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Influence of glucomannan on the bioavailability and tissue residues of doxycycline in rabbits after its oral administration

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ABSTRACT

Mycotoxins existence in rabbit fodder has toxic effects on animal health. Mycotoxin binders are feed supplements adsorb mycotoxins in the intestine to prevent oral mycotoxins from being absorbed. Concerns about an unspecified association with oral veterinary drugs were raised in 2010 by the European Food Safety Authority. This study evaluated the effect of dietary supplementation of esterified glucomannan yeast extract (0.1% for 15 days) as a mycotoxin binder (EGM) on doxycycline (DOX) bioavailability and residues in different rabbits tissues after oral administration at a dosage of 6 mg kg^{-1} b.wt; single dose for oral bioavailability study and for 15 successive days for evaluation tissue residue of doxycycline. Pharmacokinetic investigation of DOX in group received DOX alone shows that AUC_{0-inf}, T_{max} and C_{max} are diminished significantly when EGM is used with DOX as a feed additive. The 67% decline in AUC_{0-24} in the group fed EGM results in a significant decrease in oral bioavailability. This reflects its presence in different tissues, as the EGM binder significantly reduced the levels of doxycycline in different rabbit's tissues. On the basis of the obtained results of pharmacokinetic and tissue residue studies, it can be concluded that EGM mycotoxin binder can virtually reduce the DOX absorption after oral administration in rabbits, and this is may be owed to the adsorption of the EGM additive to doxycycline. Consequently, when doxycycline is administered orally to rabbits that are also actually nourished an EGM fodder additive; the potential interaction must be taken into consideration carefully.

INTRODUCTION

Doxycycline hyclate (doxycycline-HCL, is a semisynthetic tetracycline derivative commonly used in veterinary and human medication because of its relatively wide spectrum (**Rinaldi 2014**). It is the most lipophilic member in tetracycline's group-such as oxytetracycline, so it has better antimicrobial properties and more tissue diffusion (**Gutiérrez**

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et al. 2012). Doxycycline (DOX) is used more than the other tetracycline for medication of small animal's, with a perfect safety depict (**Maaland et al. 2013**). In rabbits; doxycycline is recommended for treating the gastrointestinal tract infections caused by bacteria (**EMA 2015**).

The European Commission has announced a new batch of fodder additives technology entitled "materials for reducing feed pollution with mycotoxins" (**EC 2009**). There are two types of mycotoxin detoxifiers: mycotoxin binders and mycotoxin modifiers. These modifiers convert the mycotoxins into less poisonous products, while the binders adsorb toxins on their external surface, thus reduce their toxic prospect.

Esterified glucomannan (EGM) is demarcated in the form of glucose polymers which extracted from the saccharomyces cerevisiae cell walls that have immunomodulating actions and actual in minimizing the poisonous properties of naturally polluted fodder with mycotoxins (**Huff et al. 2006**).

The European Food Safety Authority (EFSA) indicated that the mycotoxin binders' safety concerning the non-specific attachment of orally administered veterinary medicines should be studied (**EFSA 2010**).

Therefore, the purpose of this experiment is to study the effectiveness of nutritional addition of esterified glucomannan (EGM, MTB-100 ®) on the doxycycline (DOXIN® 20%) bioavailability and residues in variable rabbit's tissues after oral administration.

MATERIAL AND METHODS

Animals:

Sixty male New Zealand white healthy rabbits (weight ranged from 1.1- 1.8 kg) were given tap water and balanced rabbit's feed ad libitum. The rabbit feed pellets were formulated according to the nutrient requirements (**NRC 1977; De Blas & Weiseman 2010**) as shown in (table 1).

Table (1) Composition of the experimental rabbit feed pellets

Composition	Blank pellets %	EGM pellets %
EGM, MTB-100®	0	0.1
Soybean meal 44%	18.0	18.0
Yellow corn	7.0	7.0
Barley	9.1	9.1
Coarse wheat bran	28.0	27.9
Fennel and caraway straw	9.0	9.0
Alfalfa dehydrated meal	25.0	25.0
Dicalcium phosphate	1.0	1.0
Salt	0.4	0.4
Lime stone	1.5	1.5
Premix	0.3	0.3
Disodium phosphate	0.3	0.3
Non-Food additives	0.4	0.4

The animals were kept for adaptation for at least 15 days before the study then randomly divided into 3 groups; group of 6 rabbits served as control group (for obtaining blank samples needed for method validation), group of 12 rabbits for pharmacokinetic study and group of 42 rabbits for residues depletion study.

Conditions of animal housing were related to European experimental animals housing guidelines (**EC 2010**).

Drugs:

Esterified glucomannan (EGM, MTB-100®) was obtained from IFT Corporation, Egypt. Supplementation of feed with EGM at level of 1Kg/ Ton of feed (0.1%) (**Dönmez and Keskin 2008**). All experimental pellets were prepared (2.5 mm diameter, 9 mm length) in a commercial feed mill for rabbits. The procedure of mixing feed was performed agreeing to geometrical technique of mixing as described previously (**Earle and Earle 1983**).

Doxycycline hydrochloride (DOXIN® 20%) was obtained from PHARMA SWEDE Co., Egypt. The drug was analyzed to verify the concentration of the active principle according to **Mitić et al. (2008)**. In rabbits it is proposed for treating the digestive tract infections with a

recommended dosage of 6 mg doxycyc line/kg b.wt daily for 15 sequential days (**EMA 2015**).

Experimental design: as designed in table (2).

Table (2) Grouping of experimental animals

Gp.	No.	Drug	Dose	Administration route	Duration
A	6		Control		
B	6	DOXIN	6 mg kg^{-1} b.wt	Orally	Once
C	6	EGM + DOXIN	1 gm kg^{-1} feed 6 mg kg^{-1} b.wt		15 days before DOXIN administration Once
D	21	DOXIN	6 mg kg^{-1} b.wt		Once per day for 15 days
E	21	EGM + DOXIN	1 gm kg^{-1} feed 6 mg kg^{-1} b.wt		15 days before DOXIN administration Once per day for 15 days

Chemical reagents:

All reagents of samples preparation and extraction were analytical purity. Acetonitrile, methanol, water and trifluoroacetic acid were HPLC grade (Fisher).

Doxycycline hydrochloride (85.7% as a base); USP reference standards, Twinbrook Pkwy, Rockville, MD, USA.

Sample collection:

For pharmacokinetic study (gp. B&C); blood samples were taken from the jugular or ear veins the following time intervals after doxycycline administration [0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hours]. Centrifugation of blood samples at 1500xg at 4°C for 10 min); sera we recollected and preserved at -20°C till analysis.

For tissue residues study (gp. D&E); three rabbits were slaughtered at 1st, 3rd, 5th, 7th,9th, 15th, and 21stday following the last oral dose. Samples from blood, muscle, liver, and kidneys were taken from slaughtered rabbits for drug assay. Two hundred milligrams of tissue specimens were weighed and kept frozen at -20°C till analysis for the DOX concentrations.

Standard solutions:

Stock solution (857 ppm):

On the basis of the standard purity, the standard was weighed to the nearest 0.01 g up to 10 mL with methanol. This solution should be immediately used for fortification solution preparation.

Fortification solution (85.7 ppm):

One mL of the stock solution was diluted up to 100 mL with water for preparation of calibration standards and quality control specimens (QC).

Calibration standards:

Doxycycline calibration standards were prepared fresh daily at concentrations of 17.14, 42.85, 85.7, 171.4, 428.5, 857, 1714, 4285, 8570 and 17140 ppb in blank rabbit serum for kinetic analysis. The doxycycline calibration curves were prepared at concentrations of 42.85, 85.7, 171.4, 428.5, 857, and 1714 ppb in blank muscle, liver and kidneys for residue analysis. In the same manner, QC samples with doxycycline in serum at low (42.85 ppb), medium (857 ppb), and high (8570 ppb); for muscle (50, 100, 200 ppb), for liver (150, 300, 600 ppb) and for kidneys (300, 600, 1200 ppb) concentrations were freshly prepared to assess the precision and recovery of this assay. These calibration standards and QC samples were processed according to the extraction steps that are concise as follows.

Doxycycline assay:

Samples were analyzed using HPLC techniques for determination of doxycycline ac-

cording to **Yang et al. (2018**) with some modifications.

Two hundred fifty µl of phosphate /EDTA buffer (100 mM disodium EDTA, inclosing100mM sodium phosphate), & 50 µl of 20%Perchloric acid were added to 0.2 g of tissue or 0.2 ml of serum. After mixing for 2 minutes, Centrifuging of samples at 12.000 xg for15 minutes at 4^{o}Cand the supernatants were filtered before injection into the HPLC system.

Instrumentation with chromatographic conditions:

The HPLC instrument consists of a quad pump, model1200, an auto injector and UV-Vis detector (Agilent). The chromatographic conditions were summarized in table (3).

Table (3) The optimized chromatographic conditions

Elution solution	10 mM trifluoroacetic acid in water: acetonitrile: (7:3v/v).
Flow rate	1 ml per minute.
Volume of injection	100 µl.
Column type	LiChrospher C18 (250 mm, 4.6 mm internal diameter, 5 µm particle size).
Column thermostat	30^{o}C.
Run time	15minutes.
UV- detector	350 nm.

The analytical procedure was validated and verified according to the requirements of **ICH (2005)**and**USP (2019)**; "Linearity and Range, Precision, Specificity, Recovery, system suitability testing [Tailing factor (Tf), Theoretical plates (N)], detection limit (DL) and quantification limit (QL)".

Selectivity and specificity: It was evaluated by analyzing blank rabbit serum, muscle, liver, kidneys and spiked rabbit serum, muscle, liver, and kidneys with doxycycline; chromatograms were visually inspected for chromatographic interfering from endogenous compounds.

Linearity and range: It was evaluated by a calibration curve in the range of 17.14 to 17140 ppb doxycycline in serum, and in the range of 42.85 to 1714ppb in different matrixes. Triple injections of each level of calibration plot. The calibration standards were used to determine the regression equation and the correlation coefficient.

The linear equation: $y = a\ x \pm b$, where y is the peak area, x is the concentration (ppb). a *is* the slope and b is intercept. The correlation coefficient (R^2) was calculated for each standard curve.

The detection limits (DL) and quantification limits (QL): were calculated using SD of intercept (S); [LOD= 3.3* S/a & LOQ= 10* S/a].

Precision and recovery: QC samples were analyzed for the determination of intra-, inter-day precision and recovery %.

The intra-day and inter-day precision of the method were determined by relative standard deviation (RSD) = (Average/SD)* 100.
The recovery= (Obtained conc./ Theoretical conc.)* 100.

System Suitability Testing (SST): It evaluates the efficiency and suitability of the chromatographic system not only before usage but also during the analysis time. The SST parameters which are investigated are retention time, tailing factor (T), and theoretical number of column (N).

STATISTICS

Data showed as mean ± S.D. These data were evaluated by 2-tailed t-Test using the SPSS software program (SPSS Inc., version 22.0, Chicago, IL, USA) to assess the differences between groups (**Morgan et al. 2019**) The variances were considered significant when P< 0.05. The kinetic parameters were analyzed by PK solver, version 2 according to **Zhang et al. (2010**). Oral relative bioavailability of doxycycline (relative F %) for the study groups was calculated as follows:

$$relative\ F\% = \left(\frac{AUC0-24h, DOX\&EGM}{AUC0-24h, DOX\ alone}\right) \times 100$$

RESULTS

Method validation results:

The results were summarized in table (4) showing that the test method is accurate, precise, and sensitive with high recovery. Chromatograms of doxycycline in different matrixes in figure (1) showing no interference between the extracted different spiked matrixes and pure standard.

Table (4) Validation results of test method

Parameter	Serum	Muscle	Liver	Kidney
Range (ppb)	17.14-17140		42.85-1714	
Retention time (min.)		7.3±0.01		
Slope	4.9179	4.5026	5.0011	4.3318
Intercept	112.28	83.238	- 47.294	64.22
Coefficient of correlation (R)	0.9999	0.9994	0.9998	0.9993
DL (ppb)	1.7	4.8	15	24.8
QL (ppb)	5.2	14.3	44.7	75
Recovery %	99-100.8	92-96	89-103	85-108
Intra-day precision (RSD %)	0.21	0.8	0.74	1.1
Inter-day precision (RSD %)	0.54	1.2	1.13	1.9
Tailing factor (Tf)	1.04±0.004	1.02±0.01	1.02±0.002	1.01±0.007
Theoretical plates (N)	5406±130	5435±244	5543±104	5445±221

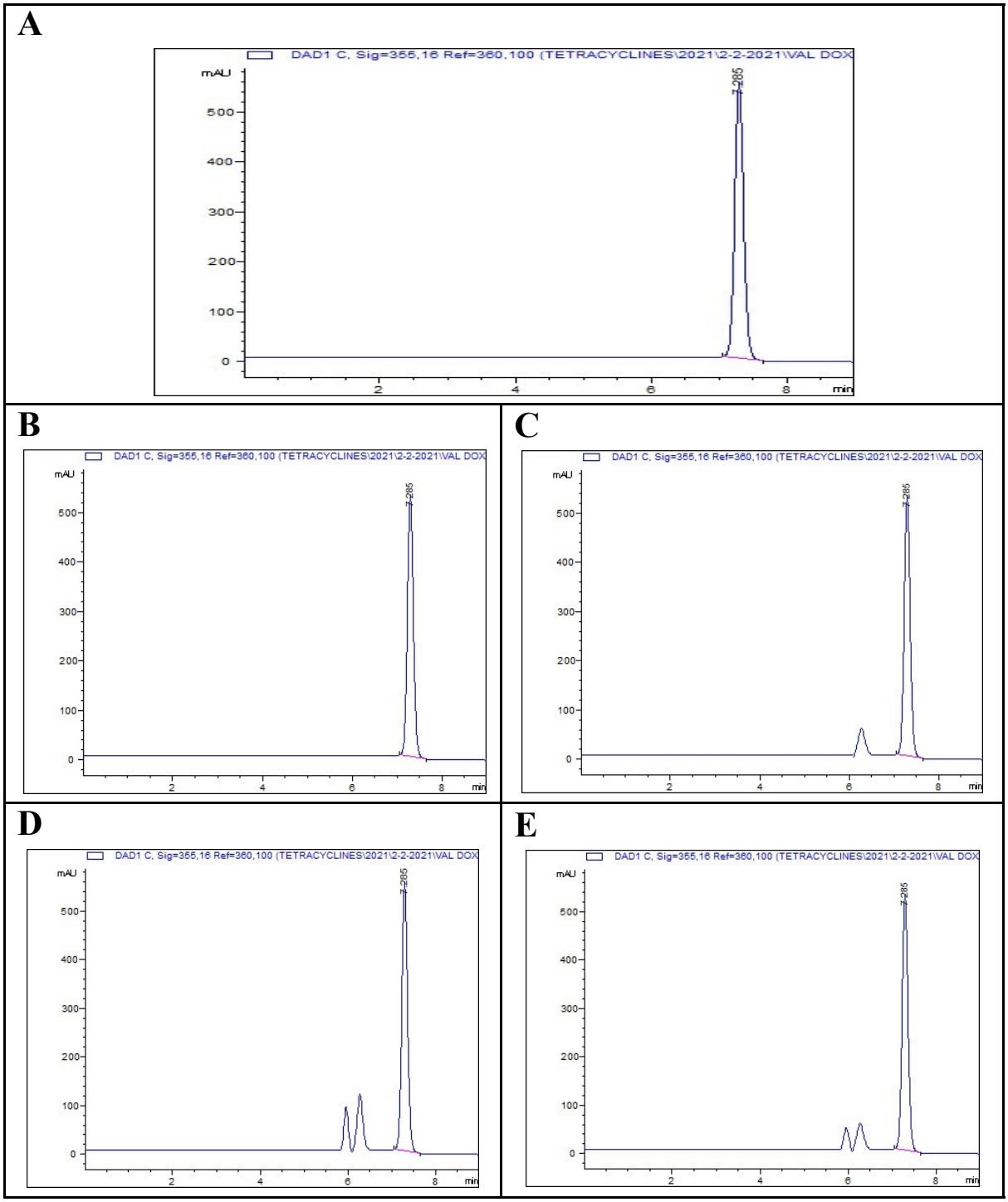

Figure (1) Chromatograms of doxycycline at 1000 ppb in (A: Deionized water, B: Serum, C: Muscle, D: Liver, E:Kidney) at a retention time 7.3 min.

Results of pharmacokinetic:

The pharmacokinetic parameters of doxycycline (DOX) in rabbits were illustrated in the table (5) showing a significant decline in DOX serum level in rabbits fed EGM as a mycotoxin binder comparing to rabbits administered DOX alone. The doxycycline in serum after oral administration in rabbits was described by a two-compartment model as appear in figure (2).

Table (5) Kinetic parameters of DOX in rabbits fed EGM as a mycotoxin binder compared to rabbits administered DOX alone [mean ± SD] (n= 6)

Parameters	Unit	DOX alone	DOX + EGM
$t_{1/2\alpha}$	hr	1.03±0.3	2.2±0.1*
$t_{1/2\beta}$	hr	4.74±0.4	2.21±0.1*
$t_{1/2ka}$	hr	0.57±0.05	0.88±0.04*
V/F	L/kg	0.096±0.01	0.128±0.002*
CL/F	L/hr/kg	0.027±0.002	0.04±0.002*
T_{max}	hr	1.35±0.07	1.94±0.02*
C_{max}	µg/ml	34.68±1.5	25.48±0.67*
AUC_{0-24}	µg/ml*h	217.35±12	149.1±5.7*
$AUC_{0-\infty}$	µg/ml*h	223.06±13	149.23±5.7*
Relative F	%	100.23±7.2	68.78±4.86*
MRT	hr	6.34±0.2	4.45±0.08*

$t_{1/2}$: half-life elimination; $t_{1/2\alpha}$:half-life disposition; $t_{1/2ka}$: half-life absorption time; V/F: apparent distribution volume after oral administration; Cl/F: Apparent total clearance of the drug from serum after administration orally; T_{max}: Time to reach maximum serum concentration after drug administration; C_{max}: maximal serum concentration; MRT: Mean residence time; AUC: area under the serum conc./time curve from time zero to 24 h(AUC_{0-24}) or zero to infinity ($AUC_{0-\infty}$); Relative F: relative bioavailability.
* Significant differences comparing with the group of DOX using T-Test ($P \leq 0.05$).

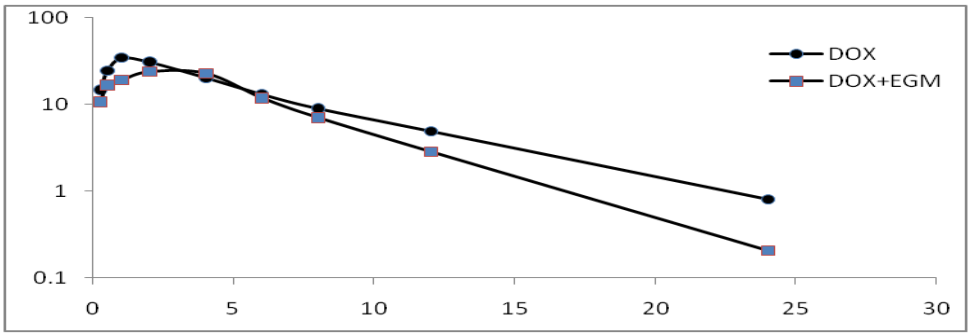

Figure (2): Semi logarithmic plot of doxycycline serum concentrations after its oral administration alone (6 $mgkg^{-1}$ b.wt) and in presence of EGM.

Results of tissue residues:

The detected concentrations of doxycycline in tissues and serum of rabbits given the drug once per day (6 mg kg^{-1}b.wt) through oral route for 15 consecutive days were significantly lower in rabbits fed EGM diet than normal rabbits. The highest doxycycline concentrations were found in serum, kidney and liver followed by muscles (Table 6). No doxycycline could be detected in the serum and liver of normal rabbits15 days after stop of medication with doxycycline through oral route; the parallel period for rabbits fed EGM diet was 9 days (figure 3).

Table (6) Doxycycline concentrations (ppb) in rabbits following administration orally, 6 mg kg^{-1} alone and in presence of EGM, once per day for 15 successive days [mean ± SD] (n=3)

Tissues	Gp.	Slaughter time after treatment cessation (day) 1st	3rd	5th	7th	9th	15th	21th	MRL
Serum	D	1400±33	619±17	293±17	138±8	86±4	Nd	Nd	
	E	817±37*	369±9*	189±12*	76±7*	Nd	Nd	Nd	
Muscle	D	138±10	68±7	32±3	Nd	Nd	Nd	Nd	100
	E	102±11*	55±2*	26±2*	Nd	Nd	Nd	Nd	
Liver	D	553±13	264±9	144±13	87±4	Nd	Nd	Nd	300
	E	376±9*	207±11*	96±7*	Nd	Nd	Nd	Nd	
Kidney	D	1246±75	567±24	256±25	107±7	83±6	Nd	Nd	600
	E	710±43*	318±14*	173±14*	66±4*	Nd	Nd	Nd	

Nd: not detected.
*Significant differences in comparison to the group of DOX using T-Test ($P \leq 0.05$).

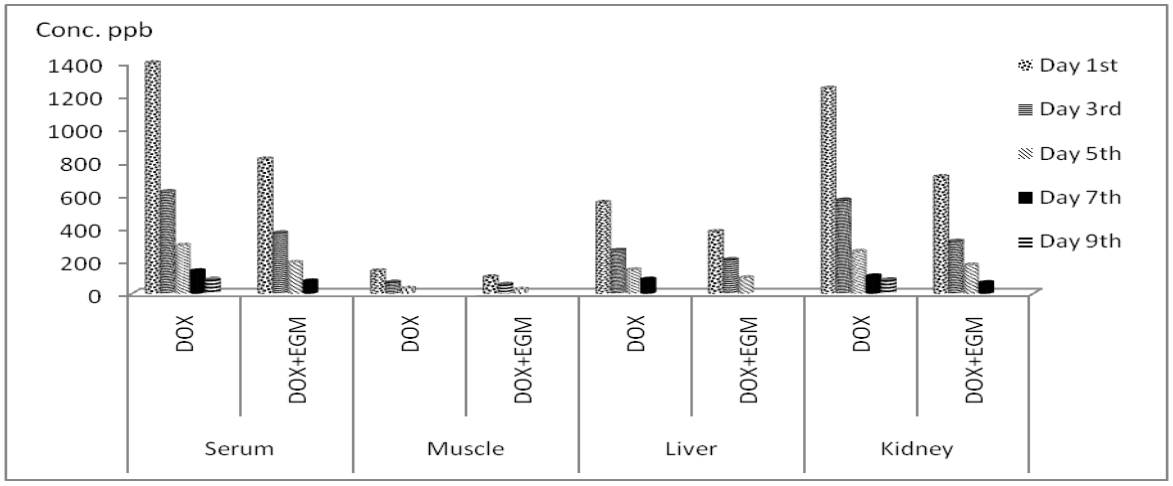

Figure (3) Doxycycline concentrations in rabbits after oral administration of 6 mg kg^{-1} alone and in presence of EGM, once daily for 15 consecutive days

DISCUSSION

These findings showed that serum concentrations of doxycycline are best analyzed by a two -compartment open model post a single oral dose of 6 mg kg^{-1} in rabbits alone and in presence of EGM. The same findings were previously described in rabbits by **DeMil et al. (2017)**, and also in chickens by **Atef et al. (2002)**.

In-vivo oral bolus pattern was applied to describe the interfaces from pharmacokinetic (**Devreese et al. 2012**) and tissue distribution perspectives; this is following the recommendations of EFSA for safety testing of this group of feed additive (**EFSA 2010**).

After single administration of 6 mg of doxycycline/kg b.wt orally, it was highly absorbed with C_{max} of 34.68 µg/ml achieved at 1.35 hr. It was slightly near from that investigated in rabbits orally medicated with5 mg doxycycline/kg (32.77 µg/ml) by **Elazab et al. (2020)**, but higher than that previously observed in broiler chicken administered 20 mg doxycycline orally/kg (5.55 µg/ml) (**DeMil et al. 2015**) and in claves received 10mg/kg doxycycline orally (3.3µg/ml) (**Meijer et al. 1993**). These disparate findings may be related to species variations.

The Kinetic investigation of the group obtained DOX alone obviously found that $AUC_{0-\infty}$, T_{max}, and C_{max} are declined significantly when EGM is used with DOX as a feed

additive. The 67 % decline inAUC_{0-24}in the group fed EGMlead to a lower ratio of AUC/ minimal inhibitory concentration, which is of great concern for the clinical concern (**Ismail & El-Kattan 2004; De Mil et al. 2015**). Consequently, a reduction in medication efficacy could be anticipated because the decline in oral bioavailability correlated linearly with a decline in AUC and thus also with the AUC/ minimal inhibitory concentration ratio. Antibiotics whose effectiveness depends on when plasma concentration is higher than minimal inhibitory concentration (MIC), such as tylosin, may be more sensitive to decreased oral bioavailability because time above MIC decreases in a logarithmic fashion as a role of bioavailability. Furthermore, sub-therapeutic dose of tetracycline's could motivate resistance of microbes (**Phillips et al. 2004**).

This interaction is consistent with previously described interactions of mycotoxin binder as feed additives with tylosin (**Devreese et al. 2012**), tilmicosin (**Shryock et al. 1994**), and with doxycycline (**De Mil et al. 2015, De Mil et al. 2016**), but disagree with **Goossens et al. (2012)** who found that diet with binder induced increased plasma concentrations of doxycycline administered as single bolus in pigs compared to diets containing blank feed, and also opposing the results obtained by **De Mil et al. (2017)** who found that no significant effects of any mycotoxin binders on the relative oral bioavailability of the antimicrobials doxycycline and tylosin in broiler chickens. These differences could be due to different animal's species, mycotoxin binder type and dose.

The pharmacokinetic data reflects its presence in different tissues, as the EGM binder considerably decreased the doxycycline levels in different rabbit's tissues. After administration of 6 mg doxycycline /kg/day for 15 successive days, the highest concentration was detected in kidneys followed by liver then muscle, and this is in line with reliable results in the European Medicines Agency (**EMA 2015**). In the two experimental groups; the residue levels declined rapidly and were below the MRL values (600, 300 and 100 µg/kg for kidney, liver, and muscle; correspondingly) at 3 days after treatment to be safe for human consumption.

Based on the in vivo results of pharmacokinetic and tissue residue studies, it can be concluded that EGM mycotoxin binder can virtually reduce oral doxycycline absorption in rabbits, and this is due to the adsorption of the EGM additive to doxycycline. Consequently, when doxycycline is given to rabbits orally that are also fed EGM fodder additive, the potential interaction must be taken into consideration carefully

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