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Effect of Bromhexine on the Pharmacokinetic of Tilmicosin in Broiler Chickens

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Concurrent administration of drugs may alter their pharmacokinetic parameters, so; investigation to what extent bromhexine hydrochloride affects the pharmacokinetic behavior of tilmicosinwas our aim of this work. Ten broiler chickens were classified intotwo groups as follow, the firstone (tilmicosin group) was given single oral dose of tilmicosin(20 mg/kg.b.wt.) while the 2nd(pre-treated group) wasgiven single oral dose of bromhexinehydrochloride (1 mg/kg.b.wt.) followed by single oral dose oftilmicosin(20 mg/kg.b.wt.) one hour later. The serum concentration of tilmicosin was measured usingHigh Pressure Liquid Chromatography (HPLC) method. The results revealed that the mean serum concentrations of tilmicosinwere significantly lower in pre-treated group when compared with tilmicosinalone group at the corresponding time intervals. Pharmacokinetic parameters were significantly differed($p<0.001$) between bothgroups.The maximum serum concentration were (C_{max} 0.70±0.02, 0.81±0.04μg/ml), achieved at $T_{max}$of (t_{max}0.89±0.16,and 2.10±0.06h),absorption half-life ($t_{0.5ab)}$ of 0.16±0.08,and 0.37±0.01hour, area under curve (AUC) of 12.96±0.42and 16.73±0.42μg.h/ml)in tilmicosin-bromhexineand tilmicosinalonegroups respectively. In conclusion,based on the obtained pharmacokinetic parameters, these findings showed that bromhexine accelerates the tilmicosin penetration into body tissues, achieving higher and faster concentrations than when given tilmicosin alone.

Keywords: Tilmicosin, Bromhexine, pharmacokinetic, HPLC, broilers.

Broilers production is considered as one of the largest and fastest growing industries in the world for providing the opportunity of animal protein needs for humans. However, poultry production has been facing the critical problems that require great efforts by the research institutions and the different studies to be explored and solved[1]whichencouraged us to choosebroilers chickens in this research work.

Macrolides antibiotics are composed of macrocyclic lactone rings to which one or more sugar residues are attachedby glycosidic linkages[2].The kinetic behavior of macrolides is characterized by high volume of distribution

enabling them to reach a high concentration in the target tissue even after administration of a small dose[3].Tilmicosin is one of the most importantbroad-spectrum macrolides developed for veterinary use*especially for treatment of respiratory infections in cattle and poultry because of its extensive accumulation in pulmonary tissues.Tilmicosin is a semi synthetic macrolide antibiotic of tylosin derivatives commonly used by veterinaries, has been shown to reveal beneficial pharmacological activities*.It suppressed bacterial protein synthesis by penetrating the cell membrane of sensitive microbes and binding to the 50s ribosomal subunit, Moreover, the translocation of immature peptide chains between the 50s and 30s ribosomal subunits is interfered leading to early detachment and synthesis of incomplete peptide chains[4]. *It inhibits* Gram-positive bacteria, such as*Corynebacterium* and *Listeria* species, some Gram-negative bacteria, such as *Pasteurella* and *Haemophilus* species, as well as atypical bacteria as *Mycoplasma*species[3].

Bromhexine is a mucolytic expectorant used in the treatment of respiratory disorders alone or in combination with other antimicrobials because it has ability to disturb the muco-polysaccharide of bronchial secretion enhancing the penetration power of antimicrobials. In addition, it produces an increase in immunoglobulin levels in airway secretions.Besides, it was recently recommended as a new drug for pathological states, such as alcoholic chronic pancreatitis where there is an increased pancreatic secretion[5].

In veterinary medicine, co-administration of bromhexine hydrochloride and antibiotics can increase antibiotic concentrations in lung tissue[6], nasal mucus[7]and sputum[8]. It promotes intra-tracheal mucus and stimulates secretion of pulmonary surfactant particles[9] to enhance their efficiency in the treatment of respiratory infections[10].Based on above data, the present study was planned to explore the effect of bromhexine hydrochloride on the disposition kinetic of tilmicosin after single oral administration in normal healthy broilers.

MATERIAL AND METHODS

Drugs

Tilmicosin phosphate was kindly provided by *Pharma-sweede* pharmaceutical company, *Egypt* as a white powder (80 %) withgood solubilityin water. It was used at a dose level of 20 mg kg^{-1} b.wt.Bromhexine hydrochloride was kindly provided by *Pharma- sweede* pharmaceutical company,*Egypt* as a white powder (98%) with poor solubilityin water but soluble in N-methyl pyridine/ propylene glycol (NMP/PG) (50%: 50%) solvent.

Animals and Experimental Design

The study was carried out on broiler chickens of both sexes with an average body weight from 2.5 to 3 kg. b.wt. and 45 days old.These birds were obtained from a special poultry farm atBeni-suef Governorate. The birds were kepton balanced commercial ration and water *ad-libitum*. They were kept under good hygienic conditions and left without treatment for two weeks before the experiment for acclimatization and ensuring complete clearance of any antibacterial agents. The experimental protocol was designed according to EthicalCommittee of the Faculty of Veterinary Medicine, Beni-suef University, in accordance with the Guide for the Care and Use of Laboratory Animals.Feed was withheld 12 hours before giving drugs. They divided into two groups each of 5 chickens.the firstone (tilmicosin group) was given single oral dose of tilmicosin (20 mg/kg.b.wt.) while the 2nd (pre-treated group) was given single oral dose of bromhexine hydrochloride (1 mg/ kg.b.wt.) followed by single oral dose of tilmicosin (20 mg/kg.b.wt.) one hour later.

Blood samples (1-1.5 mL) were collected from wing vein into test tubes at 15, 30 minutes, 1, 2, 4, 8, 12, 24, 48 and 72 hours post administration. All blood samples were left to clot for 30 minutes, centrifuged at 3000 *r.p.m*for 15 minutes and the obtained clear sera were transferred to eppendorff's tubes and kept in deep freeze (-20 C^{o}) till assayed by HighPressure Liquid Chromatography (HPLC).

Analytical procedure

Chemicals and Reagents

Reagent grade methanol, acetonitrile, n-hexane (Merck, Nogent–Sur–Marne, France), de-Ionized water or HPLC grade water, Ammonium acetate, di-potassium hydrogen phosphate (Merck) and calcium chloride (Sigma, USA). Trifluoroacetic acid: - UV grade (Merck). The solvents used during the chromatographic analysis of the drug were HPLC grade.

Chromatographic condition

Serum tilmicosinconcentrations were measured using HPLC method. The HPLC

system[12](in Animal Health Research Institute, *Dokki, Giza, Egypt*) which isconsisted of: Agilent series 1200 quaternary gradient pump,Series 1200 auto sampler, Series 1260 UV Vis detector, HPLC 32D Chemstation software (Hewlett-Packard, Les Ulis, France), Analytical column:the chromatographic column was a reversed-phase column (Extend-C18, Zorbax (5µm, 250mm x 4.6mm) column (Agilent Company), Acrodises (syringe filters), Millex HV13 filters (0.45 µm (tilmicosin), 13 mm id) (Millipore, Saint Quentin Yvelines, France).

Sample preparation

Plasma protein in each collected sample was precipitated by adding acetonitrile to chicken plasma or a standard sample (1:1). The mixture was mixed using the vortex for 30 seconds, and then centrifuged for 5 minutes at 1000xg. The clear supernatant was evaporated using nitrogen evaporator (0.5ml). The dried residue was dissolved in equivalent volume of dipotassium hydrogen phosphate buffer (0.5ml). The sample was injected directly into HPLC system after filtration with a fit acrodisc 0.45 ìm.

Liquid chromatography operating conditions

Injection volume, 50µl: flow rate, 0.7 ml/min; wave length, 287 nm; column temperature, ambient; stop time, 20 min; post time, 5min; mobile phase A, 0.05% trifluoroacetic; mobile phase B, acetonitrile.

Liquid chromatography gradient conditions

The gradient mobile phase consisted of (A): 0 min, acetonitrile –0.05% trifluoroacetic acid (22:78 v/v). (B): 6 min, acetonitrile –0.05% trifluoroacetic acid (45:55 v/v). (C): 10 min, acetonitrile –0.05% trifluoroaceticacid (22:78 v /v). The mobile phase was filtered using 0.45 µm membrane filter and degassed. The mobilephase was eluted at a flow rate of 0.7 ml/min with UV detection wave length of 287 nm.

Pharmacokinetic analysis of data obtained

Serum concentration (log_{10}) versus time curve were generated and best fitted by the aid of computer poly-exponential curve stripping program (*R-strip*, Micromath, Scientific software, USA). Data from each chicken were fitted individually and the pharmacokinetic variables were computed by the aid of the software program. The hybrid rate constants of the first order absorption and elimination rate constants(K_{ab} and K_{el}), absorption and elimination half-lives $t_{0.5(ab),}$ $t_{0.5\ (el),}$ area under the curve from zero to infinite time (AUC 0-œ), mean residence time (MRT), maximum serum concentration (C_{max}) and time to be achieved (t_{max}) were calculated. The results were expressed as Mean±SE and the obtained data statistically analyzed using student *T-test"*.

Statistical Analysis

The results were expressed as mean ± standard error of mean (S.E). Statistical significance was determined by student *(T-test)*using SPSS (version 20.0) software (IBM SPSS Statistic 20.0, Armonk, NY, USA). The *P* values less than 0.05 were considered statistically significant[13].

Table 1. The concentrations of tilmicosin standard (ug/ml) and their corresponding peak response

Retention time	Level	Concentration (ug/ml)	Area
10.88	1	0.03	46.344
	2	0.06	97
	3	0.15	240.33
	4	0.3	476.91
	5	0.6	964.33
	6	1.5	2409.3
	7	3	4595.4

Table 2. Mean Serum concentrations of tilmicosin and tilmicosin-bromhexine hydrochloride in healthy broiler chickens after single oral administration of 20 and 1 mg/kg. b.wt. respectively (n = 5)

Time	Mean±S.E	
	Tilmicosin group	Tilmicosin+ bromhexine group
15 min	0.19±0.01	0.28±0.01***
30 min	0.34±0.01	0.60±0.05***
1h	0.75±0.02	0.75±0.04
2 h	0.85±0.02	0.60±0.02***
4 h	0.73±0.02	0.54±0.02***
8 h	0.55±0.01	0.44±0.003***
12 h	0.45±0.01	0.35±0.002**
24 h	0.30±0.004	0.20±0.004***
48 h	0.11±0.01	0.07±0.002**
72 h	0.03±0.002	0.015±0.003**

** Significant at $p \leq 0.01$, *** Significant at $p \leq 0.001$

RESULTS

Standard curve of tilmicosin

Tilmicosin standard concentrations of 0.03, 0.06, 0.15, 0.3, 0.6, 1.5 and 3 ìg/ml and their corresponding peak responses are illustrated in Table (1) and Fig.(1),and Typical Chromatogram of tilmicosinareillustrated inFig. (2). thecalibration curve was calculated by linear regression equation method as y= 1538.5x + 21.755 where 'y' indicates the area under peak and 'x' indicatestilmicosin concentrations. Linearity existed within the range of 0.03 and 3 ìg/ml with a correlation coefficient r^2=0. 9994. The LOD for tilmicosin was 0.001 ìg/ml, while, LOQ was 0.003 ìg/ml.

Single oral administration of tilmicosin in healthy broiler chickens

The mean serum concentrations of tilmicosin at different time intervals following single oral dose (20 mg.kg^{-1} body weights) in broiler chickens are tabulated in Table (2). The drug was firstly detected (0.19±0.01µg/ml) after 15 minutes and the peak serum concentration (0.85±0.02µg/ml) was reached at 2 hours post drug administration and the lowest drug concentration (0.03±0.002 µg/ml) was reached at 72 hours post drug administration.

The pharmacokinetic parameters of tilmicosin following its oral administration are tabulated in Table (3). The calculated value of maximum concentration (C_{max}) was 0.81±0.02µg/ml and the time (t_{max}) taken to reach the peak was 2.10±0.06 hours. The drug was rapidly absorbed from broilers gut with absorption half-life ($t_{0.5ab}$) of 0.37±0.01hour but slowly eliminated with elimination half-life ($t_{0.5el}$) of 13.49±0.54 hours, the area under curve (AUC) was 16.73±0.42 µg.h/ml and mean residence time (MRT) was 19.4±0.74 hours.

Single oral administration of tilmicosin pre-treated with bromhexine hydrochloride in control healthy broiler chickens

The mean serum concentrations of tilmicosin (20 mg/kg b.wt.) pre-treated with bromhexine hydrochloride (1 mg/kg b.wt.) at

Table 3. Pharmacokinetic parameters of tilmicosin and tilmicosin-bromhexine hydrochloride in healthy broiler chickens after single oral administration of 20 and 1 mg/kg.b.wt. respectively (n = 5)

kinetic parameters	Unit	Tilmicosin	Tilmicosin+ bromhexine
K_{ab}	h^{-1}	1.89±0.05	8.13 ± 2.94
$t_{0.5ab}$	h	0.37±0.01	0.16 ± 0.08**
K_{el}	h^{-1}	0.05 ± 0.002	0.05 ± 0.002
$t_{0.5el}$	h	13.49±0.54	13.78 ± 0.65
C_{max}	µg/ml	0.81±0.02	0.70 ± 0.01***
t_{max}	h	2.10±0.06	0.89 ± 0.16***
AUC	$\mu g.h^{-1}.ml^{-1}$	16.73±0.42	12.96 ± 0.42***
AUMC	$\mu g.h^{2}.ml^{-1}$	18.80±0.25	13.8 ± 0.13***
MRT	h	19.4±0.74	19.57 ± 1.05

** Significant at $p \leq 0.01$, *** Significant at $p \leq 0.001$.

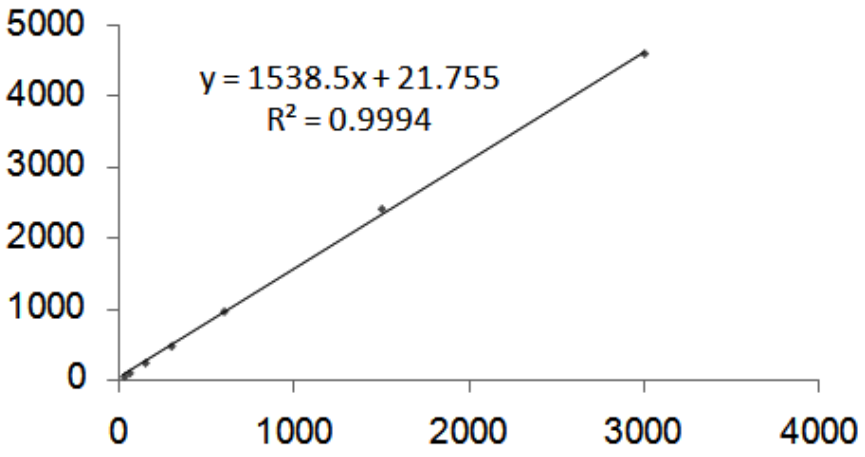

Fig. 1. Standard curve of tilmicosin

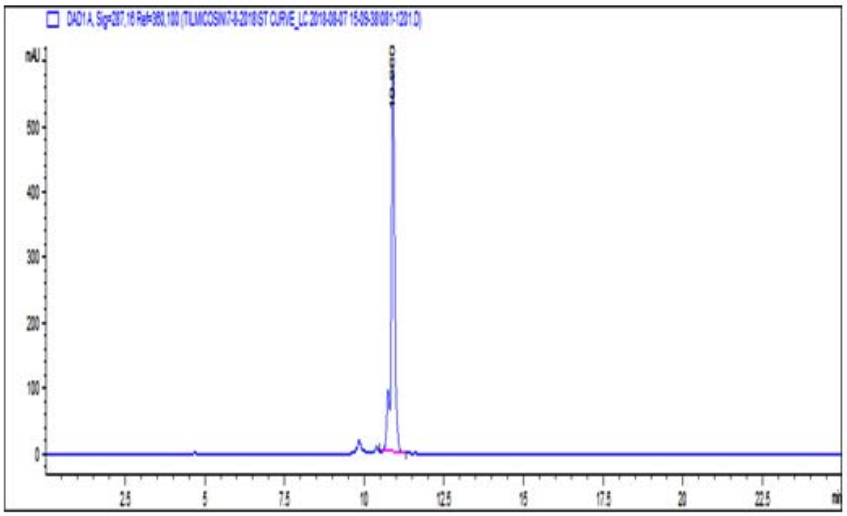

Fig. 2. Typical Chromatogram of Tilmicosin

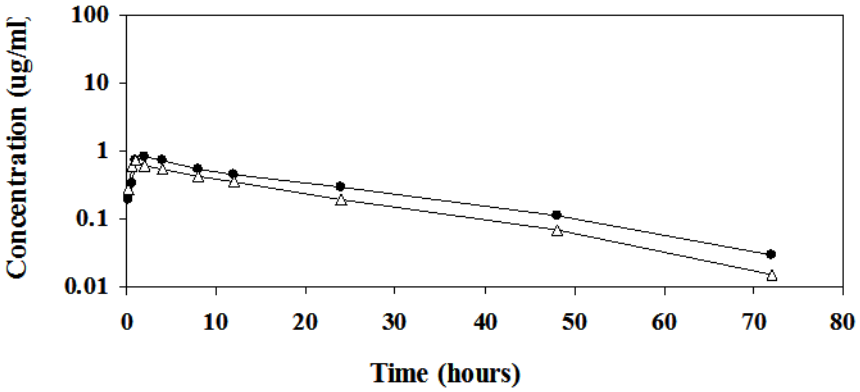

Fig. 3. Mean serum concentrations of tilmicosin (•) and tilmicosin-bromhexine hydrochloride (Δ) (µg/ml) in healthy broiler chickens following a single oral administration of 20 and 1 mg/kg.b.wt. respectively (n=5)

different time intervals post single oral dose in five broiler chickens are tabulated in Table (2). The drug was firstly detected (0.28±0.01 µg/ml) after 15 minutes and the maximum serum concentration (0.75±0.04 µg/ml) was reached at 1 hour post drug administration and the lowest serum concentration (0.015±0.0003 µg/ml) was reached at 72 hours post drug administration.

The pharmacokinetic parameters of pre-treated group are tabulated in *Table (3)*. The calculated value of maximum concentration (C_{max}) was (0.70±0.01µg/ml) and the calculated value of (t_{max}) was 0.89 ± 0.16 hour. The drug was rapidly absorbed from healthy broilers gut with absorption half-life ($t_{0.5ab}$) of 0.16±0.08 hour but slowly eliminated with elimination half-life ($t_{0.5el}$) of 13.77±0.66 hours, the area under curve (AUC) was 12.96±0.42 µg.h/ml and mean residence time and (MRT) was (19.57±1.05 hours).

Comparison pharmacokinetic between tilmicosinand tilmicosin pre-treated group after single oral administration in healthy broiler chickens

The mean serum concentrations of tilmicosin in control and pre-treated groupsafter single oral administration in healthy broiler chickens at different time intervals are shown in Table (2)anddepictedin Fig.(3).The drug was firstly detected (0.28±0.01, 0.19±0.01µg/ml) at 15 minutes post single administration of tilmicosin pre-treated

and tilmicosin alone respectively. The peak serum level(0.75±0.04 µg/ml) was higher in the first one hour then become lower in pre-treated group while the peak serum level of tilmicosin(0.85±0.02µg/ml) was reached at 2 hours post drug administration and the lowest concentration (0.015±0.0003, 0.03±0.02 µg/ml) were determined at 72 hours post single oral administration of tilmicosin in pre-treated and control groups respectively.

Pharmacokinetic parameters were significantly different *(p<0.01)* in both groups and recorded in Table (3). The maximum serum level (C_{max}) was lower in pre-treated group (0.70±0.02, 0.81±0.04µg/ml), while calculated (T_{max}) was shorter than control group (0.89±0.16, 2.10±0.06 hours) respectively, The drug was rapidly absorbed in pre-treated group with absorption half-life (t_{ab}) (0.16±0.08, 0.37±0.01hour), Area under the curve (AUC) (12.96±0.42, 16.73±0.42µg.h/ml) and Area under the maximum concentration curve (AUMC) (13.8 ± 0.13, 18.80±0.25 µg.h/ml^2) in pre-treated and non- treated groups respectively.

DISCUSSION

Tilmicosin is commonly used in veterinary field for treatment of respiratory diseases, so evaluation of the effect of bromhexine hydrochloride on the dispositionkinetics of tilmicosin is our aim in this research. The adverse effects of tilmicosin including cardiovascular toxicity as well as deaths after intravenous administration in broiler chickens had been previously mentioned[14].The pharmacokinetics of tilmicosin (20 mg/kg body weight) alone or pre-treated with bromhexine hydrochloride (1 mg/kg body weight) following a single oral administration were detected in this study. Tilmicosin was detected in serum 15 minutes post administration (0.19µg/ml) and increased gradually thereafter to reach its peak (0.81µg/ml) at 2.10 hours post administration then decreased gradually till reach its lower level (0.03µg/ml) at 72 hours in tilmicosin only group. Concerning of pharmacokinetic parameters,the result of C_{max}0.81 µg/mlisconsistent with that reported for azithromycin in broilers (0.95 µg/ml)[15], in calves(0.97 µg/ml)[16], and in cows (0.86 µg/ml)[17], but lower than that reportedin sheep (1.29,1.19 ìg/ml)[18], in goat (1.56 ìg/ml)[19],in swine (2.03ìg/ml)[20], in broilers for Pulmotil AC® at a single dose of 30 mg/kg (2.12 ìg/ml)[14],and that reported in rabbits forPulmotil® at a single dose of 12.5 mg/kg (1.31 µg/ml)[21].These differences might be attributed to dose, species and age variations, difference in formulations and/or the method used for assaying of the drug.On the other hand, time to peak serum level (t_{max} 2.10 hours) is similar to that reportedin broiler chickens for azithromycin (1.9 hours)[15], also that reported for tylosin in chicken (2.36 h)[22] but lower than that recorded in broilers (5.82 hours)[14] for (Pulmotil AC®)at a single dose of 30 mg/kg, which mightbe the cause of variation while itwaslonger than that detectedin rabbits (0.66 h)[21], in calves and cows (1 h)[16,17]which might be credit to species and dose variation, routes of drug administration and presence of food in the crop of chicken, that would affect the crop movements as well as the consistency of the feed might be affecting on the emptying of the crop.In addition; the presence of Lactobacillus ûora in the crop which lead to inactivation of the macrolides may be attributed[23].

Tilmicosinwas rapidly absorbed with an absorption half-life ($t_{0.5ab}$) 0.37 h. Our findingis nearly similar to that reported for azithromycin in broiler chickens ($t_{0.5ab}$ 0.57 h)[15]. Tilmicosin has been slowly eliminated with elimination half-life ($t_{0.5el}$) of13.49 h. This outcomeis higher than that reported for erythromycin (1.9 h) [24]which may beattributed to that tilmicosin was detected in the serum till 72 h, but lower than that reported in sheep, swine and goat (29.3, 25.26 and 29.4 h)[19,20,25]. In this study, the calculated area under serum concentration-time curve (AUC) was 16.73 µg.h/ml which come in agree with that stated for tylosin in broilers (18.60 ìg.h.ml^{-1}) [26] while it is lower than that detected in chicken (21.82 ìg.h.ml^{-1})[14] for tilmicosin but higher than that recoded in pigs (9.68 ìg.h.ml^{-1}) [27]. These varieties might be credit to the species and dose variation.

This study was planned to evaluate whether there is a pharmacokinetic connection amongst tilmicosin and bromhexine hydrochloride in broiler chickens after single oral administration, the mean serum concentrations of tilmicosin (C_{max}) were significantly lower in bromhexine pre-treated (0.70±0.02 µg/ml) broilers contrasted with tilmicosin alone(0.81±0.04µg/ml).Similar finding indicated higher concentration of oxytetracycline within the secreted mucous when used in

combination with bromhexine hydrochloride[28]. Also, patient given amoxicillin-bromhexine combination showed a significant reduction in symptoms such as cough frequency, cough discomfort, sputum volume and had favorable clinical response at the end of the course of treatment [29]. Similar results revealed that the bioavailability of erythromycin and its concentration in bronchial fluid were increased after its administration as combined with bromhexine[30]. Furthermore, injection of bromhexine with spirmycin resulted in an increase in concentration of spiramycin in bovine nasal secretion [31]. The value of C_{max} in both groups is higher than the minimum inhibitory concentrations (MICs) for *Mycoplasma gallisepticum*and *Mycoplasma synoviae* (0.0125-0.1 ìg/ml)[32], *Corynebacteriumpyogenes* in cattle (0.04 ìg/ml) [33] and*Ornithobacteriumrhinotracheale* (0.06–1 ìg/ml) [34] but lower than the MICs for *Clostridumperfringens* strains isolated from commercial broiler farms[32] as well as *Pasteurella multocida* and *Mannheimiahaemolytica*(3.125 and 6.25 ìg/ml) respectively[33]. TheNational conference of constituency leaders(NCCLS) guidelines for tilmicosin susceptibility list a breakpoint of (d•8 ìg/ml) [35]. This revealed that the serum concentrations of tilmicosin are lower than the MICs for some susceptible bacteria. Nevertheless, previous studies have reported that administration of tilmicosin at the recommended dose is effective for control of respiratory diseases [36, 37]because of its prolonged duration in lung tissues at therapeutic level[38]. Tilmicosin is rapidly absorbed when given in birds pretreated with bromhexine as appeared shorter $t_{0.5ab}$ (0.16±0.08hour)compared to (0.37±0.01 hour). Tilmicosin concentration is rapidly reached to the peak in pre-treated group than control group as appeared shorter t_{max}(0.89±0.16) compared to 2.10±0.06 hours respectively.Similar finding was previously reported for enrofloxacin in sheep [39].They reported that, $t_{0.5ab}$was found to be 0.53 ± 0.11h for enrofloxacin alone in sheep compared to 0.33 ± 0.09h, whenenrofloxacin given in combination with bromhexine.

The data of our experiment reported that C_{max} and AUC in pre-treated group are significantly lower than that for control group as reported that excipients are considered inert components of a drug formulation affecting only the physicochemical characters of the product (e.g. dissolution and drug stability) [40].However, there wereprevious studies revealed that some excipients are able to produce its own direct action for example mannitolwhich decreases gastrointestinal transit time *via* its osmotic activity [41], surfactants, which can change membrane characteristics [40, 42] and vitamin E which can change the activity of multi-drug resistance proteins thereby affecting drug bioavailability [43].Moreover the changes in the serum concentration and pharmacokinetic parameters induced by pre-treatment with bromhexine may be attributed to enhancing the absorption of tilmicosin and the distribution of tilmicosin to different tissues and body secretionsbybromhexine. Similar results were reported previously for furaltadone into tracheobronchialsecretions in broilers[44].

CONCLUSION

The obtained results explain that concurrent administration of tilmicosin and bromhexinealtered serum concentration but improve pharmacokinetic parameters. Pre-treatment with bromhexine enhanced the absorption of tilmicosin and the distribution oftilmicosin to different tissues and body secretions by bromhexine, which reflects enhanced efficacy the combination of bromhexine as compared with tilmicosin alone.

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