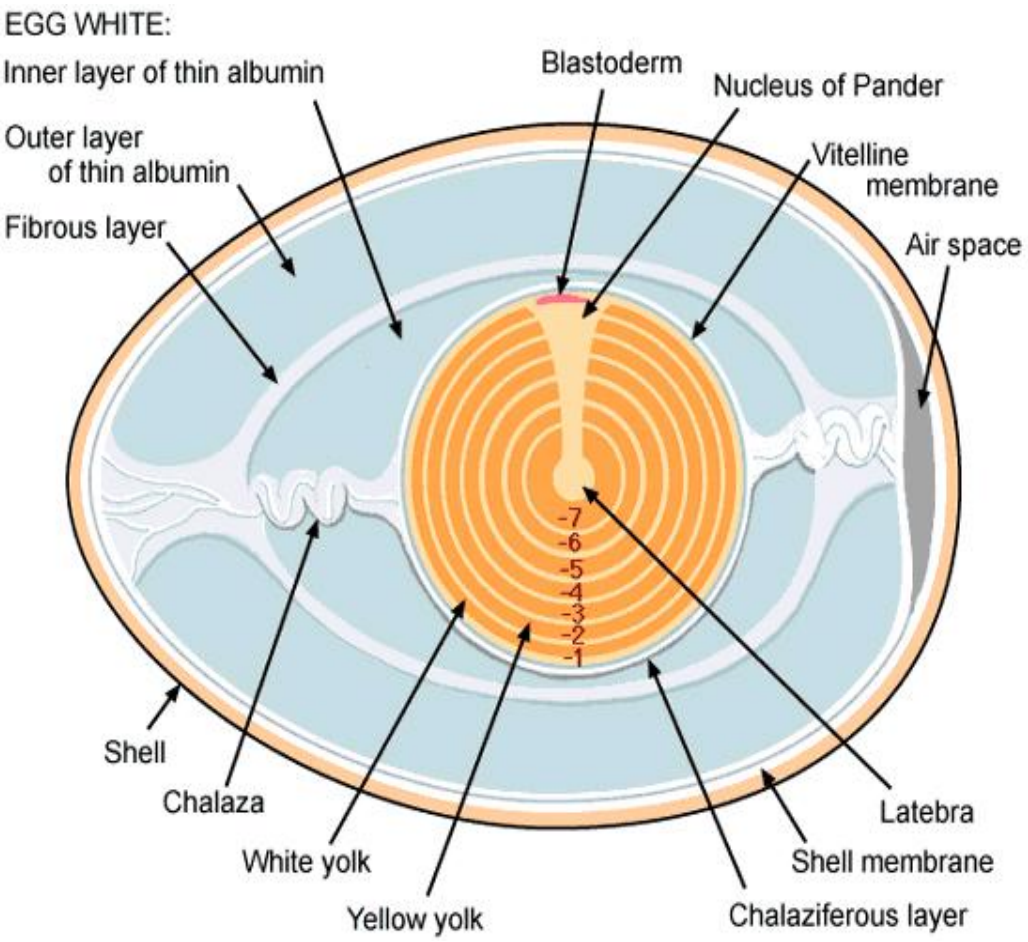


Fig. 5 Components of egg

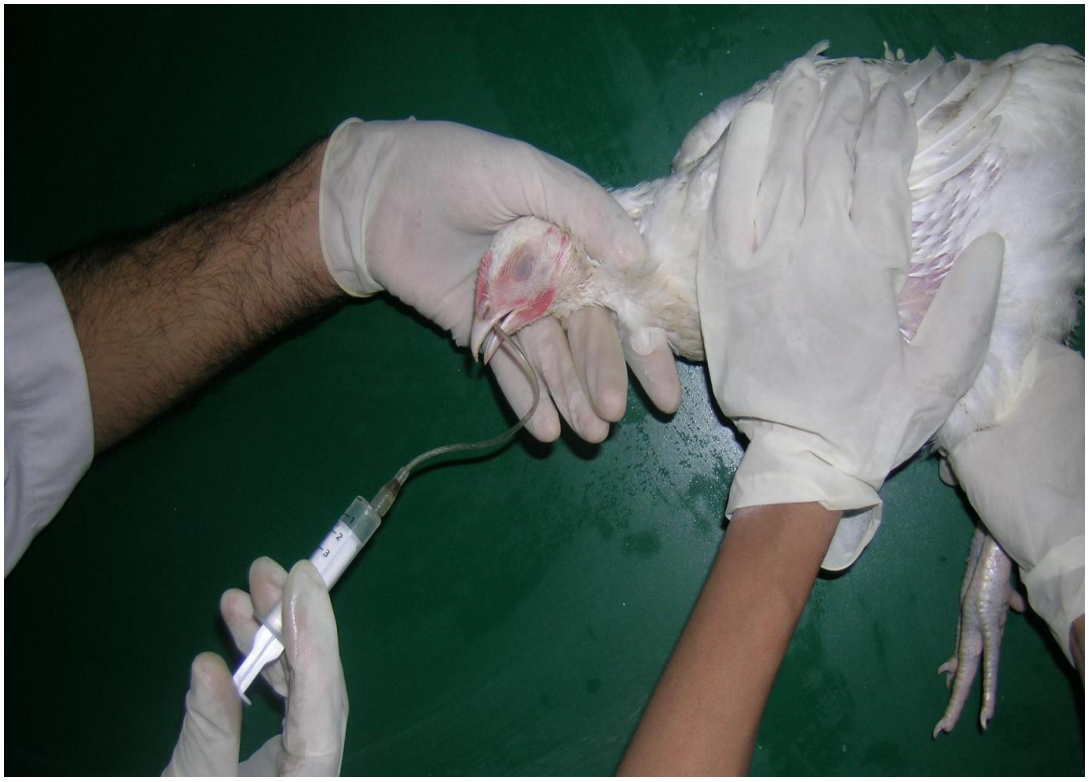


Fig. 7 Oral administration of Florfenicol to bird using oral gavage

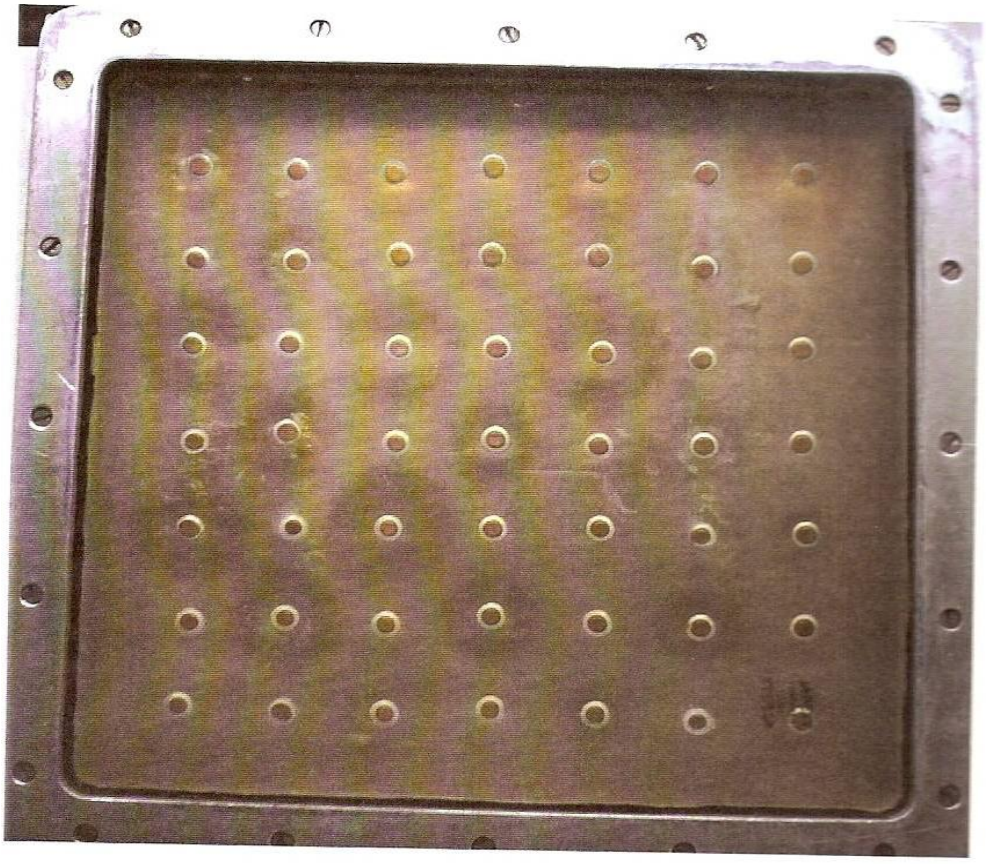


Fig. 8 Large glass plate showing zones of inhibition by Florfenicol

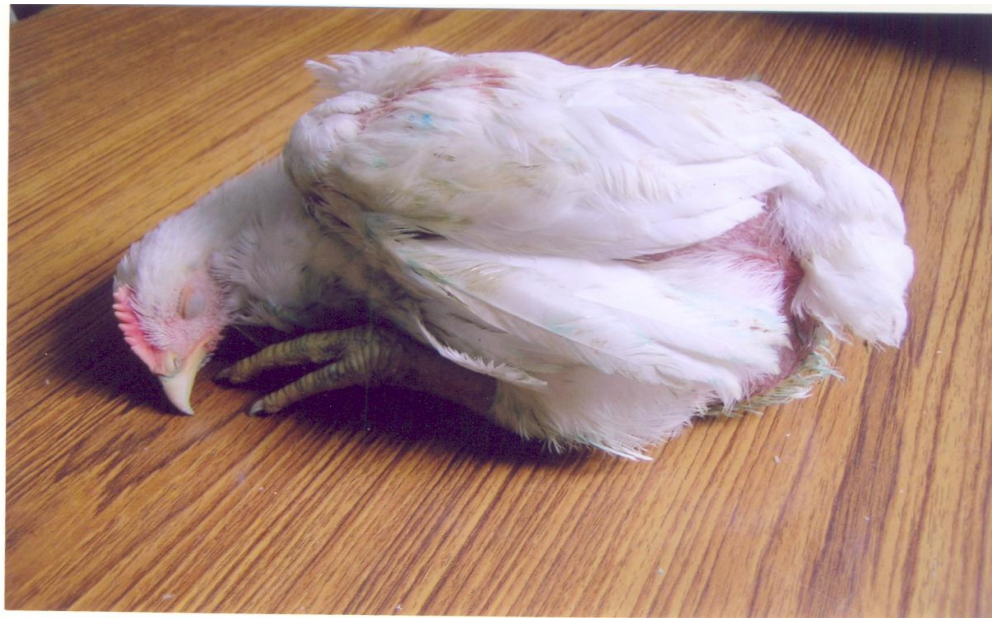


Fig. 10 Twisting of neck after administration of limit dose (2000mg/kg) in chicken.

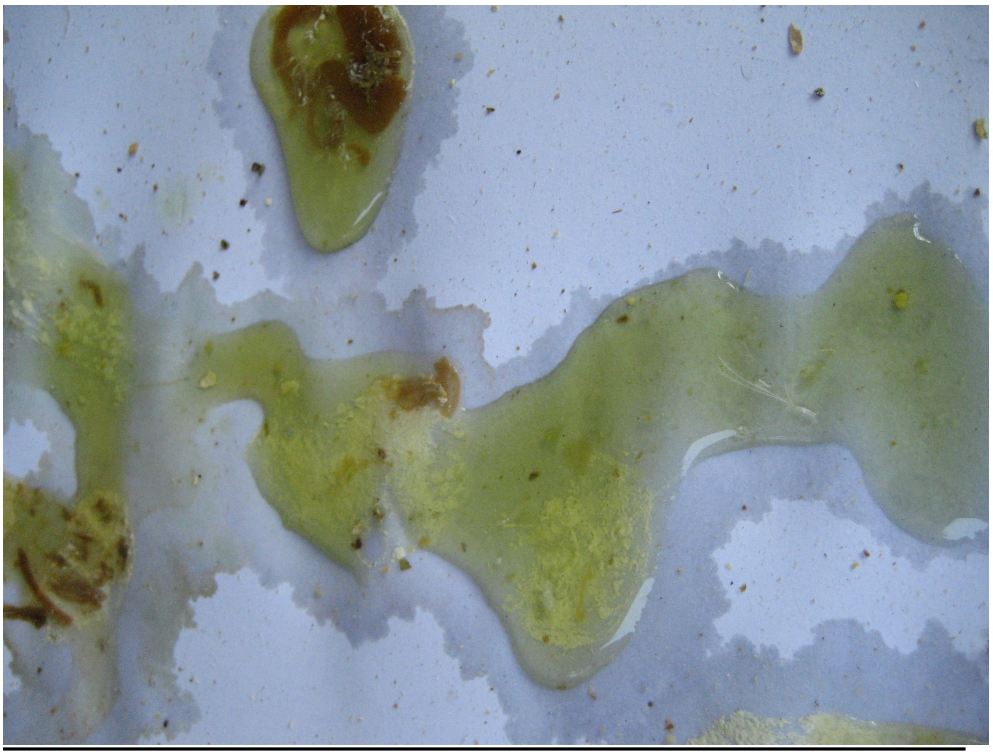


Fig.11 Greenish watery diarrhoea after administration of limit dose (2000mg/kg) in chicken.

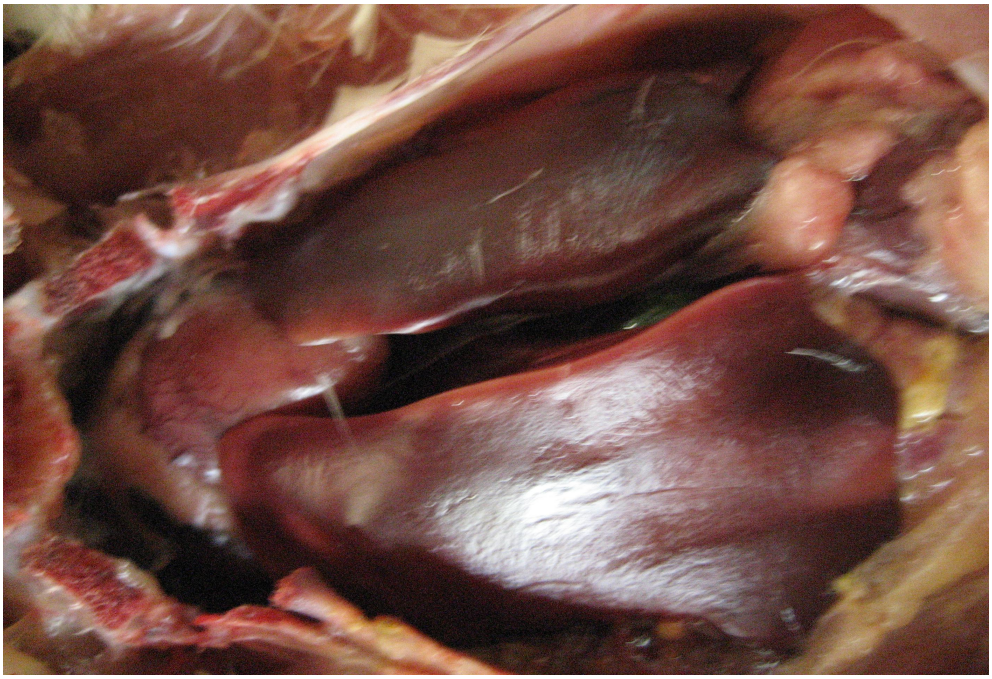


Fig. 12 Pale areas of necrosis on the liver

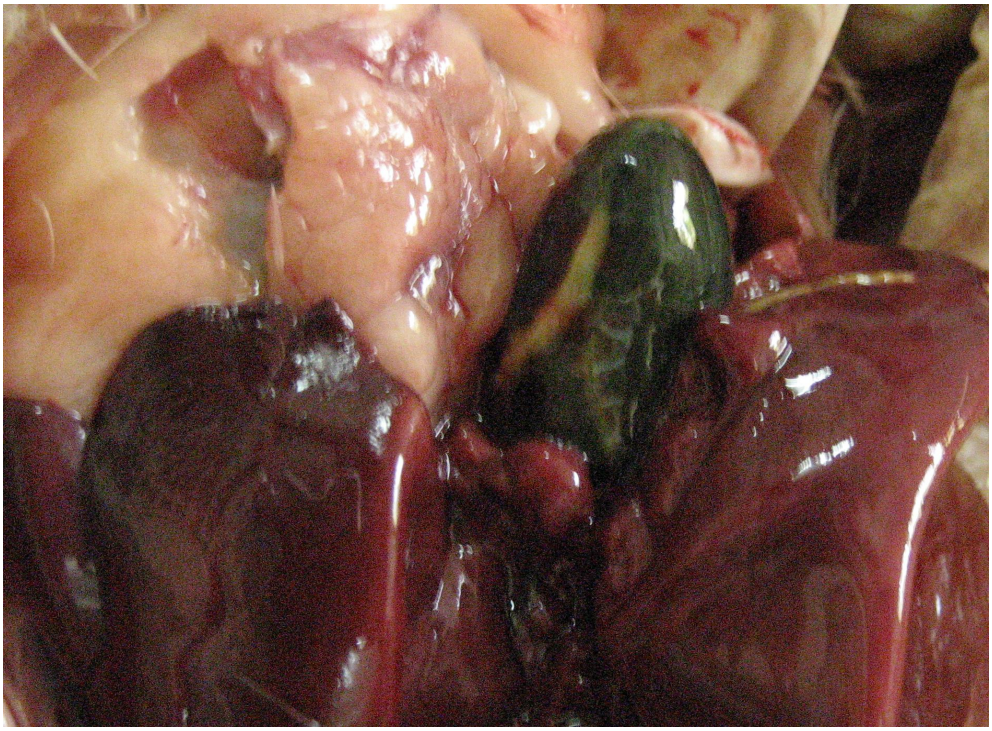


Fig. 13 Gall bladder engorged with bile.

Fig. 14 Hemorrhage and congestion on intestines

Table 3: Functions of the oviduct (Ensminger, 1992)

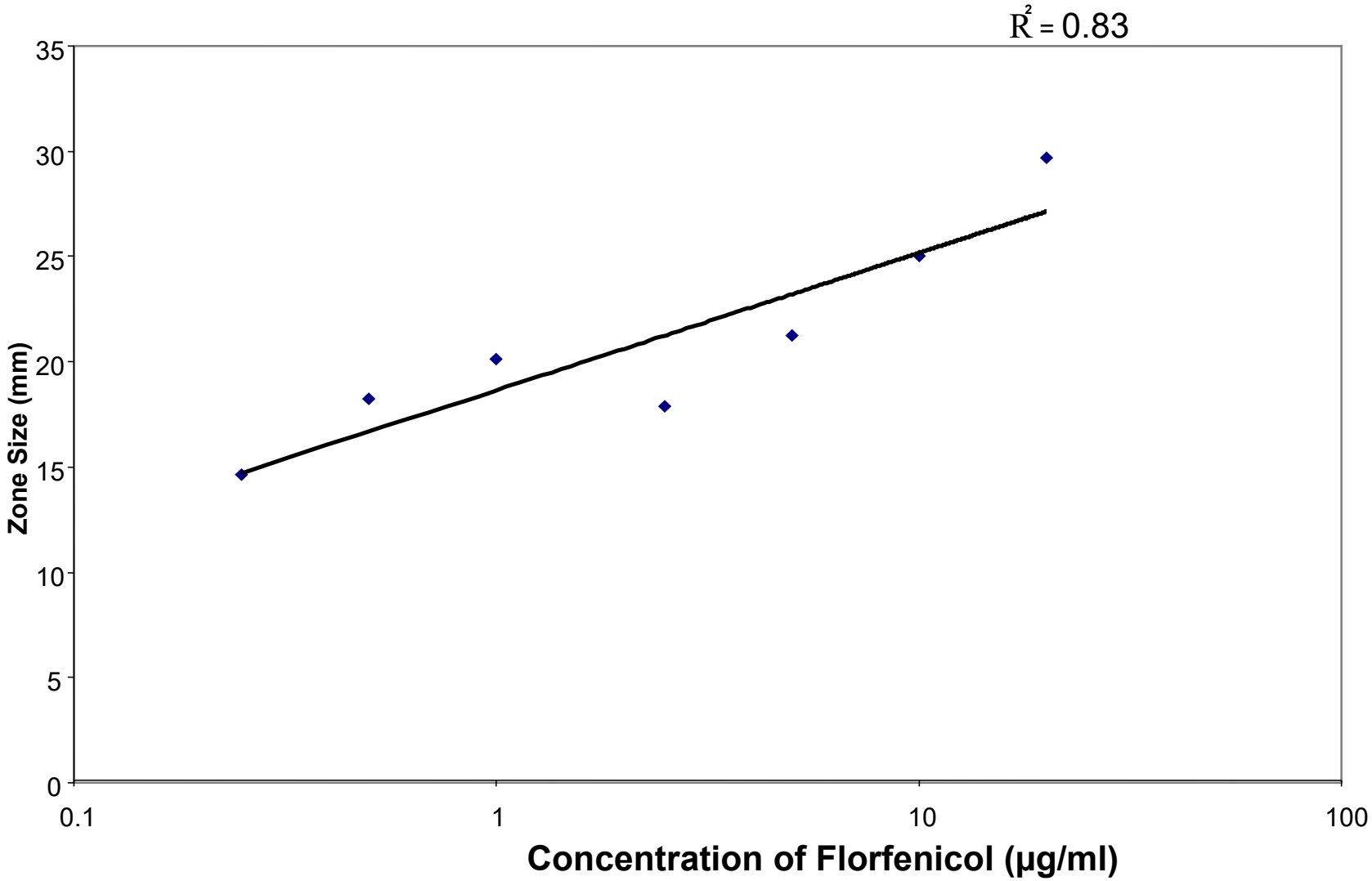
Sl. No.	Part	Approximate time egg spends in section	Functions
1	Infundibulum (funnel)	15 minutes	Picks up yolk from the cavity after it is released fro the follicle. If live sperm are present, fertilization occurs in this section.
2	Magnum (albumen secreting region)	3 hours	Thick white (albumen) is deposited around the yolk. This layer later forms the chalaziferous layer, the chalaza, and inner thin and thick white
3	Isthmus	1½ hours	Inner and outer shell membranes are added and some water and mineral salts. These membranes give some protection to the egg contents from outside contamination
4	Uterus (shell gland)	21 hours	During the first part of the egg's stay in the shell gland, water and minerals pass through the shell membranes into the white, inflating the egg and giving rise to the outer layer of the thin white. Soon after the egg is inflated, the shell gland starts to add calcium over the shell membranes, containing this process until just prior to laying. If the shell I going to be colored, pigment is added in this section.
5	Vagina	Entire time from ovulation to laying is slightly more than 24 hours	The egg passes into this section just prior to laying. function is not known.

Table 4: Literature data on drug residues in white and yolk of egg (Kan, 2003)

Compound name	pKa value	Content in white (mg/kg)	Content in yolk (mg/kg)	Ratio white/yolk	Exposure way	Author
Chloramphenicol	5.5	2	10	0.2	400 mg/l water for 10 days	Arnold and Somogyi (1986)
		0.35	2	0.17	500 mg/kg feed for 14 days	Furusawa (2001)
		0.05	0.2	0.3	200 mg/kg feed for 5 days	Samouris *et al.* (1998)
		0.5	1.5	0.3	500 mg/kg feed for 5 days	
		0.5	2.5	0.2	800 mg/kg feed for 5 days	
		1.2	4	0.3	1000 mg/kg feed for 5 days	
		0.15	0.2	0.8	40 mg/L water for 5 days	Sisodia and Dunlop (1972)
Sulfonamides						
Sulfanilamide	10.5	35	43	0.8	1000mg/l water for 8 days	Blom (1975)
Sulfadiazine	6.5	0.22	0.14	1.6	200mg/l water for 5 days	Atta and El-zeini (2001)
		0.32	0.18	1.8	400mg/l water for 5 days	
Sulfadimidine	7.5	56	45	1.2	1000 mg/l water for 8 days	Blom (1975)

Compound name	pKa value	Content in white (mg/kg)	Content in yolk (mg/kg)	Ratio white/yolk	Exposure way	Author
		19	19	10	500 mg/kg feed for 14 days	Furusawa (2001)
Tetracyclines						
Chlortetracycline	3.4; 7.4; 9.3	0.1	0.4	0.25	600 mg/kg feed for 5 days	Roudaut *et al.* (1989)
		0.25	0.25	1	8000 mg/kg feed for 7 days	Yoshida *et al.* (1973)
Oxytetracycline	3.3;7.3; 9.1	0.10	0.06	1.6	400 mg/kg feed for 7 days	Furusawa (1999)
		0.21	0.25	0.8	500 mg/kg feed for 1 days	Furusawa (2001)
		1.9	2.9	0.7	2g/l water for 7 days	Nagy *et al.* (1997)
		0.05	0.5	0.1	600mg/l water for 7 days	Omija *et al.* (1994)
		0.13	0.3	0.4	0.25 g/l water for 5 days	Roudaut *et al.* (1987)
		0.08	<0.20	>0.40	300 mg/kg feed for 7 days	
Tetracycline	8.3; 10.2	0.11	0.5	0.2	0.25 g/l water for 5 days	Roudaut *et al.* (1989)
		0.17	0.9	0.2	300 mg/kg feed for 7 days	
Doxycycline		11	3.5	3	0.5 g/l water for 7 days	Yoshimura *et al.* (1991)
Quinolones						
Enrofloxacine	6.2	1.1	0.3	3.7	5 mg/kg/day in water for 5 days	Gorla *et al.* (1997)
Ciprofloxacine	6.3	<0.15	0.18	<0.8		

FIG. 9: STANDARD GRAPH OF CONCENTRATION OF FLORFENICOL (mcg/ml) VS ZONE OF INHIBITION (mm)



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APPENDIX I

Details of standard feed used in poultry

Sr. No.	Item used	Quantity (kg)
1	Maize	306
2	Soya	130
3	Oil	3.75
4	Fat	1.25
5	Dicalcium Phosphate	1.50
6	Minerals	5
7	Gold Mohar	50
8	NaCl	1.5
9	Choline	0.5
10	Vitamin A	0.5
11	Tylosine	0.25
Total amount		**500**

APPENDIX II

Phosphate Buffer Saline composition

Solution A: 0.025 M $Na_2 HPO_4$

($Na_2 HPO_4$; mol. Wt. = 141.97;

$Na_2 HPO_4 .12 H_2O$; mol.wt. = 358.16)

Solution B: 0.025 M KH_2PO_4; mol.wt. =136.09

PBS (pH: 6.8) = Solution A (50 ml) + Solution B (50 ml)

APPENDIX III

Composition of Mueller Hinton Agar

Sr. No.	Ingredients	g/L
1	Beef infusion form	300.00
2	Casein acid hydrolysate	17.50
3	Starch	1.50
4	Agar	17.00

Final pH of the medium was adjusted to 7.3±0.2 at 25^{o}C

APPENDIX IV

Weekly egg production record of layer birds on percentage basis

Sr. No.	Age of chickens (weeks)	Percent production (%)
1	49	70.68
2	50	70.48
3	51	68.48
4	52	67.64
5	53	72.26
6	54	73.52
7	55	68.90
8	56	63.44

APPENDIX V

Calculation for the dose estimation for step 2:

As there were mortalities in three out of five birds in Step 1, it indicates 60% mortality .As per the Draft guidelines initial estimate of LD_{50} was taken as 1780 mg/kg. While proceeding for the doses calculation of Stage 1, LD_{50} was taken as 1780 mg/kg.

The following parameters are used in this paragraph:

l dose--- The lowest dose used during a particular stage (mg/kg).

h dose--- The highest dose used during a particular stage (mg/kg).

Step--- The multiplication factor used in calculating the individual doses.

k_{1}---- The number of doses in Stage 1. (typically 4).

Stage1:

A series of k doses is equally placed on a log scale around the initial estimate of the LD_{50}.

(1) Calculation of *l* dose:

l dose = 0.1414 x LD_{50}

= 0.1414 x 1780

= **251.69 mg/kg.**

Calculation of h dose:

h dose = 7.071 x LD_{50}

= 7.071 x 1780

= 12586.38 mg/kg.

However h dose was much greater than the capacity of the digestive tract of the bird As per the guidelines, the upper limit of the dose for any drug/ chemical being tested for LD_{50} in birds is 3330mg/kg. Thus h dose was taken as **3330 mg/kg.**

(2) Calculation of Step:

Step = $50^{(1/k_1-1)}$

= $50^{(1/3)}$ ……since k_1= 4

= **3.68**

(3) Calculation of k_1 doses:

Dose i = *I* dose x step $^{(i-1)}$, for i = 1 to k_1 = 1 to 4 since total doses selected in Stage 1 are four

Therefore,

Dose 1 = *I* dose x step $^{(1-1)}$

= 251.69 x 3.68 $^{(0)}$

=**251.69 mg/kg**

Dose 2 = *I* dose x step $^{(2-1)}$

=251.69 x 3.68 $^{(1)}$

=793.74 mg/kg

Dose 3 = *I* dose x step $^{(3-1)}$

= 251.69 x 3.68 $^{(2)}$

= 251.69 x 13.54

=**2920.96 mg/kg**

Thus the four k_1 doses are 251.69, 793.74, 2920.96 and 3330.00 mg/kg. Each of the k1 doses of the Florfenicol liquid calculated above was given to a single bird, orally considering individual body weight of the birds.

Calculation for total volume to be administered to birds according to individual body weight.

a) Bird No.1:

Florfenicol liquid was administered at dose 1 i.e. @ 251.69 mg/kg.
Body weight of this bird was 1120 gm, therefore total administered was

$$\frac{1120 \times 251.69}{1000} = 281.90 \text{ mg}$$

The formulation of Florfenicol provided is 20% (w/v), thus each ml of Florfenicol liquid contains 200 mg of florfenicol. Now we require 281.90 mg, thus total volume to be administered is

$$\frac{281.90}{200} = \textbf{1.44 ml}$$

b) Bird No.2:
Florfenicol liquid was administered at dose 2 i.e. @ 793.74 mg/kg.
Body weight of this bird was 1100 gm, therefore total administered was

$$\frac{1100 \times 793.74}{1000} = 873.11 \text{ mg}$$

The formulation of Florfenicol provided is 20% (w/v), thus each ml of Florfenicol liquid contains 200 mg of florfenicol. Now we require 873.11 mg, thus total volume to be administered is

$$\frac{873.11}{200} = \textbf{4.37 ml}$$

c) Bird No.3:
Florfenicol liquid was administered at dose 3 i.e. @ 2920.96 mg/kg.
Body weight of this bird was 1000 gm, therefore total administered was

$$\frac{1000 \times 2920.96}{1000} = 2920.96 \text{ mg}$$

The formulation of Florfenicol provided is 20% (w/v), thus each ml of Florfenicol liquid contains 200 mg of florfenicol. Now we require 2920.96 mg, thus total volume to be administered is

$$\frac{2920.96}{200} = \textbf{14.61 ml}$$

d) Bird No.4:

Florfenicol liquid was administered at dose 4 i.e. @ 3330 mg/kg.

Body weight of this bird was 907 gm, therefore total administered was

$$\frac{907 \times 3330}{1000} = 3023.03 \text{ mg}$$

The formulation of Florfenicol provided is 20% (w/v), thus each ml of Florfenicol liquid contains 200 mg of florfenicol. Now we require 3023.03 mg, thus total volume to be administered is

$$\frac{3023.03}{200} = \textbf{15.12 ml}$$

Calculation for the estimation of LD_{50}:

As there were mortalities observed at the last two doses i.e. h dose (@3330 mg/kg) and a dose prior to h dose i.e. @ 2920.96 mg/kg, according to OECD guidelines 223 (October 2002) the LD 50 can be calculated by the formula:

$$\textbf{(dose2 x dose3)}^{1/2}$$

Therefore, $LD_{50} = (873.11 \times 2920.96)^{1/2}$

$$= \textbf{1596.97 or 1597 mg/kg}$$

APPENDIX VI

Concentration of Florfenicol in organs of each of the bird detected at eight hours post-treatment

Bird No		Concentration of Florfenicol (µg/g)			
		Liver	Kidney	Lung	Muscle
Group A	1	0.58	0.66	0.44	0.66
	2	0.44	0.66	0.38	0.38
	3	0.84	0.66	0.58	0.44
	4	0.58	0.44	0.66	0.46
	5	0.58	0.44	0.44	0.44
	6	0.58	0.46	0.84	0.66
Group B	7	0.44	0.66	0.44	0.30
	8	0.44	0.44	0.38	0.70
	9	0.58	0.70	0.44	0.30
	10	0.70	0.44	0.44	0.58
	11	0.30	0.44	0.70	0.58
	12	0.46	0.58	0.44	0.70

VITA

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