AN *INVITRO* STUDY ON THE EFFECT OF POTENTIATING AGENTS ON SELECTED FLUOROQUINOLONE ANTIBIOTICS

Sumi Sebastian, V.M. Subrahmanyam*, J. Venkata Rao, N. Sivagurunathan and P.Vasanth Raj

Manipal college of Pharmaceutical Sciences, Manipal University, Manipal -576 104

Summary

Development of resistance to antimicrobial agents is a major problem in chemotherapy. Finding agents which potentiate antimicrobial activity could circumvent this problem. Present study was envisaged to study the *in vitro* action of potentiating agents viz., EDTA, caffeine, citric acid, theophylline, tartaric acid, tri-sodium citrate in combination with selected fluoroquinolone antibiotics – ciprofloxacin, sparfloxacin, levofloxacin against both Gram-positive(*B. subtilis, S. aureus*) and Gram-negative bacteria (*E. coli, P. aeruginosa*). Effect of potentiating agents on antibiotics was studied by determination of MIC, zone diameter and turbimetric analysis. All potentiating agents were used in concentrations at which they do not have any antimicrobial activity. EDTA showed antibacterial activity at lower concentrations. In combination with antibiotics- EDTA, caffeine, citric acid exhibited considerable potentiation of the activity. Tri-sodium citrate exhibited least potentiation effect.

Key words: Chemotherapy, fluoroquinolone antibiotics, Potentiating agent, Minimum inhibitory concentration (MIC).

Address of communication, *corresponding author
Dr.V.M.Subrahmanyam, Associate Professor,
Department of Pharmaceutical Biotechnology
Manipal College of Pharmaceutical Biotechnology
Manipal College of Pharmaceutical Sciences
Manipal University, Manipal-576104, Karnataka, India.
Email: vmsmanyam@yahoo.com, vmsubrahman@gmail.com
Telephone: 0091 820 2922482. Fax: 0091 820 2571998

Introduction

Currently the antibiotic era is threatened by emergence of three adverse circumstances *viz*, high levels of antibiotic resistance of important pathogens, an uneven supply of novel classes of antibiotics and a dramatic reduction in discovery and development of anti infective agents**.** Bacterial resistance to an antimicrobial agent could be due to the drug not reaching its target [1], inactive drug [2] or alteration in the target [3].

Development of antibiotic resistance due to indiscriminate use in last 60 years necessitated the development of new antibiotics or combinations which improve antibiotic activity. Concerted and systematic programmes to discover and to develop new antimicrobials have enabled to a considerable extent, the battle against the resistance factor. However cost involved in such programs is huge and can be a limitation. Alternatively, it can be envisaged to augment the activity of the already existing ones by using potentiating agents [4]. The present work is a step in this direction.

Most of the potentiating agents act as permeabilizing agents, which are the chemicals that increase the permeability of antimicrobials into bacteria [5]. Impermeability to antimicrobials is a major mechanism of intrinsic bacterial resistance and consequently, chemical agents that enhance intracellular accumulation could have a useful role to play in the clinical environment.

In the present study, action of selected fluoroquinolone antibiotics along with different potentiating agents was assessed.

Material and Methods

Antimicrobial and potentiating agents

Standard laboratory powders of ciprofloxacin, sparfloxacin (Ranbaxy laboratories, Delhi), Levofloxacin (Wockhardt, Mumbai) were used in the study. The potentiating agents used were caffeine (Loba Chemie, Mumbai), citric acid (Sigma Aldrich, Germany), theophylline (Genuine Chemical co., Mumbai), disodium EDTA (S.D.Fine Chem Ltd., Mumbai), trisodium citrate (Nice Chemicals pvt.,Ltd.,) and tartaric acid (E.Merck Ltd., Mumbai).

Microorganisms and Media

The microorganisms employed were *Staphylococcus aureus*(NCIM 2079), *Pseudomonas aeruginosa*(NCIM 2036**)**, *Bacillus subtilis*(NCIM 2063) and *Escherichia coli*(NCIM 2345). The identities of the microorganisms were confirmed by standard biochemical tests and are maintained as per standard guidelines.
Antibiotic assay medium No.1, Mueller Hinton broth (MH broth), Nutrient broth, Nutrient agar, Mueller Hinton (MH) agar, EMB agar, Mannitol salt agar (Hi-media lab Pvt Ltd, Mumbai) were used in the study.

Determination of Minimum Inhibitory Concentration for Antimicrobial and potentiating agents

Minimum Inhibitory Concentration (MIC) of both antimicrobial agents and potentiating agents alone were determined by tube dilution method. The inoculum contained 5×10^5 CFU/ml. The concentration ranges were: 0.015- 4 μg/ml, ciprofloxacin; 0.03125-8 μg/ml, sparfloxacin; and 0.078- 10 μg/ml, levofloxacin; 156.25-10000 μg/ml, theophylline; 3.12- 200 μg/ml, EDTA; 78.125- 5000 μg/ml, caffeine; 156.25- 10000 μg/ml, citric acid; 156.25- 10000 μg/ml, tartaric acid; 156.25- 10000 μg/ml, trisodium citrate. Antimicrobial solutions were prepared and diluted freshly on the day of testing. Each test was performed in triplicate. Concentration of potentiating agents has to be selected based on their MIC values against the particular organism. The selected concentration should be lesser than the obtained MIC values to avoid the chances of these agents themselves showing the anti-bacterial activity.

Agar diffusion method

In the agar diffusion method, the bacterial organism was uniformly swabbed onto a nutrient agar plate and the compounds to be checked are placed in wells (6 mm dia). Initially, potentiating agents in three different concentrations (200, 100 and 50μg/ml, EDTA; 1000, 500 and 250μg/ml, citric acid; 1000, 500 and 250μg/ml, tartaric acid; 2000, 1000 and 500μg/ml, trisodium citrate; 2000, 1000 and 500μg/ml, caffeine; 2000, 1000 and 500μg/ml, theophylline) were

checked by agar diffusion method. Concentrations at which no zone of inhibition was observed were used for further study.
The study was carried out by two designs:
In the first design, antibiotic mixed with potentiating agent was added to wells bored into seeded agar medium, while in the second design, potentiating agents were mixed with the medium and antibiotics alone were put into the wells. The concentration of antimicrobial agents used was 4µg/ml. The plates were kept at 4^0 for 1 h for diffusion followed by incubation at 37° for 12 h. Zone diameters were measured by using an antibiotic zone reader.

Turbidimetric analysis

Turbidity of samples was measured employing both the potentiating agent and antibiotic alone and in combination. Potentiating agents in half concentrations of their MIC values were added to 50 ml nutrient broth medium contained in 250 ml Erlenmeyer flasks. This concentration of potentiating agent was chosen as it would give turbidity almost close to the turbidity on medium without any potentiating agent. Antibiotics in concentrations just below their MIC values were then added. The concentrations of potentiating agents and antibiotics used for turbidimetric analysis are given in Table 4a. Flasks having antibiotics without potentiating agents were also made. All these flasks were inoculated and kept for incubation at 37° for 12 h. Turbidity was measured using a nephelometer.

Results and Discussion

This study was important for selecting promising potentiating agents. The first step envisaged was to determine the MIC values for chosen antimicrobial agents and potentiating agents, both alone and in combination. MIC was determined for potentiating agents so that in combination with antibiotics, their concentrations are fixed at lower than their MIC values. This is to ensure that the potentiating agent in the combination has no antibacterial activity. EDTA showed antibacterial activity at lower concentrations and in combination with antibiotics it reduced MIC considerably (Table1a and 1b). Caffeine showed the highest potentiating effect on ciprofloxacin against *P. aeruginosa* with eight times reduction in MIC. Most significant effects were observed against *B. subtilis* where EDTA and caffeine in combinations with ciprofloxacin

showed eight-fold decrease in MIC. Tartaric acid and theophylline did not show any potentiation.
Ayres H M *et al*., [5] showed that EDTA has significant potentiating effect on novobiocin and fusidin against *P.aeruginosa*. In the present case it was found that potentiation of activity with caffeine was more than that of EDTA on ciprofloxacin for the organism.
In case of levofloxacin, Caffeine showed maximum potentiation (four-fold reduction) against *E. coli*. Against *S. aureus* except tri-sodium citrate and EDTA all combinations showed increase in activity of levofloxacin. The activity was maximum with caffeine (four fold) .Against *P. aeruginosa* EDTA, caffeine, citric acid, and theophylline showed a two-fold decrease in MIC of levofloxacin. Tri-sodium citrate did not show any potentiation in activity of levofloxacin for *B. subtilis*. Caffeine showed maximum potentiation. Tartaric acid, citric acid and theophylline reduced MIC to half.

With Sparfloxacin, citric acid showed potentiation of four times against *E. coli*. EDTA, caffeine, and tri-sodium citrate showed a decrease in MIC value to half. Tartaric acid and theophylline did not show any potentiation in this combination. Sparfloxacin and EDTA combination was most effective against *S. aureus* with an 8-fold decrease in MIC. Caffeine, citric acid, and theophylline showed a four-fold decrease in MIC. Tartaric acid and tri-sodium citrate reduced the MIC to half. Here all combinations tested showed potentiation. Only EDTA and tartaric acid showed potentiation of sparfloxacin activity against *P. aeruginosa viz*., four fold and two fold decrease in MIC respectively. Sparfloxacin showed a high MIC value against *P. aeruginosa* compared to other organisms**.** Against *B. subtilis* EDTA, tartaric acid, citric acid, and theophylline showed potentiation of sparfloxacin activity with a decrease in MIC to half. Caffeine and tri-sodium citrate did not show any potentiation.
E. coli was found to be the most susceptible organism to the combined action of antibiotic and potentiating agent. EDTA was found to be the most effective potentiating agent against *P. aeruginosa*. This is in concurrence with the study on EDTA having a direct bactericidal action against *P. aeruginosa* by chelating metal ions important for the integrity of bacterial cell wall [6]. It stimulates the release of cell wall polysaccharides, proteins and other cell contents. It has been found that other potentiating agents tested showed very less increase in activity against the organism. Citric acid was established as an agent that can increase biocidal activity in

Gram-negative bacteria. [7, 5]. Caffeine showed increased potentiation than theophylline and it was effective in many combinations against both Gram-positive and Gram-negative. Also caffeine and theophylline inhibit *Staphylococcus* penicillanase enzyme [8].

There was no significant change in zone diameters in the agar diffusion method when the antibiotic and potentiating agent mixture was kept into the wells (table 2). A maximum increase of 3 mm was observed in some cases. However when potentiating agents were added to the medium before the antibiotics were added the zone diameter was more (table 3). It showed that when cells were pretreated with a potential permeabilizer the effects were more. Here the permeabilizer might sequester ions from the medium which could become nutritionally deficient or weaken cells [5].

In the next stage, potentiation of antibiotics was studied by turbidimetric analysis.

Potentiating agents and antibiotics were used in concentrations below their MIC values. Except tri-sodium citrate all other potentiating agents showed a reduction in turbidity (tables 4a and 4b). EDTA showed maximum reduction in turbidity of *E. coli, S. aureus, P. aeruginosa* and caffeine showed maximum reduction in *B. subtilis* growth.

EDTA caused a marked reduction in *P. aeruginosa* when combined with sparfloxacin, while *E. coli* showed maximum reduction with citric acid. Tartaric acid and tri-sodium citrate did not show much reduction in turbidity when combined with sparfloxacin in all four organisms.

Among all the combinations levofloxacin and caffeine showed maximum reduction in turbidity of *E. coli.* Trisodium citrate was not effective.

Most of the potentiating agents used in conjunction with fluoroquinolone antibiotics reduced the minimum inhibitory concentration, increased the zone diameter and reduced growth. The study can be extended using clinical strains. This approach of using a combination of antibiotic and potentiating agent can be cost effective and an effective way to circumvent growing antimicrobial resistance.

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Table 1a: MIC values for antimicrobial and potentiating agents (both alone and combined)

Antimicrobial/potentiating agents (alone or combination) Range of MIC values (μg/ml) against test organisms				
	E.coli	*S.aureus*	*P. aeruginosa*	*B.subtilis*
Caffeine(CA)	≤ 2500 >1250	≤ 1250 > 625	≤ 5000 > 2500	≤ 5000 > 2500
Citric acid(CI)	≤1250 > 625	≤1250 > 625	≤1250 > 625	≤ 625 > 312.5
EDTA(E)	≤ 50 > 25	≤ 50 > 25	≤ 50 > 25	≤ 50 > 25
Tartaric acid(TA)	≤625 > 312.5	≤ 1250 > 625	≤ 1250 > 625	≤625 > 312.5
Theophylline(TH)	≤2500 >1250	≤625 >312.5	≤2500 >1250	≤1250 >625
Trisodium citrate(TRC)	≤ 10,000 >5000	≤5 000 >2500	≤ 10,000 >5000	≤5 000> 2500
Ciprofloxacin(C)	≤0.125>0.0625	≤2 >1	≤4 >2	≤0.5 >0.25
(C)+(CA)	≤0.0625>0.031	≤1 >0.5	≤0.5 >0.25	≤0.0625>0.031
(C) + (CI)	≤0.0625>0.031	≤2 >1	≤2>1	≤0.25 >0.125
(C) + (E)	≤0.031>0.015	≤0.5 >0.25	≤1 >0.5	≤0.0625>0.031
(C) + (TA)	≤0.0625>0.031	≤1 >0.5	≤4 >2	≤0.5 >0.25
(C) + (TH)	≤0.0625>0.031	≤2>1	≤4>2	≤0.25 >0.125
(C) + (TRC)	≤0.125>0.0625	≤2 >1	≤4 >2	≤0.5 >0.25

Table 1b: MIC values for antimicrobial and potentiating agents (both alone and combined)

Antimicrobial/potentiating agents (alone or combination) Range of MIC values (μg/ml) against test organisms				
	E.coli	*S.aureus*	*P. aeruginosa*	*B.subtilis*
Levofloxacin(L)	≤5 >2.5	≤5 >2.5	≤2.5 >1.25	≤10 >5
(L)+(CA)	≤1.25 >0.625	≤1.25 >0.625	≤1.25 >0.625	≤1.25 >0.625
(L) + (CI)	≤2.5 >1.25	≤2.5 >1.25	≤1.25 >0.625	≤5 >2.5
(L) + (E)	≤2.5 >1.25	≤5 >2.5	≤1.25 >0.625	≤2.5 >1.25
(L) + (TA)	≤2.5 >1.25	≤2.5 >1.25	≤2.5 >1.25	≤5 >2.5
(L) + (TH)	≤2.5 >1.25	≤2.5 >1.25	≤1.25 >0.625	≤5 >2.5
(L) + (TRC)	≤5 >2.5	≤5 >2.5	≤2.5 >1.25	≤10 >5
Sparfloxacin(S)	≤0.5 >0.25	≤1 >0.5	≤8 >4	≤0.5 >0.25
(S) + (CA)	≤0.25 >0.125	≤0.25 >0.125	≤8 >4	≤0.5 >0.25
(S) + (CI)	≤0.125 >0.0625	≤0.25 >0.125	≤8 >4	≤0.25 >0.125
(S) + (E)	≤0.25 >0.125	≤0.125>0.0625	≤2>1	≤0.25 >0.125
(S) + (TA)	≤0.5 >0.25	≤0.5 >0.25	≤4 >2	≤0.25 >0.125
(S) + (TH)	≤0.5 >0.25	≤0.25 >0.125	≤8 >4	≤0.25 >0.125
(S) + (TRC)	≤0.25 >0.125	≤0.5 >0.25	≤8 >4	≤0.5 >0.25

Table 2: Zone diameters after addition of potentiating agents to the medium

Antibiotic	**Zone diameters(mm)**						
	Antibiotic (A)	**A+EDTA**	**A+Caffeine**	**A+Tartaric acid**	**A+Citric acid**	**A+Tri-sodium citrate**	**A+ Theophylline**
				Bacillus subtilis			
Ciprofloxacin	30	36	35	38	34	35	39
Levofloxacin	30	39	36	37	35	32	41
Sparfloxacin	31	39	35	39	36	35	39
				Escherichia coli			
Ciprofloxacin	29	29	35	34	33	36	33
Levofloxacin	25	30	34	34	31	36	31
Sparfloxacin	30	31	31	38	32	36	32
				Pseudomonas aeroginosa			
Ciprofloxacin	26	30	32	28	27	26	29
Levofloxacin	26	29	30	28	28	31	27
Sparfloxacin	26	30	27	28	31	31	29
				Staphylococcus aureus			
Ciprofloxacin	26	26	32	37	26	24	28
Levofloxacin	25	25	32	37	25	25	29
Sparfloxacin	27	27	32	36	26	29	29

Table 3: Zone diameters when antibiotics were mixed with potentiating agents

Organism	Zone diameters(mm)						
	Antibiotic (A)	**A+EDTA**	**A+Citric acid**	**A+Tartaric acid**	**A+Trisodium citrate**	**A+Caffeine**	**A+Theophylline**
				Ciprofloxacin			
E. coli	32	33	34	33	32	32	34
S. aureus	36	36	36	37	36	36	38
P. aeruginosa	27	29	28	29	29	27	28
B. subtilis	32	34	32	32	33	33	32
				Sparfloxacin			
E.coli	30	31	32	33	30	30	30
S. aureus	27	27	28	28	27	27	29
P. aeruginosa	27	29	30	29	29	28	28
B. subtilis	31	32	33	33	32	31	31
				Levofloxacin			
E.coli	25	27	26	27	25	26	26
S. aureus	24	26	23	24	25	24	24
P. aeruginosa	26	28	27	26	26	27	27
B. subtilis	30	30	31	32	32	30	30

Table 4a: Concentration of Potentiating agents and antibiotics used for turbidimetric analysis

Organism	**Concentration of potentiating agents and antibiotics (µg/ml)**								
	EDTA	**Caffeine**	**Citric acid**	**Tartaric acid**	**Trisodium citrate**	**Theo-phylline**	**Cipro-floxacin**	**Spar-floxacin**	**Levo-floxacin**
E.coli	12.5	625	312.5	156.25	2500	625	0.0625	0.25	2.5
S. aureus	12.5	312.5	312.5	312.5	1250	156.25	1.0	0.5	2.5
P. aeruginosa	12.5	625	312.5	312.5	2500	625	2.0	4.0	1.25
B. subtilis	12.5	1250	156.25	156.25	1250	312.5	0.25	0.25	5.0

Table 4b: Turbidity measurements of antibiotics and potentiating agents

Organism	Turbidity (NTU) nephelo turbidimetric unit														
	EDTA	Caffeine	Citric acid	Tartaric acid	Theophylline	Trisodium citrate	Antibiotic (A)	A+ EDTA	A+ Caffeine	A+ Citric acid	A+ Tartaric acid	A+ Theo-phylline	A+ Trisodium citrate	Control +	Control -
Ciprofloxacin															
E. coli	20.4	23.1	30.1	26.1	23.6	24.6	13.1	1.1	3.4	1.9	3.2	4.1	10.3	33.2	0.6
S. aureus	20.8	22.4	26.4	27.3	22.6	23.6	12.6	0.9	1.6	9.1	9.1	6.0	13.3	36.4	0.1
P.aeruginosa	21.4	22.8	30.8	24.4	22.4	20.8	14.2	1.2	1.3	6.8	12.8	13.0	13.2	34.2	0.4
B. subtilis	20.1	23.4	29.6	26.4	21.8	22.7	12.9	0.9	0.1	3.9	11.9	11.6	13.1	33.8	0.8
Sparfloxacin															
E. coli	20.8	24.2	29.8	28.2	23.8	24.6	16.1	2.7	0.8	0.1	15.2	16.0	10.9	38.2	0.3
S. aureus	21.4	21.9	27.1	25.1	20.9	23.8	14.8	0.6	1.3	4.1	10.6	8.2	14.4	34.6	0.6
P.aeruginosa	20.8	22.4	29.2	26.1	22.6	20.5	15.2	0.2	10.6	9.1	10.1	13.6	15.6	35.8	0.1
B. subtilis	19.8	24.2	30.1	27.1	22.2	22.4	14.5	1.2	11.2	4.2	6.8	2.2	14.2	40.1	0.5
Levofloxacin															
E. coli	19.1	23.9	30.1	28.1	23.0	22.6	13.1	9.2	2.4	10.6	8.0	10.5	12.9	35.6	0.6
S. aureus	20.9	22.1	26.3	25.2	21.2	22.1	12.8	10.2	6.6	9.8	10.2	10.9	13.2	34.4	0.4
P.aeruginosa	20.7	22.3	32.2	26.9	23.4	24.4	16.5	15.2	9.2	9.8	9.6	9.2	15.9	36.2	1.1
B. subtilis	20.6	22.2	29.6	25.3	21.8	21.9	14.5	7.6	6.9	8.2	10.2	8.9	14.2	38.9	1.3

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