International Journal of Livestock Research, Vol. 11 (9) Sep' 2021
http://www.ijlr.org
eISSN: 2277-1964

Original Research

Effect of Efflux Protein Inhibitors on Pharmacokinetics and Pharmacodynamics of Enrofloxacin in Chicken

Srividya Gullapudi[1]*, G. Srinivasarao[2], P. Ravikumar[3], Muralidhar Metta[4] and V. Rama Devi[5]

[1]Assistant Professor, Dept of pharmacology, CVSc, Proddatur, SVVU, Andhra Pradesh, INDIA
[2]Professor, Dept of pharmacology, NTRCVSc, Gannavaram, SVVU, Andhra Pradesh, INDIA
[3]Professor, Dept of pharmacology, NTRCVSc, Gannavaram, SVVU, Andhra Pradesh, INDIA
[4]Assistant Professor & Head, Department of Animal Genetics & Breeding, Andhra Pradesh, INDIA
[5]Professor, Dept of pathology, NTRCVSc, Gannavaram, SVVU, Andhra Pradesh, INDIA

*Corresponding Author: vidyaprasad125@gmail.com

How to cite this paper: Gullapudi, S., Srinivasarao, G., Ravikumar, P., Metta, M., & RamaDevi, V. (2021). Effect of Efflux protein inhibitors on Pharmacokinetics and Pharmacodynamics of Enrofloxacin in Chicken. *International Journal of Livestock Research*, 11(9), 40-47. https://dx.doi.org/10.5455/ijlr.20201015090255

Received : Oct 15, 2020
Accepted : Jul 11, 2021
Published : Sep 30, 2021

Abstract

The present study was designed to investigate the effect of efflux protein inhibitors glycyrrhizicacid and glycyrrhetenic acid on pharmacodynamics and pharmacokinetics of enrofloxacin in chicken. The pharmacokinetic study was carried out in broiler chicken by randomly dividing eighteen birds into three groups of six each. Birds with enrofloxacin @10 mg.kg-1body weight orally served as control. Treatment groups received glycyrrhizic acid and glycyrrhetenic acid orally along with enrofloxacin. Blood samples were collected at predetermined time intervals upto 48 h and plasma concentrations of enrofloxacin were determined by HPLC with UV detection at 277 nm. The pharmacodynamic study was carried out by determining the MIC of enrofloxacin against E. coli ATCC25922 as per the CLSI guidelines. Cmax and AUC of enrofloxacin was enhanced in treatment groups. The MIC of enrofloxacin was improved in the presence of phytochemicals. In conclusion, glycyrrhizic acid and glycyrrhetenic acid enhanced the antibacterial activity of enrofloxacin.

Keywords: Enrofloxacin, Glycyrrhizic Acid, Glycyrrhetenic Acid, MIC, Pharmacokinetics

Introduction

Antimicrobial resistance (AMR) is threatening millions of lives worldwide, and is rightly declared as a global risk by the World Economic Forum (World Economic Forum, 2013). Intrinsically, AMR is more prevalent and severe in Gram negative bacteria than their Gram-positive counterparts mainly due to the outer membranes serving as permeability barrier for drug-influx into the Gram-negative bacteria (Silhavy *et al.,* 2010, Exner *et al.,* 2017). To enhance their rate of survival to biocidal compounds, gram-negative bacteria reduce the membrane permeability by reducing the number of proteins and inducing drug efflux pumps for outward transport of drugs, in a non-specific manner making the bacterial cells resistant to traditional antibiotics (Masi *et al.,* 2017). Some of the gram-positive bacteria like methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA), coagulase negative Staphylococci members including *S. epidermidis* and *S. haemolyticus*, *Streptococcus pneumonia*, *Enterococcus faecalis*, *E. faecium* and *Clostridium difficile* also develop resistance (Schindler and Kaatz 2016).

The four modes of resistance development of bacteria to antibiotics are target alteration, drug inactivation, decreased permeability and increased efflux. Among these, the drug extrusion by the multidrug efflux pumps serves as an important mechanism of MDR. Due to the development of resistance to antimicrobial agents to microbial infections phytochemicals became complementary and alternative medicine. Phytochemicals which possess efflux pump inhibitory activity if combined with classical antimicrobial agents reduces the development of resistance and also improves their efficacy.

Enrofloxacin, a fluoroquinolone group of antibiotics is most commonly used against gram negative bacterial infections in majority of livestock species. Due to indiscriminate usage of enrofloxacin resistance had been developed to this quinolone group of drug.

Phytochemicals in combination with antimicrobial agents alters their antimicrobial activity which were studied as herb drug interactions. Some of the research reports revealed that Plumbagin, Mangiferin and `Quercitin enhances the antibacterial activity by acting on efflux pumps (Ohene-Agyei *et al.,* 2014). Piperine in combination with ciprofloxacin potentiated the antibacterial effect by reducing the MIC values of Staphylococcus aureus (Khan IA, et al., 2006). Naringenin, a flavonoid obtained from grape juice inhibits the transport of fexofenadine due to its inhibitory effect on Organic Anion Transporting Polypeptides (OATP). Based on the above interested reports on natural efflux pump inhibitor combination with antibiotics, the present study was designed to determine the effect of putative efflux pump inhibitors glycyrrhizic acid and glycyrrhetenic acid on pharmacokinetic and pharmacodynamic behaviour of enrofloxacin

Materials and Methods

Drugs and Chemicals

Enrox 10% oral solution was obtained from M/S Alembic Pharmaceuticals Ltd, Gujarat, India, was used for oral administration to determine the Enrofloxacin Kinetics. Pure technical grade powder of enrofloxacin, glycyrrhizicacid, glycyrrhetenic acid and were procured from Sigma-Aldrich, St. Louis, MO, USA. Heparin 20,000 IU/vial were obtained from M/S LobaChemie, Mumbai. Acetonitrile, Methanol, Glacial acetic acid, Triethyl amine, Orthophospharic acid and other chemicals of HPLC grade that are used in the experiment were procured from M/s Merck, Mumbai, India. Water for HPLC was obtained by Millipore water purification system and was filtered, through 0.2 µm filter prior to use. Muller-Hinton Broth was obtained from M/S HiMedia Laboratories Pvt. Ltd. Mumbai, India. Magnesium chloride was obtained from M/S Fisher Scientific; Mumbai, India and Calcium chloride was from M/S SD Fine - Chem Ltd, Mumbai, India. *p* - iodonitrotetrazolium (INT) was procured from M/S SRL, Mumbai, India. *E. coli* ATCC 25922 was procured from Himedia. The ATCC cultures of *E. coli* was obtained from principal investigator of RKVY project, Dept of Pharmacology & Toxicology, NTRCVSc, Gannavaram.

Experimental Animals

Eighteen broiler chicken of 6-8weeks age and 1.5 to2.0 kg were obtained from local hatcheries and kept for acclimatization for two weeks prior to the experimentto avoid any drug residual effect. The experimental protocol was approved by Institutional Animal Ethics Committee vide Proc.No.3/IAEC/NTRCVSc/2017dtd 15.07.2017.

Experimental Design

Eighteen broiler chicken were randomly divided into three groups of six birds each. Birds in Group I received Enrofloxacin @10 mg.kg^{-1}B.wt *p.o* which serves as enrofloxacin control. Group II and III birds were coadministered with glycyrrhizic acid and glycyrrhetenic acid @100 mg.kg^{-1} *p.o* respectively one hour before administration of enrofloxacin@10 mg.kg^{-1}B.wt.Blood samples were collected at predetermined time intervals from the tarsal vein at 0,0.166,0.33,0.5,0.75,1,1.5,2,4,6,8,12,24,36 and48h in all three groups. Plasma was harvested by centrifugation at 2000rpm for 5min and stored at -20^{o}C until analysis.

Analysis of Enrofloxacin

The serum concentrations of enrofloxacin were determined by a reverse phase high-performance liquid chromatography (HPLC) using the method described by Rao *et al.* (2002), with few modifications. 20 µL supernatant was injected into the HPLC system for analysis. Chromatography was carried out using a SHIMADZU C18 column (particle size 5 µm, 4.6 mm x 250 mm); the mobile phase consisted of 70% 10mM KH_2PO_4(0.3% orthophosphoric acid, and 0.3% triethylamine), 20% acetonitrile, 10% methanol at a flow rate of 0.8ml/min and UV detector was operated at a wavelength of 277 nm. The analytical method was validated by limit of quantification, linearity for a range of concentrations, accuracy, precision. The temperature of column oven was maintained thermostatically at 40^{0}C.

Calibration Curve

The calibration curve of serum was prepared with seven different concentrations between 0.001 and 10 µg/ml using blank chicken plasma. A calibration curve was obtained by plotting the peak area versus the nominal concentrations. The equation was calculated by the least-squares method using linear regression. The standard curve of enrofloxacin in chicken plasma was linear between 0.003 and 4 µg/ml (R^2> 0.99). The peak area of unknown sample was compared with that of the standard enrfloxacin.

Validation of the Assay Method

The concentrations of enrofloxacin estimated eight times in a day and eight occasions at least 24 hours gap (inter-day variation) for two standard concentrations of 0.125 µg/ml and 2.0 µg/ml in broiler chicken plasma The intra-day coefficients of variation for two concentrations were below 10% (7.3% and 4.5%) with accuracy percent of 98.09 to 98.68%. The inter-day coefficients of variation for two concentrations were below 10% (7.2% and 4.3%) with accuracy percent of 96.30 to 98.83%. Plasma concentrations of enrofloxacin after oral administration were subjected to a noncompartmental analysis with the help of a PK Solver, (Zhang *et al.*, 2010). Pharmacokinetic parameters were directly calculated from the data by the software program.

MIC by Broth Microdilution Method

The broth microdilution method was used to determine the MIC of enrofloxacin against *E. coli* ATCC25922. Working standard of 1µg/ml enrofloxacin was prepared by diluting the stock solution with normal saline. Two-fold serial dilution of enrofloxacin in CAMHB (Cation Adjusted Mueller Hilton Broth) was prepared in 96 well microtiter plate, so that final volume in each well was 100µl.The bacterial culture incubated in CAMHB at 37±1°C for 6 to 8 h was taken and its turbidity was adjusted to 0.5 McFarland turbidity standard (1 X 10^8 CFU/ml) which was then diluted 1:20 in CAMHB. When 0.01 ml of this suspension was inoculated into the broth, the final concentration of bacteria was approximately 5 X 10^5 CFU/ml (range 2 - 8X 10^5 CFU/ml or 5 X 10^4 CFU/well). Each plate was sealed properly to prevent drying during incubation. Inoculated microdilution trays were then incubated at 35±2°C for 16 to 20 h in an ambient air incubator.

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye or microplate reader (Multiskan^{TM} GO, Thermo fisher scientific^{TM}) to discern growth in the wells. The amount of growth in the wells containing antimicrobial agent was compared with that of growth-control wells (no antimicrobial agent) used in each set of tests. Alternatively, bacterial growth and inhibition was detected by adding 25 µl of INT to each well and incubation for 30 min at 35±2°C. INT is reduced to a red formazan compound by biologically active organisms; in this case the dividing bacteria. Bacterial

growth was considered to be inhibited when the solution in the well remained clear. This concentration was considered as MIC. Solvent controls and growth controls were included in each experiment (CLSI, 2012).

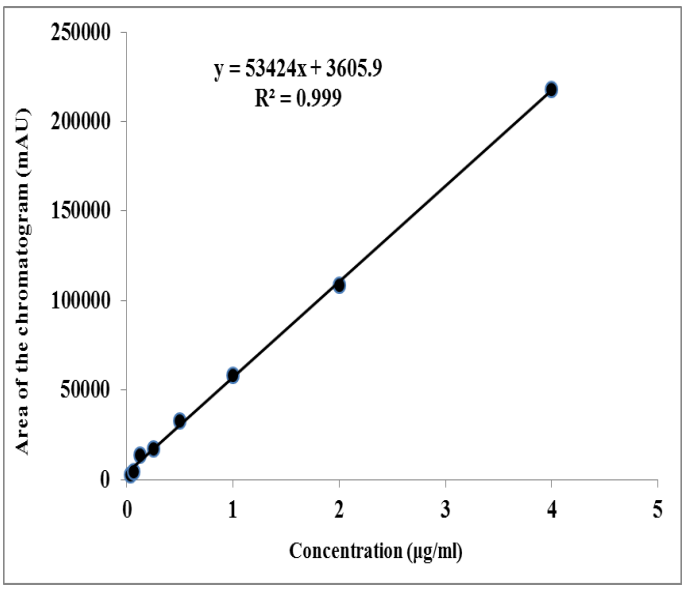

Figure 1: Standard calibration curve for enrofloxacin in chicken

Statistical Analysis

Plasma concentrations, pharmacokinetic variables of enrofloxacin and all other data were expressed as Mean±SEM. The variation among the pharmacokinetic parameters were analyzed using non- parametric approach (Kruskal – Wallis test). The analysis was performed using graphpad Instat software. $P < 0.05$ is considered as significant.

Results

Enrofloxacin plasma concentration–time curves in normal and efflux protein inhibitors co-administered chicken following oral administration were shown in figure 2 and 3. The plasma concentrations of glycyrrhizic acid and glycyrrhetenic acid coadministered chickens were greater than in control birds. Plasma concentration of enrofloxacin following oral administration of enrofloxacin and enrofloxacin coadministered with glycyrhizic acid and glycyrrhetenic acid were presented in table 1. Pharmacokinetic parameters following oral administration of enrofloxacin and enrofloxacin coadministered with glycyrhizic acid and glycyrrhetenic acid were presented in table 2. The pharmacodynamic variable MIC was determined as per CLSI guidelines against *E. coli* ATCC 25922. The MIC of enrofloxacin alone against *E. coli* is 0.02 µg/ml and in the presence of glycyrrhizic acid and glycyrrhetenic acid it was reduced to 0.012µg/ml. The PK- PD index was calculated and presented in table 3.

Table 1: Effect of oral coadministration of glycyrrhizic acid and glycyrrhetenic acid with enrofloxacin on plasma concentrations of enrofloxacin in chicken (n=6) Mean + SEM.

Time(h)	Group I	Group II	Group III
	Enrofloxacin	Glycyrrhizic acid +enrofloxacin	Glycyrrhetenic acid +enrofloxacin
0.166	0.23±0.11	0.18±0.16	0.11±0.01
0.333	0.53±0.17	0.27±0.01	0.35±0.03
0.5	0.81±0.24	0.36±0.01	0.97±0.04
0.75	0.99±0.24	0.89±0.04	1.60±0.02
1	1.24±0.30	1.02±0.04	2.03±0.06
1.5	1.64±0.34	2.17±0.16	2.38±0.09
2	1.76±0.35	2.74±0.15	2.64±0.11
4	2.02±0.32	2.60±0.09	2.75±0.08
6	1.85±0.20	2.08±0.06	2.64±0.12
8	1.48±0.13	1.49±0.04	2.25±0.05
12	1.06±0.13	1.34±0.03	1.91±0.06
24	0.58±0.09	0.69±0.05	1.52±0.04
36	0.34±0.06	0.35±0.01	0.45±0.03
48	0.11±0.01	0.16±0.02	0.18±0.01

Table 2: Effect of oral co-administration of glycyrrhizic acid and glycyrrhetenic acid with enrofloxacin on pharmacokinetic parameters of enrofloxacin in chicken (n=6 Mean+SEM)

Parameter	Unit	Group I	GroupII	Group III
		Enrofloxacin	Glycyrrhizic acid+ enrofloxacin	Glycyrrhetenic acid + enrofloxacin
β	h^{-1}	0.06±0.00	0.06±0.00	0.06±0.00
$t_{1/2\beta}$	h	11.05±0.27	11.49±0.46	11.42±0.32
T_{max*}	h	4.25±0.68	2.67±0.42	4.00±0.73
C_{max*}	µg. mL^{-1}	2.17±0.30	2.96±0.04	2.94±0.03
AUC_{0-t*}	µg.h. mL^{-1}	36.28±4.80	43.37±0.98	63.35±1.08
$AUC_{0-\infty*}$	µg.h. mL^{-1}	633.04±85.53	800.16±47.39	1174.61±29.76
$AUMC_{0-t*}$	µg.h^2. mL^{-1}	16.60±0.42	17.28±0.69	17.70±0.25
MRT	h	4.47±0.43	3.59±0.10	2.49±0.09
V_d/F	$L.kg^{-1}$	0.28±0.03	0.22±0.01	2.489±0.078
Cl_B/F	$L.kg^{-1}.h^{-1}$	0.282±0.044	0.217±0.020	0.151±0.013

Table 3: Effect of efflux protein inhibitors on PK/PD indices of enrofloxacin in chickens on *E. coli* ATCC29922

	C_{max}/MIC	AUC/MIC
Group I (Enrofloxacin)	86.92	1813.93
Group II	118.48	2165.4
(Enr + glycyrrhizicacid acid)		
Group III	117.36	3167.3
(Enr + glycyrrtetenic acid)		

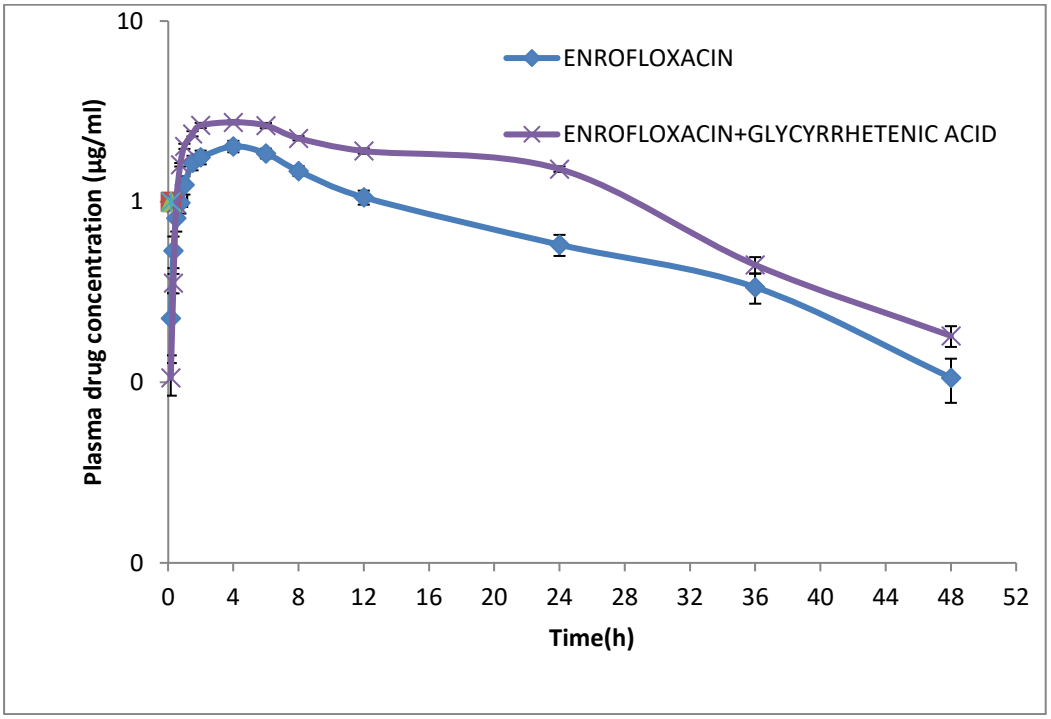

Figure 2: Semilogarithmic plot of enrofloxacin concentration (µg/ml) in plasma versus time after co-administration of glycyrrhetenic acid (100 mg.kg-1) with single oral administration of enrofloxacin (10 mg.kg-1). Each point represents Mean+SEM of six chickens.

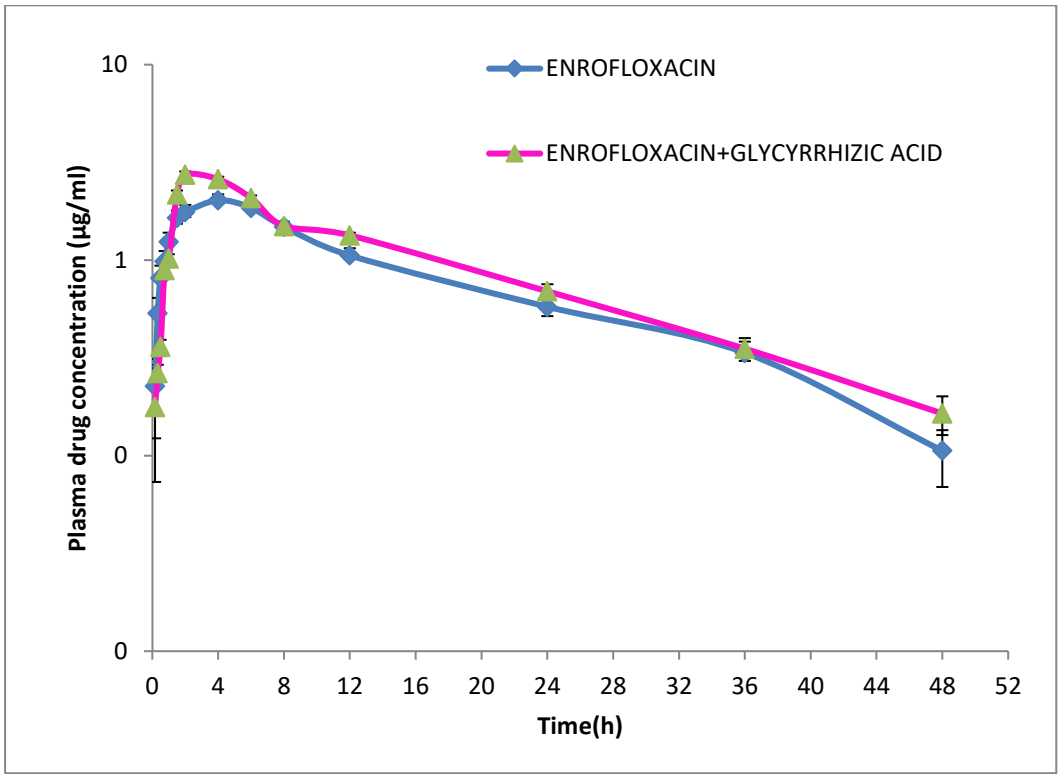

Figure 3: Semilogarithmic plot of enrofloxacin concentration (µg/ml) in plasma versus time after co-administration of glycyrrhizic acid (100 mg.kg-1) with single oral administration of enrofloxacin (10 mg.kg-1). Each point represents Mean+SEM of six chickens

Discussion

Effect of glycyrrhetenic acid on the plasma levels and pharmacokinetics of enrofloxacin was investigated by co-administration of glycyrrhetenic acid @100 mg. Kg^{-1}p.o with enrofloxacin. Upon co-administration with

glycyrrhetenic acid, C_{max} of enrofloxacin increased significantly from 2.17+ 0. 14µg.ml^{-1} to 2.94+ 0. 04µg.ml^{-1}($p<0.05$) (Table 2). The bioavailability which was denoted by AUC_{0-t} showed a significant rise to 63.35+0.17µg.h.mL^{-1}from 36.28+0.57µg.h.mL^{-1} ($p<0.01$) level of significance. The increased C_{max} and bioavailability may be due to potent inhibitory activity of P-gp efflux protein by 18β-glycyrrhetenic acid (Feng *et al.*, 2015) as well as competitive inhibition of CYP3A4 (Li QL *et al.*, 2016). Katoh *et al.* (2009) reported that glycyrrhetenic acid inhibited the glucuronidation of β-estradiol and SN-38in human liver microsomes. Some of reports revealed that glycyrrhetenic acid significantly inhibited UGT2B7 activity (Nakagawa *et al.*, 2009 and Huang *et al.*, 2013). AUMC of enrofloxacin was increased significantly from 633.05+2.41 to 1174+1.42µg.h^2.mL^{-1} upon co-administration of glycyrrhetenic acid.

Upon co-administration of glycyrrhizic acid the mean C_{max} was increased significantly to 2.96+0.06µg.ml^{-1}from 2.17+0.14µg.ml^{-1}($p<0.05$) (Table 2). The enhanced C_{max} may be due to its inhibitory effect on OATPs as reported by Ismair *et al.*, 2003 being enrofloxacin a substrate of OATPs (Xiao *et al.*, 2014). Xu *et al.* (2012) reported that due to the inhibitory effect of MRP_2 secretion of methotrexate was reduced, glycyrrhizic acid reduced the drug transport by inhibiting the transport proteins which may be attributed to higher plasma levels of the administered drug. Time required to reach maximum plasma concentration (T_{max})was reduced almost to 50% due to increased rate of drug absorption by glycyrrhizic acid. There is no statistically significant change in elimination rate constant, half-life, MRT, volume of distribution and clearance upon co-administration of glycyrrhizic acid ($p<0.05$) level of significance. Upon coadministration with glycyrrhetenic acid C_{max} and AUC of enrofloxacin increased significantly ($p<0.05$ and $P< 0.01$)

The MIC (µg/ml) of enrofloxacin was reduced to 0.012µg/ml against *E. coli*0.02µg/ml, in presence of glycyrrhizic acid and glycyrrhetenic acid. The markers for dose optimisation are Cmax/MIC>10 was considered for better clinical effect and AUC/MIC>125will prevent the development of resistance (Haritova *et al.*, 2008). In the present study for enrofloxacin dose optimisation, *E. coli* ATCC 25922 was selected as its sensitive. As Cmax/MIC and AUC/MIC are more than 10 and 125 respectively. The dose 10mg/kg is effective orally in chicken to produce effective antibacterial action.

Conclusion

Based on the above results it can be concluded that glycyrrhizic acid and glycyrrhetenic acid enhances plasma concentrationand bioavailability of the enrofloxacin. These putative efflux pump inhibitors also potentiated the antibacterial activity of enrofloxacin *invitro.* The PK-PD integration values revealed that the enrofloxacin dose of 10mg/kg in combination with selected phytochemicals reduces the chances of development of resistance. It also revealed that the enrofloxacin dose 10mg/kg is effective against *E. coli*.

Conflict of Interests

There is no conflict of interest.

Publisher Disclaimer

IJLR remains neutral concerning jurisdictional claims in published institutional affiliation.

References

1. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P and Hartemann P (2017) Antibiotic resistance: what is so special about multidrug-resistant Gram-negative bacteria? *GMS Hygiene and Infection Control*12:Doc05. 10.3205/dgkh000290.
2. Feng X, Ding L and Qiu F (2015) Potential drug interactions associated with glycyrrhizin and glycyrrhetinic acid. *Drug Metabolism Reviews*. Vol 47(2): 229-38.
3. Haritova AN, Rusenova A, Rusenov J, Schrickx L, Lashev and Fink-Gremmels J (2008). Effects of fluoroquinolone treatment on MDR1 and MRP2 mRNA expression in Escherichia coli-infected chickens. Avian Pathology 37:465–470.
4. Huang YP, Cao YF, Fang ZZ (2013) Glycyrrhetinic acid exhibits strong inhibitory effects towards UDP-glucuronosyltransferase (UGT) 1A3 and 2B7. *Phytotherapy Research* (27): 1358–1361.

5. Katoh M, Yoshioka Y and Nakagawa N (2009). Effects of Japanese herbal medicine, Kampo, on human UGT1A1 activity. *Drug Metabolism and Pharmacokinetcs*. 24:226–234.
6. Khan IA, Mirza ZM, Kumar A, Verma V and Qazi GN (2006). Piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy*. 50(2):810–812.
7. Lv QL, Wang GH, Chen SH, Hu L, Zhang X, Ying G and Zhou HH (2015). In Vitro and in Vivo Inhibitory Effects of Glycyrrhetinic Acid in Mice and Human Cytochrome P450 3A4. *International Journal of Environmental Research and Public Health.*13(1): 84.
8. Masi M, Refregiers M, Pos KM and Pages JM (2017). Mechanisms of envelope permeability and antibiotic influx and efflux in Gram-negative bacteria. *Nature Microbiology*2:17001.
9. Nakagawa N., Katoh M and Yoshioka Y (2009). Inhibitory effects of Kampo medicine on human UGT2B7 activity. *Drug Metabolism and Pharmacokinetics* 24:490–499.
10. Ohene-Agyei T, Mowla R, Rahman T and Venter H. (2014). Phytochemicals increase the antibacterial activity of antibiotics by acting on a drug efflux pump. *Microbiology Open*3(6):885-896.
11. Rao GS, Ramesh S, Ahmad AH, Tripathi HC, Sharma LD and Malik J K(2002). Pharmacokinetics of enrofloxacin andits metabolite ciprofloxacin in goats given enrofloxacinalone and in combination with probenecid. *Veterinary Journal*163(1):85-93.
12. Schindler BD and Kaatz GW (2016). Multidrug efflux pumps of Gram-positive bacteria. *Drug Resistance Updates*27:1–13
13. Silhavy TJ, Kahne D, and Walker S (2010). The bacterial cell envelope. *Cold Spring Harbor perspectives in biology*2(5): a000414.
14. World Economic Forum (2013). *Global Risks* Eighth Edition 28–33.
15. Zhang Y, Huo M, Zhou J, Xie S and PKSolver (2010). An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs Biomedicine.* 99(3):306-14.
16. Ismair MG, Stanca C, Ha HR, Renner EL, Meier PJ and Kullak-Ublick GA (2003) Interactions of glycyrrhizin with organic anion transporting polypeptides of rat and human liver. *Hepatology Research* 26(4):343-347.
17. Xiao, Y., Deng, J., Liu, X., Huang, J., Sun, Y., Dai, R., & Hong, M. (2014). Different Binding Sites of Bovine Organic Anion–Transporting Polypeptide1a2 Are Involved in the Transport of Different Fluoroquinolones. *Drug Metabolism and Disposition*, 42(8), 1261-1267.
18. Xu R, Zhang X, Yang J, Liu X, Davey AK, Wang J (2012). Effects of glycyrrhizin on biliary transport and hepatic levels of glutathione in rats. *Biopharmaceutics Drug Disposition* 33(5):235-45

****