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Interplay of selected efflux protein inhibitors on the antibacterial activity and pharmacokinetics of enrofloxacin in chicken

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Abstract
The present study was designed to determine the interplay of efflux protein inhibitors on the antibacterial activity and pharmacokinetics of enrofloxacin in chicken. Capsaicin and theobromine were used as efflux protein inhibitors. Birds weighing about 1.5 to 2.0 kg were allocated to 3 groups of six at random. Group I served as enrofloxacin control, whereas Group II and III birds were coad ministered with capsaicin and theobromine along with enrofloxacin. The plasma levels of enrofloxacin was determined by HPLC. The pharmacokinetic parameters were estimated by using Pksolver. Coadministration theobromine improved the bioavailability of enrofloxacin. The antibacterial activity was assessed by using microbroth dilution technique as per CLSI guidelines using MIC as determinant against ATCC strains of *E. coli, S. aureus, K. pnemoniae, P. aureginosae*. The results obtained in the study reveaed that combination of enrofloxacin with capsaicin and the obrominne enhanced the antibacterial activity of enrofloxacin by reducing the MIC values. The combination may be beneficial in reducing the development of resistance after conducting further studies.

Keywords: Enrofloxacin, capsaicin, theobromine, MIC, PK-PD

1. Introduction
Enrofloxacin, a fluoroquinolone group of drug is most commonly used antibacterial agent against Gram positive and Gram negative bacterial infections. Due to over the counter availability of oral syrups and long acting formulation of enrofloxacin, its usage of in livestock practice has increased, and resistance had been developed by the microorganisms to this quinolone drug [1, 2]. Due to the potential development of resistance to antimicrobial agents by the microbes, phytochemicals have been thought as a complementary or alternative medicine to combat microbial infections. The resistance of bacteria to antibiotics basically have been attributed to alteration in their receptors, inactivation by enzymes, reduced permeability and enhanced efflux. Among these, the drug extrusion by the multidrug efflux proteins serves as an important mechanism of multi drug resistance (MDR). Inhibition of efflux proteins will result in increase in contact time of antibiotic as well as increases the intracellular concentration of antibiotic inside the bacteria, thereby making the antibiotic more effective.

Phytochemicals possess various pharmacological properties such as antioxidant, antibacterial, antiviral, anti-inflammatory and efflux pump inhibitory activity. Reports described that phytochemicals which possess inhibitory activity on efflux proteins co-administered with classical antimicrobial agents may enhance the antibacterial efficacy and reduces the development of resistance [3, 4]. Recent research reports suggested that there are no literature available on combination of phytochemicals that possess efflux inhibitory activity with enrofloxacin in chicken.

Hence, the present study was designed to determine the MIC of enrofloxacin against selected bacteria and to determine the interplay of efflux protein inhibitors like capsaicin, theobromine on antibacterial efficacy and pharmacokinetics of enrofloxacin after oral administration in chicken.

2. Materials and Methods

With an attempt to determine the effect of efflux protein inhibitors on the antibacterial activity and pharmacokinetics of enrofloxacin in broiler chicken the present study was conducted in the Department of Veterinary Pharmacology and Toxicology, N.T.R College of Veterinary Science, SVVU, Gannavaram after obtaining approval from Institutional Animal Ethics Committee of the N.T.R College of Veterinary Science, Gannavaram, vide Letter No. 3/IAEC/NTRCVSc/ 2017 dated 15.07.2017.

2.1 Experimental Animals

The broiler birds used in the experiment were brought from M/S Srinivasa Hatcheries, Vijayawada. They were provided with *Ad libitum* access to feed kept under standard management conditions.

2.2 Administration of drugs

Broiler birds were off feed 12 h prior to the experimentation. Water was given *ad libitum*. Birds were randomly assigned to three groups of six each, Enrofloxacin @ 10 mg/kg was administered orally as single bolus in Group I birds. Enrofloxacin single bolus dose @ 10 mg/kg along with capsaicin @15 mg/kg, P.O and theobromine 125 mg/kg, P.O was administerd in group II and III birds.

2.3 Collection of blood samples

Blood samples were collected into heparinized vials from tarsal vein prior to administration of enrofloxacin and at 0.166, 0.33, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 h post-enrofloxacin administration. Blood samples were centrifuged at 3000 *g* for 15 minutes and separated the plasma which was stored at -20 ºC until analyzed for enrofloxacin and ciprofloxacin by HPLC.

2.4 Assay procedure

Enrofloxacin concentrations in plasma were determined by high performance liquid chromatography (HPLC) [5].

2.5 Extraction procedure

The plasma sample and deproteinizing solution was added in the ratio of 1:1. After vortex mixing at high speed for 60 sec, the tube was subjected to centrifugation for 30 min at 10000 g. The clear supernatant thus obtained was transferred to a separate micro centrifuge tube and an equal volume of 1% acetic acid was added and mixed thoroughly. This mixture was then filtered through a 0.22 µm nylon membrane filter and 20 µl of filtrate was injected into the HPLC system.

2.6 High performance liquid chromatography (HPLC) assay

The HPLC system (M/s SHIMADZU, Japan) equipped with SPD-20A quaternary gradient pump, Rheodyne manual loop injector, with a 20 µl loop, LC-20AD UV-Vis detector and a column oven CTO-10ASVP was used. Detection of enrofloxacin was performed by using an Ascentis® C18 reverse phase column (particlesize5µm, 4.6 x 250 mm). Solution containing 10mM KH_2PO_4 (pH 2.37) with triethylamine and orthophospharic acid each at 0.3% (70), acetonitrile (20) and methanol (10) used as mobile phase with a flow rate of 0.8 ml/min. Enrofloxacin was detected at a wavelength of 277 nm by maintaining the column temperature at 40 °C.

Chromatograms obtained for blank plasma and spiked plasma standards revealed that there were no interfering peaks at the retention time of either enrofloxacin or ciprofloxacin. Standard curve prepared with areas of peak was used for enrofloxacin quantification in plasma samples.

2.7 Preparation of stock solutions for standards

Stock solution of enrofloxacin (1 mg/ml) was prepared in 0.1N NaOH. From this stock solution, working standards were prepared.

One hundred microliters of the stock solutions of enrofloxacin and ciprofloxacin was taken and added to eight hundred microliters of mobile phase, thus giving a solution of 100 µg/ml. From this, working standards (0.3125, 0.625, 1.25, 2.5, 5, 10, 20 and 40 µg/ml) were prepared daily by serial dilution with mobile phase. Twenty microliters of respective concentrations of enrofloxacin was spiked in different tubes containing 180 µl of pooled plasma to obtain working standards of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 µg/ml of enrofloxacin in plasma. These plasma samples spiked with standards were subjected to extraction as described above.

2.8 Standard calibration curve

The standard calibration curve was generated by using different known concentrations with a range of 0.03125 to 4 µg/ml of enrofloxacin. The standard curve was linear with a correlation coefficient of 0.999. The regression equation based on the standard curve was y=53424x + 3605.9. This equation is further used to determine the plasma concentrations of enrofloxacin in plasma collected from the birds of all groups in the present study. The specificity of determination of enrofloxacin was done using known enrofloxacin standards in HPLC.

2.9 Precision of assay

Analytical recovery values for enrofloxacin spiked in untreated bird plasma of two known concentration of 0.125 µg/ml and 2.0 µg/ml with the help of standard curve prepared from solvent standards were presented in table 1. As per these values the recovery percent for enrofloxacin was 99%.

Table 1: Recovery of enrofloxacin from chicken plasma

Concentration injected (µg/ml)	0.125	2.0
Concentration observed (range-µg/ml)	0.117-0.136	1.829-2.076
Mean concentration ±SE (µg/ml)	0.128±0.002	1.984±0.027
Coefficient of variation %	4.8	3.9
Recovery %	99	99

2.10 Effect of time of assay

The concentrations of enrofloxacin estimated eight times in a day and on 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 were shown in table 2. 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%.

Table 2: Intra and inter-day estimated values of enrofloxacin for precision of the HPLC assay

	Known concentration (µg/ml)	Concentration found Mean ± SEM (µg/ml)	Coefficient of variation %	Accuracy (%) on nominal concentration
Intra-day (N=8)	0.125	0.123±0.003	7.3	98.09
	2.0	1.974±0.032	4.5	98.68
Inter-day (N=8)	0.125	0.120±0.003	7.2	96.30
	2.0	1.977±0.0330	4.3	98.83

2.11 MIC by broth microdilution method

The broth dilution method was used to determine the antibacterial activity of enrofloxacin against *S. aureus* ATCC 25923, *E. coli* ATCC25922, *K. pneumoniae* ATCC700603 and *P. aureginosa* ATCC27853. Working standard of 1 µg/ml enrofloxacin was prepared by serial dilution the stock solution. 100 µl of cation adjusted Mueller Hilton Broth (CAMHB) was prepared and added in 96 well microtiter plate. To the first well 100 µl of test compound was added and two fold serial dilution was made. The 18h bacterial culture incubated in CAMHB at 37±1 °C was taken and adjusted to 0.5 McFarland turbidity standard (1 X 10^8 CFU/ml). 10 µl of the bacterial inoculums was added to all the wells and to achieve the final concentration of bacteria 5 X 10^5 CFU/ml. Microdilution plate were properly sealed and incubated at 35±2 °C for 16 to 20 h.

2.12 MIC End Point

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism. 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. Bacterial growth and inhibition was detected by adding 25 µl of INT to each well. INT is reduced to a red formazan compound by bacteria in growing phase. Bacterial growth was considered to be inhibited when the solution in the well is colourless, which was considered as MIC. Solvent controls and growth controls were included in each experiment as per CLSI guidelines [6].

2.13 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 Instate software. $p<0.05$ is considered as significant.

3. Results and Discussion

The antibacterial activity of enrofloxacin alone and enrofloxacin with efflux protein inhibitors *viz.* Capsaicin and theobromine with regards to MIC was calculated against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 700603 and *P. aureginosa* ATCC 27853 strains and were presented in table 5. Enrofloxacin's MIC value for *E. coli* ATCC 25922 was 0.02 µg.ml^{-1}, but it was decreased to 0.012 µg.ml-1 in the presence of capsaicin and theobromine, increasing *E. coli* specific antibacterial activity. Enrofloxacin's MIC for *S. aureus* ATCC 25923 was 0.2 µg.ml-1; this value was reduced to 0.090 µg.ml^{-1} (55%) and 0.110 µg.ml^{-1} (45%) when combined with capsaicin and theobromine, respectively. Capsaicin's potentiation of enrofloxacin against *S. aureus* is ascribed to its inhibitory effect on the organism's NorA efflux pump [7]. Enrofloxacin's MIC value for *K. pneumoniae* ATCC 700603 decreases from 1.650 µg.ml^{-1} by 83.8% in the presence of capsaicin and by 75% in the presence of theobromine. Enrofloxacin's MIC value for *P. aureginosa* ATCC 27853 was 2.433 µg.ml^{-1}, which was decreased to 0.450 µg.ml^{-1}.

Capsaicin, major constituent of hot chilli, possesses NorA efflux pump inhibitory activity. Research reports revealed that NorA efflux pump, NorB and NorC contribute to the quinolone resistance especially in *S.aureus* [8]. Research reports suggest that Capsaicin disrupts the membrane channels in the lipid bilayer through increased rate of absorption [9]. Pre-treatment with the Capsaicin at 3 mg. Kg^{-1} for 7 days improved the bioavailability of cyclosporine in rats by reducing the activity of P-gp and CYP3A1/2[10]. Capsaicin inhibits P-gp which may give lead to P-gp mediated drug interaction of enrofloxacin. Plasma levels of enrofloxacin are inverlsy proportional to the expression of P-gp [11].

Co-administration of capsaicin has produced an increasing C_{max} and AUC of enrofloxacin as documented in the capsaicin enrofloxacin combination study was reported [12]. They reported an increase in C_{max} of enrofloxacin by administration of capsaicin at 20 µg. Kg^{-1} p.o. Conversely capsaicin co-administration does not produce any significant changes in the pharmacokinetics parameters of enrofloxacin. It shows slight reduction in the Cmax values from 2.17±0.30 to 2.01±0.28 µg.ml^{-1} (table 4). The t_{max} values obtained after oral co administration of capsaicin with enrofloxacin was slightly delayed from 4.25±0.68 h to 5.00±0.86 h, which implies slow rate of drug absorption. Capsaicin produces pain which may retard gastric emptying and reduced the drug absorption [13].

Co-administration of theobromine had significantly reduced the elimination rate constant ($p<0.05$) (table 4). The AUC, which determines the amount of drug absorbed, was enhanced to 48.93±1.29 µg.h.mL^{-1} from 36.28±4.80 µg.h.mL^{-1} suggesting increased bioavailability of enrofloxacin in the presence of theobromine. Theobromine has also increased half-life of enrofloxacin, which indirectly enhances the duration of action of the enrofloxacin. The MRT was significantly increased from 16.60±0.42 h to 19.05±0.42 h at $p<0.01$ suggesting that theobromine is likely to enhance the enrofloxacin residence time in the body when theobromine is combined together with enrofloxacin. Clearance (Cl_B/F) was significantly slowed down to 0.19±0.01 L.kg^{-1}.h^{-1} from 0.28±0.03 L.kg^{-1}.h^{-1}. The results together indicate that theobromine co-administered with enrofloxacin increases bioavailability of enrofloxacin and maintains the drug in the plasma for a longer duration of time. This increased bioavailability may be due to down regulation of efflux pump ABCG2 expression by theobromine [14] as enrofloxacin and danofloxacin are transported by ABC transporters [15].

The ratio AUC to MIC is generally used for optimisation of the dose and clinical effectiveness of the drug. The PK-PD integration data AUC/MIC > 400 against *E. coli* revealed that the combination with capsaicin and theobromine reduced the development of resistance.

Table 3: Effect of oral coadministration of capsaicin (15 mg.kg^{-1}) and theobromine (125 mg.kg^{-1}) with enrofloxacin on plasma concentrations (µg/ml) of enrofloxacin (10 mg.kg^{-1}) in chickens

Time (H)	Group I	Group II	Group III
0.166	0.23±0.11	0.36±0.11	0.13±0.00
0.333	0.53±0.17	0.61±0.15	0.38±0.02
0.5	0.81±0.24	0.90±0.17	0.94±0.03
0.75	0.99±0.24	1.16±0.27	1.32±0.04
1	1.24±0.30	1.37±0.25	1.48±0.07
1.5	1.64±0.34	1.21±0.45	1.85±0.16
2	1.76±0.35	1.60±0.24	2.38±0.18
4	2.02±0.32	1.76±0.28	2.43±0.14
6	1.85±0.20	1.69±0.20	2.07±0.11
8	1.48±0.13	1.50±0.18	1.76±0.08
12	1.06±0.13	1.17±0.10	1.41±0.04
24	0.58±0.09	0.75±0.08	1.04±0.01
36	0.34±0.06	0.25±0.04	0.39±0.02
48	0.11±0.01	0.09±0.01	0.20±0.01

The values were expressed as Mean ± SEM of six chickens.

Table 4: Effect of oral co-administration of capsaicin (15 mg.kg^{-1}) and theobromine (125 mg.kg^{-1}) with enrofloxacin (10 mg.kg^{-1}) on pharmacokinetic parameters of enrofloxacin in chickens

Parameter	Unit	Group I	GroupII	GroupIII
β	h^{-1}	0.06±0.00	0.07±0.00*	0.06±0.00
$t_{1/2\beta}$	h	11.05±0.27	10.00±0.42*	12.65±0.33*
t_{max}	h	4.25±0.68	5.00±0.86	3.33±0.42
C_{max}	µg.mL^{-1}	2.17±0.30	2.01±0.28	2.62±0.14*
AUC_{0-t}	µg.h.mL^{-1}	36.28±4.80	37.18±3.60	48.93±1.29*
$AUMC_{0-t}$	µg.h^{2}.mL^{-1}	633.04±85.53	621.61±70.89	1003.45±33.77**
MRT	h	16.60±0.42	16.00±0.68	19.05±0.42**
V_d/F	L.kg^{-1}	4.47±0.43	3.89±0.31	3.48±0.12
Cl_B/F	L.kg^{-1}.h^{-1}	0.28±0.03	0.28±0.03	0.19±0.01*

The values were expressed as Mean ± SEM of six chickens, $^{*}p<0.05$.

Table 5: Minimum Inhibitory Concentration (MIC, µg/ml)) of enrofloxacin alone and in combination with efflux protein inhibitors against the selected bacteria

Name of the test compound	*S. aureus* ATCC25923	*E. coli* ATCC25922	*K. pneumoniae* ATCC700603	*P. aureginosa* ATCC27853
Enrofloxacin	0.202	0.020	1.650	2.433
Capsaicin + Enrofloxacin	0.090	0.012	0.266	0.404
Theobromine+ Enrofloxacin	0.110	0.012	0.258	0.450

Table 6: Effect of Capsaicin and theobromine on PK/PD indices of enrofloxacin in Chickens on *E. coli* ATCC29922

	C_{max}/MIC	AUC/MIC
Group II (Enrofloxacin)	86.92	1813.93
Group III (Enr + Capsaicin)	80.2	1859.15
Group IV (Enr + Theobro)	104.72	2446.45

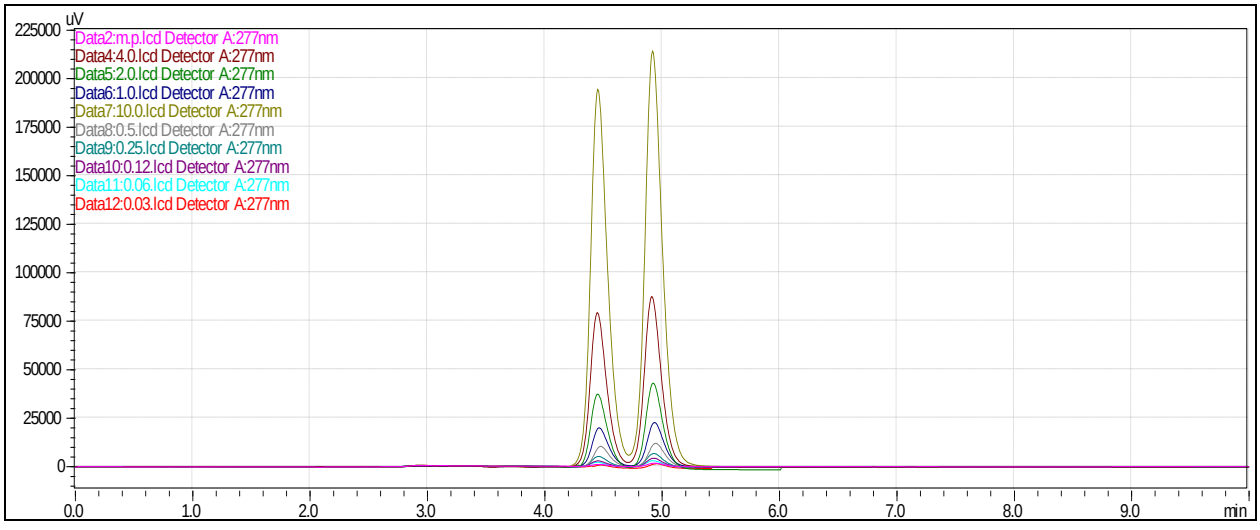

Fig 1: Overlay chromatograph of enrofloxacin and ciprofloxacin (metabolite of enrofloxacin) spiked in chicken plasma

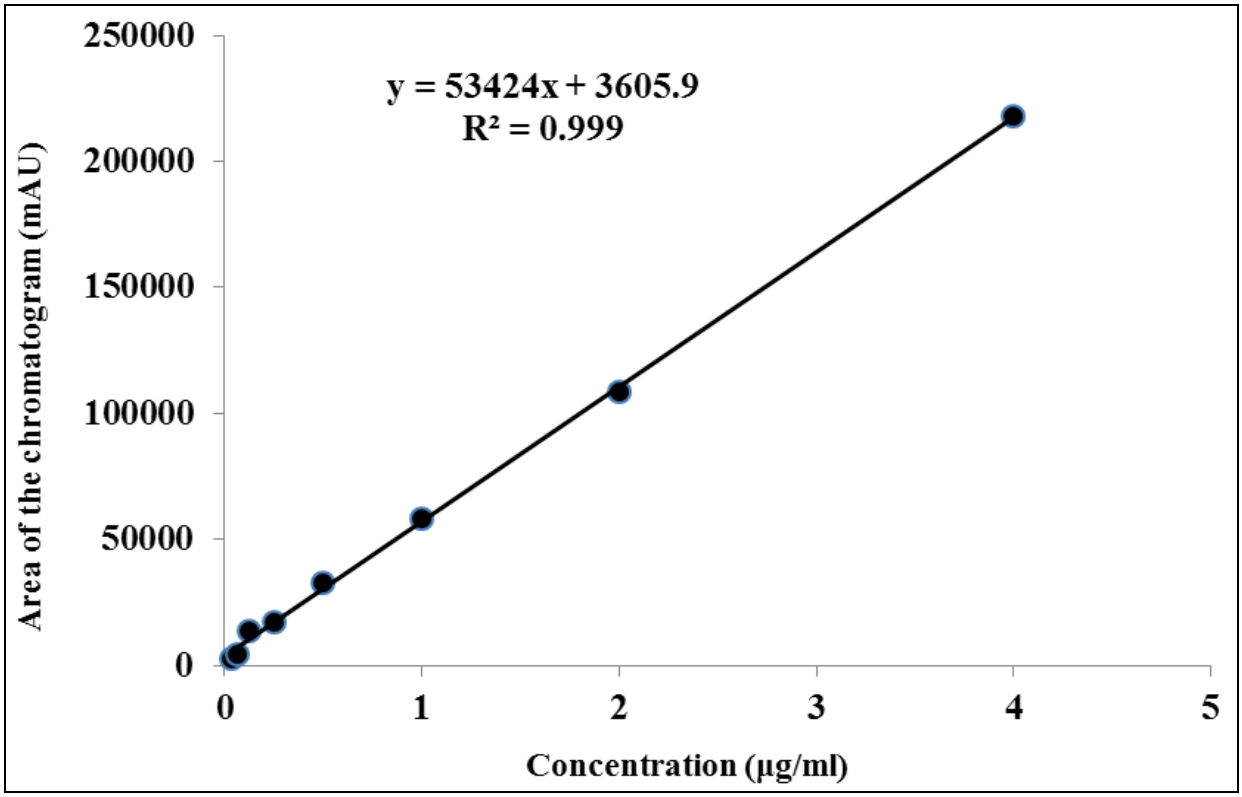

Fig 2: Standard calibration curve for enrofloxacin in chicken plasma

4. Conclusion

It is concluded from the results obtained from the above study that theobromine increases the oral bioavailability of enrofloxacin in chicken. It is also evident that capsaicin and theobromine increased the antibacterial activity of enrofloxacin.

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