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Determination of Florfenicol and Doxycycline Residues in Chickens by Microbiological Assay

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ABSTRACT: *Background:* Florfenicol (Ff) is a synthetic antibiotic with a broad antibacterial spectrum and high therapeutic effectiveness that was specifically developed for veterinary use as well as, doxycycline is an antibiotic synthetically derived from oxytetracycline.
Methods: In the present study, the in-vitro efficacy of Ff and doxycycline against *Salmonella entertidis* and *Escherichia coli (*Serotype O78) *pathogens* was determined using disc diffusion technique and Minimum inhibitory concentrations (MICs). In the other hand serum and tissue residual levels of Ff and doxycycline after intramuscular (i.m.) administration of 30 mg/kg and 250 mg/kg orally respectively to 5 weeks old chicken were determined using microbiological assay method with *Bacillus subtilis* ATCC 6633 as a reference organism.
Results: The results showed that both microorganisms were highly susceptible to Ff with lower MIC value than those of doxycycline. The peak concentration of florfenicol in serum was 5.34 ± 0.01 µg/ml and reached at 10 hr post medication and the drug was detected up to 48 hr while of doxycycline the peak concentration was 7.35±0.14 µg/ml at 12 hr post medication then declined gradually. Liver was the highest tissue concentration of florfenicol and the kidney was for doxycycline while muscle showed the lowest tissue concentration for both drugs.
Conclusions: The results concluded that florfenicol was more effective *in-vitro* against the tested microorganisms as will as it persisted lower time than doxycycline in the tissue and sera of the chickens.

KEYWORDS: Florfenicol, Doxycycline, *Bacillus subtilis,* Drug residues.

INTRODUCTION

Florfenicol has retained the broad spectrum and strong antibacterial activity of chloramphenicol and possesses the more favorable toxicity profile of thiamphenicol because it also lacks the aromatic nitro group. However, it is more likely than chloramphenicol to cause a reversible, non-life-threatening hematopoietic depression [1].

Ref [1] found that florfenicol has potent activity against some chloramphenicol resistant strains of bacteria possibly because it is less affected by the major enzyme produced in plasmid-mediated bacterial resistance against chloramphenicol and thiamphenicol.

Florfenicol, a structural analogue of thiamphenicol, is a synthetic broad spectrum, primarily bacteristatic, antibiotic against gram-negative and gram- positive bacteria isolated from domestic animals. It is approved for use in cattle to treat the bovine respiratory disease complex, in swine for the treatment and control of swine respiratory disease [**2**]**.**

Florfenicol is a bacteristatic antibiotic that inhibits protein synthesis by binding to the 50S ribosomal subunits of susceptible bacteria, leading to inhibition of peptidyl transferase and preventing the transfer of amino acids to the growing peptide chains and subsequent protein formation [**3**].

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Ref [**4**] Investigated the bioavailability and pharmacokinetic disposition of florfenicol in broiler chickens after intramuscular and oral administrations of 15 and 30 mg/kg body weight (b.wt.). The intramuscular bioavailability and the oral bioavailability of florfenicol were 95, 98 and 96, 94%, respectively, indicating that florfenicol was almost absorbed completely after intramuscular and oral administrations of 15 and 30 mg/kg b.wt.

Ref [**5**] Compared the pharmacokinetics of florfenicol, thiamphenicol and chloramphenicol after single intravenous and oral administration in broiler turkeys. All the fenicol antibiotics were administered at a dose of 30 mg/kg b.wt. The mean residence time values of florfenicol, thiamphenicol and chloramphenicol after intravenous injection were 3.37±0.63, 2.43±0.29 and 2.12±0.21 h, respectively. The bioavailability of florfenicol, thiamphenicol and chloramphenicol after oral administration were 82%, 69%, and 45%, respectively.

Ref [**6**] Investigated that the prolonged presence of residues of florfenicol and florfenicol-amine in edible tissues could give rise to a possible health risk in human food.

Doxcycline is an antibiotic synthetically derived from oxytetracycline and is available as doxcycline hyclate (alpha-6-deoxy-5-oxytetracycline [7].

Doxcycline and minocycline appear to offer advantages that would render them useful in certain situations in veterinary medicine. Their major advantage lies in their greater lipid solubility relative to other tetracyclines. This characteristic probably accounts for their enhanced antimicrobial effectiveness for some organisms, more efficient absorption after oral administration, and enhanced distribution in the body. The principal excretory organ for doxcycline is the intestine, where the drug diffuses through the intestinal mucosa into the intestinal tract. This unique characteristic makes this drug useful in cases of preexisting renal dysfunction and may render this drug superior to other tetracyclines in the treatment of intestinal infections. While the usefulness of doxcycline and minocycline in food-producing animals may be limited because of persistent drug residues [8].

Doxcycline has a special position in the tetracycline group because it is more lipid soluble than other tetracyclines. Probably therefore it has a better bioavailability from the gastro-intestinal tract. The lypophylic nature has significant consequences for egg residues which can be high in concentration and relatively very long-lasting has the longest elimination half life also it shows the strongest binding to plasma proteins, as well as it is relatively much more stable in watery solution, These properties make doxcycline theoretically very attractive for treatment of systemic diseases of poultry [9].

The goals of the present study were to determine the in-vitro activity of Ff and doxcycline against *Salmonella enteritidis* and Pathogenic *Escherichia coli* (Serotype O78) and to compare the serum and tissue concentrations of both drugs after their single dose to chickens.

MATERIAL AND METHODS

Florfenicol (Floromed®) 30% injectable solution was supplied by Arabcomed Company for Medical products, Cairo, Egypt.

Dose: 30 mg/kg intramuscular injection [10].

Doxcycline (Egy-Doxcenl®) 20% oral solution was supplied by Arabcomed Company for Medical products, Cairo, Egypt.

Dose: 250 mg\kg body weight [11].

Bacillus subtilis* (*ATCC 6633) was used as a tested organism in microbiological assay method for assaying florfenicol and doxcycline residues [12] it was obtained from Animal Health Research Institute, Dokki, and Cairo, Egypt.

Salmonella enteritidis and **Pathogenic *Escherichia coli* (Serotype O78)** were used as tested organisms in estimation of minimum inhibitory concentration (MIC) and sensitivity test of the tested drugs. It was obtained from Animal Health Research Institute, Dokki, Cairo, Egypt.

EXPERIMENTAL DESIGN

A total number of 99 clinically healthy Hy-line chickens, approximately 5 weeks old and weighing 1.53 ± 0.14 kg were used. The chickens were housed indoors in hygienic conditions, fed an antibacterial-free diet and given free access to water. The chicken was divided in two main experiments:

The first experiment, 9 birds were used to determine the peak of the tested medicament in sera of the tested birds. Birds were divided equally in to 3 groups. Group (1) kept control non treated chickens, while chickens of group (2) were administered a single intramuscular dose of Floromed® solution at 30 mg⁄ kg body weight, and group (3) given single oral

dose of 250 mg\kg body weight Egy-Doxcenl®. Blood samples were collected from wing vein of 3 birds in each group at 30 min and 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120 hrs after drug administration. Sera were separated and stored frozen till assayed microbiologically.

The second experiment, 90 birds were used to determine tissue concentration and elimination of the tested drugs from the body of chickens. Birds were divided equally in to 3 groups. Group (1) kept control non treated chickens, while chickens of group (2) were achieved a therapeutic dose of Floromed® solution at 30 mg/ kg body weight intramuscularly for 3 successive days , group (3) given oral dose of 250 mg\kg body weight Egy-Doxcenl® for 5 successive days. Three chickens from each group were slaughtered for collection of liver, kidney and muscles at 30 min and 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120 hrs after drug administration.

Extraction of drug from tissues:

One ml of phosphate buffer (pH 7.2) was added to 1 g of the sample, tissue samples were homogenized thoroughly using sterile mortar with pestle then centrifuged at 3000 rpm for 10 minutes, then the supernatant was placed in individual marked plastic bags and stored in deep freezer at -20 ºC till assayed microbiologically .

Microbiological analysis of the drug:

The collected samples (sera and tissues) were assayed for determination of florfenicol and doxcycline concentrations by the microbiological assay method according to [13] and [12] using *Bacillus subtilis* (ATCC 6633) as a tested organism. Comparisons were made against standard solutions in antibiotic-free chicken serum, from the standard curve, concentrations of the two tested drug were obtained corresponding to the corrected average values of inhibition zones [14].

Sensitivity test (disc diffusion method): The in-vitro antibacterial effect of florfenicol and doxcycline against *Salmonella enteritidis* and *Escherichia coli* was carried out using disc diffusion [15]. The technique was standardized by the National Committee for Clinical Laboratory Standards [16] and the interpretation of the results was done according to [17]. **Determination of Minimum Inhibitory Concentrations (MICs):** MICs of Ff and doxcycline against *Salmonella entertidis* and *Escherichia coli* were carried out using macro dilution technique (dilution broth method). The lowest concentration of each antimicrobial that inhibit visual growth of bacteria in incubated tubes was determined as MIC [18].

Statistical analysis:

Data obtained in this study were statistically analyzed for variance using one way (ANOVA), and least significant difference (LSD) as described by [19] using computerized SPSS program, version 10.0.

RESULTS

In-vitro sensitivity test:

The *in-vitro* antibacterial effect of florfenicol and doxcycline against *Salmonella enteritis* and *Escherichia coli* using agar disc diffusion method showed that both microorganisms were highly susceptible to florfenicol and doxcycline with clear zone of inhibition (Table, 1).

Minimum inhibitory concentrations (MICs):

The results showed that tested strains of microorganisms were highly susceptible in broth medium to florfenicol with MIC lower than that of doxcycline as shown in Table (2).

Standard curves of florfenicol and doxcycline

Standard curves of florfenicol and doxcycline in antibacterial free chicken's serum, liver, kidney and muscle using *Bacillus subtilis* (ATCC 6633) as a tested organism showed the diameters of inhibition zones were proportionally related to the concentration of florfenicol and doxcycline. The diameter of inhibition zones (mm) were linear when plotted against the logarithm of the tested drug concentrations (µg/ml) as shown in table (3, 4), and Figure (1, 2).

Detection of florfenicol and doxcycline in chickens of control group

Analysis was applied for serum and tissues (Liver, kidney and muscles) of non– medicated control group. No antimicrobial residues could be detected.

Tissue concentrations of florfenicol and doxcycline in chickens

The concentrations of florfenicol and doxcycline in serum and tissue (liver, kidney and muscle) of chickens were shown in table 5& 6.

After single i.m. injection of florfenicol to chickens at 30 mg\kg b.wt, the observed initial serum concentration was 1.31± 0.02µg/ml at 30 minutes post dosing and reached the peak concentration of 5..34±0.01µg/ml at 10 hours post dosing and disappeared from the blood at 24 hrs post dosing. Also it was found that after a therapeutic dose of florfenicol (30 mg\kg b.wt i.m. injection for 3 successive days), liver has the highest concentration (2.81±0.08) followed by kidney (1.36±0.06) the lastly muscle (1.06±0.01) µg/gm, and the drug was eliminated from liver and kidney after 48 hrs post dosing where the drug still in muscle till 96 hrs.

While single oral dose of doxcycline (250 mg/kg b.wt) showed that the initial serum concentration was 0.64±0.17 µg/ml at 1 hr post dosing and reached the peak concentration of 7.35±0.14 µg/ml at 12 hr post dosing then declined gradually and disappeared after 72 hr. Also it was found that after a therapeutic dose of doxcycline (250 mg/kg b.wt for 5 successive days), drug was eliminated from kidney and liver after 96 hrs and kidney showed the highest concentration (6.61±0.05) followed by liver (5.51±0.21) and lastly muscle (4.80 ± 0.11) µg/g which showed persistence of the drug till 120 hrs post dosing.

DISCUSSION

Our studies were carried out to reveal the in-vitro efficacy of florfenicol and doxcycline against *Salmonella enteritis* and *Escherichia coli* pathogen, special attention was directed to determine the residues of these drugs in serum and different organs (liver, kidney and muscle) of chickens.

The low value of MIC indicates that florfenicol was highly active against *Salmonella enteritis* and *Escherichia coli* pathogen. The obtained results declared that florfenicol tissue concentrations were more than the minimum inhibitory concentration MIC (0.09 and 0.28 µg/ml) for *Salmonella enteritis* and *Escherichia coli* pathogens respectively.

As florfenicol and its putative active metabolites might have different antimicrobial activities and because the ratio of the parent drug to its metabolites might not remain constant through the dosing interval, the movement of the metabolites from the body may not be the same as for the parent drug. This confounds interpretion of the concentration derived from the microbiological assay for the purpose of establishment of minimally effective concentrations.

Florfenicol amine is the longest-lived major metabolite and this was therefore used as a marker residue for withdrawal calculation in cattle [20]**,** while in calves most of the dosed Ff drug is excreted in urine in the parent form, indicating a major kidney clearance [21].

The relatively low extent of serum protein binding of Ff is consistent with its large steady state volume of distribution which represents extensive disposition of the drug in tissues. This is also consistent with the presence of higher concentrations of Ff in the highly perfused organs / tissues such as liver after the disappearance of Ff from the blood [22]**.**This observation supported those of [23] for broiler chicken and of [24] for male veal calves who concluded that higher doses or multiple doses may result in drug residues being detected for a longer period of time and the withdrawal time should therefore be extended.

Florfenicol drug level showed a rapid distribution and a slow elimination phase with greater AUC, volume of distribution at steady state (Vss), and elimination half-life values than those for chloramphenicol in pigs after a single intravenous bolus [25]**.** This likely resulted from the replacement of the hydroxyl group by a fluorine atom that postponed the in vivo metabolic glucuronidation [26].

Doxcycline is a semi-synthetic bacteristatic tetracycline and a broad-spectrum antibiotic against Gram-negative and Gram-positive aerobic and anaerobic bacteria, *Rickettsiae, Chlamydiae, Mycoplasmas* and some protozoa [27] and [28]**.**

Pharmacokinetics properties of doxcycline is superior than older tetracycline; in terms of higher lipid solubility, complete absorption, better tissue distribution, longer elimination half life and lower affinity for calcium [29]. As the protein-bound part of doxcycline is microbiologically not active and the free part has like the other tetracyclines in poultry, a relatively short elimination half life, only relatively low microbiologically active concentrations can be achieved; this probably accounts for the fact that no therapeutic advantage could be found [30].

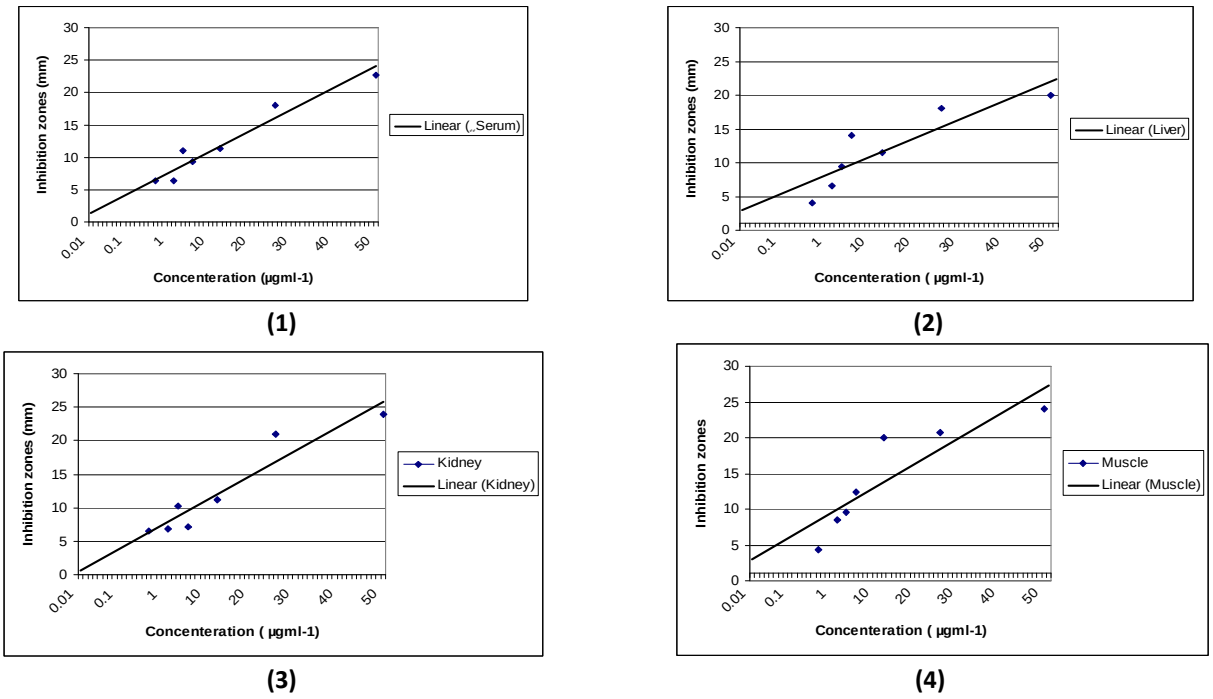

Fig. 1. The Corrected reading of inhibition zones (mm) for the standard curves of florfenicol in serum (1), liver (2), kidney (3) and muscle (4) of chicken.

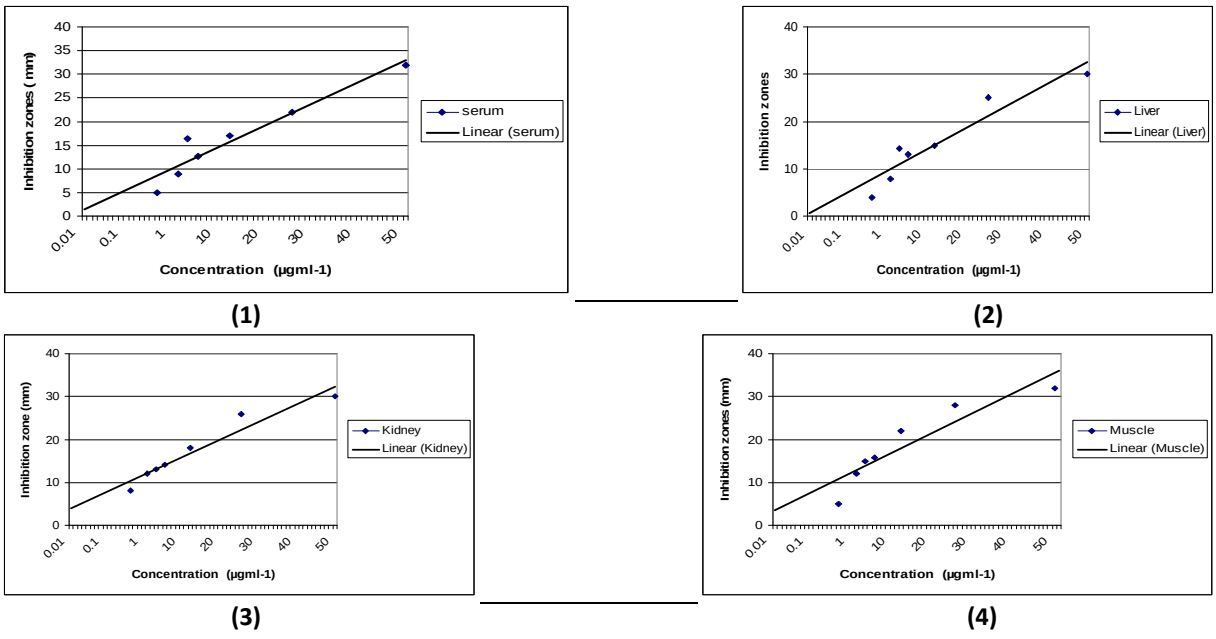

Fig. 2. The Corrected reading of inhibition zone (mm)for the standard curves of doxcycline in serum(1), liver(2), kidney(3) and muscle(4) of chicken.

Table 1. Interpretation chart for the size of growth inhibition zones according to Quinn et al. (1994):

Drug	Diamerter of inhibition zones (mm)	Interpretation
Florfenicol	22	Susceptible
Doxcycline	18	Susceptible

Susceptible" indicates the pathogen was inhibited by generally achievable blood levels of the tested drug.

Table 2. Minimum inhibitory concentrations (MICs) of the tested drugs on Salmonella enteritidis and E.coli.

Drug	*Salmonella enteritidis*	*E. coli*
Florfenicol	0.09	0.28
Doxcycline	0.8	4.0

Table 3. The Corrected reading of inhibition zone (mm) for the standard curves of florfenicol in serum, liver, kidney and muscle of chicken

Concentration (µgml^{-1})	Inhibition zones (mm)			
	Serum	Liver	kidney	Muscle
50	22	20	23	24
25	20	18	20	19
12.50	16	15	18	16
6.25	14	12	16	15
3.12	12	10	14	13
1.5	10	8	12	10
0.78	8	7	8	8

Table 4. The Corrected reading of inhibition zone (mm) for the standard curves of doxcycline in serum, liver, kidney and muscle of chickens

Concentration (µgml^{-1})	Inhibition zones (mm)			
	Serum	Liver	kidney	Muscle
50	28	30	27	30
25	22	27	24	28
12.50	16	21	16	22
6.25	14	18	14	20
3.12	12	15	12	17
1.5	10	12	11	12
0.78	8	9	7	10

Table 5. The mean concentration of florfenicol in serum (µg/ml) and tissues (µg/g) at different time intervals of chickens. (M±S.E)

Time of sampling (hr)	Serum	Liver	Kidney	Muscle
0.50	1.31± 0.02	ND	ND	ND
1 hr	1. 55± 0.03	ND	ND	ND
2 hr	1.94± 0.02	1.39±0.002	1.04± 0.01	ND
4 hr	2.06± 0.04	1.43± 0.01	1.15± 0.01	ND
6 hr	2.52±0.02	1.58± 0.02	1.17±0.01	1.02± 0.01
8 hr	3.03± 0.01	1.65±0.22	1.26± 0.01	1.02±0.01
10 hr	5..34±0.01	2.26± 0.03	1.29±0.002	1.06±0.001
12 hr	3.21±0.13	2.81±0.08	1.36±0.06	1.08±0.01
24 hr	1.05± 0.35	1.78±0.02	1.14±0.02	1.06±0.01
48 hr	ND	0.95±0.05	0.92±0.05	1.04± .005
72 hr	ND	ND	ND	0.21±0.01
96hr	ND	ND	ND	ND

ND: not detected.

Table 6. The mean concentration of doxcycline in serum (µg/ml) and tissues (µg/g) at different time intervals in chicken (M±S.E)

Time of sampling (hr)	Serum	Liver	Kidney	Muscle
0.50	0.34±0.07	ND	ND	ND
1 hr	0.64±0.17	ND	0.94±0.11	ND
2 hr	1.32±0.20	0.37±0.17	1.93±0.01	ND
4 hr	2.82±0.20	1.90±0.04	2.38±0.15	ND
6 hr	3. 48±0.03	2.37±0.23	3.17±0.05	0.08±0.11
8 hr	4.16±0.03	3.98±0.01	4.07±0.01	0.59±0.02
10 hr	5.18±0.01	4.01±0.001	4.82±0.03	0.50±0. 23
12 hr	7.35±0.14	4.31±0.05	5.15±0.08	1.87±0.02
24 hr	6. 90±0.07	5.51±0.21	6.61±0.05	3.84±0.12
48 hr	4.04±0.14	3.82±0.09	3.12±0.09	4.80 ± 0.11
72 hr	1.66 ±0.21	1.86 ±0.21	1.52±0.01	3.52±0.14
96 hr	ND	0.76±0.08	0.97±0.01	1.67±0.05
120 hr	ND	ND	ND	0.88 ±0.05

ND: not detected.

CONCLUSION

Florfenicol was more effective *in-vitro* against the tested microorganisms as will as it persisted lower time than doxcycline in the tissue and sera of the chickens. However the representative disposition of the both drugs in sera and tissues of chickens indicating that chickens must be left for a certain period (withdrawal time) before being released to the market to allow the elimination of antimicrobial from their body.

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CONFL ICT OF INTEREST

None declared

ETHICAL APPROVAL

The study was approved by the Institutional Animal Ethics Committee

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