CHAPTER - III

BIOLOGICAL ACTIVITY OF s-TRIAZINE CEPHALOSPORINS

INTRODUCTION:-

Once the drug is synthesized it should be tested for its medicinal value. It can be tested in two ways as,
i) in vivo and ii) In vitro.

The disease were produced by many microorganisms in the body of man like bacteria, viruses etc.

The bacteria contributes major component of micro-organisms. Depending upon their staining properties they are classified as a) Gram positive and b) Gram negative "Antagonism" of one microorganism by another has been noticed from early times, cornil and Babes wrote "If the study of mutual antagonism of bacteria were sufficiently far advanced a disease caused by one b acterium could probably be treated by another." E. Duchesne, found that a penicillium reduced the virulence of E. coli and Salmonella when injected into an animal with them, and concluded. "In pursuing the study and facts of biological anatagonism between molds and bacteria one may arrive perhaps at ... other facts ... applicable to prophylactic hygiene and to therapy". Several instances of microbial antagonism were studied before the recent great development of antibiotics began. One arose from the discovery that the damping-off fungus, Rhizoctonia solani, which kills many seedlings, is itself killed by a common soil

organisms, Gliocladium fimbriatum. This led to the isolation of Glitoxin,[2] the first natural antibiotic to be prepared pure.

Penicillin is remarkable nontoxic to man and other mamals, As an antibiotic it is especially effective against cocci, both gram-positive and gram-negative, including pneumo cocci, streptococci and gonococci.

Since then several antibiotics were discovered and they were tested for antibacterial activity using cultures of certain bacteria and inhibition to their growth.

ANTIBACTERIAL ACTIVITY:-

Assessment of antibacterial activity of cephalosporins

There are many methods of assessing the antibacterial activity of antibiotics. The aim of in vitro 'Susceptibility testing is to predict as accurately as possible the 'in vivo activity of an agent. Cephalosporins provide peculiar difficulties in assessment because of their complex mode of action and because of the various mechanisms of resistance to them. 'In vitro', if there is a readily penetrable barrier, the antibiotic may diffuse so rapidly into the cell that the latter is killed before any Beta-lactamase present has had the opportunity to act.

But 'in vivo' the antibiotic may be delivered to the cells more slowly than in a laboratory experiment, and the Beta-lactamase may be able to in-activate the antibiotic for

example, ampicillin may inhibit penicillinase producing H. influenzae in vitro at low concentrations when small inocula are tested because there is little barrier to penetration. However, when large inocula are tested, as would be present in vivo for example in meninges, the organisms are not responsive to ampicillin.

The effect of the Beta-lactamase contribution to the outcome of cephalosporin organism interaction is difficult to assess and varies considerably with the test conditions. In table 3·1 where small inocula were tested, it appears that the presence of a Beta-lactamsase, which is capable of hydrolysing many cephalosporins, does not produce a major change in MIC or in zone size in disc diffusion method.

In order to compare the activity of the cephalospoorins against a variety of organisms, the MIC values expressed in Table 3·2 and 3·3 gives parenterally and orally - adsministered cephalosporins against Gram-positive cocci. In general the newer cephalosporins tend to be slightly less active than cephalothin and cephaloridine. There is little difference between the MIC values for Beta lactamase producing and non-producing strains of staph, aureus. Gram positive Beta lactamases have relatively little activity against cephalosoporins compared with their activity against penicillins.

Table 3·4 shows the activity of nine cephalosporins against H. influenzae. It can be seen that Beta lactamase has a role to play in the resistance of H. influenzae to

cephalosporins. The MICs of enzyme sensitive cephalosporins for Beta lactamase producing strains are approximately 4 times greater than those for Beta lactamase non producers. The enzyme resistant cephalosporins cefamandole, cefuroxime, cefoxitin and cefotaxime show equally good activity in vitro against Beta lactamase producing and non producing strains of H. infl luenzae.[14-18]

Tables 3·5 and 3·6 show the activity of parenterally and orally administered cephalosporins against aeroric Gram negative bacilli. Here the effects of the enzyme stability of the newer cephalosporins can b e seen most clearly. Until recenply Ps. aeruginosa has remained unaffected by the cephalosporin group of antibiotics but recent modifications of the cephalosporin molecule have resulted in new agents which have antipseuudoomonal activity.

Cefotaxime when tested[19] against 245 clinical isolates of Ps. aeruginosa gave MIC values ranging between those of azlocillin and carbenicillin. Against 31 strains of gentamicin resistant Ps. aeruginosa the MICs ranged from 4 µg/ml-64 µg/ml. with a mode MIC value of 16 µg/ml. Cefsulodin is a new cephalosporin with activity directed primarily against Ps. aeruginosa and Gram-positive cocci. It is less active than other cephalosporins against Gram negative becilli other than Ps. aeruginosa. This high antipseudomonal activity is thought to be related partly to a high affinity of the agent for the penicillin binding proteins in the pseudomonas cell membrane and partly to the resistance of the drug to pseudomonal Beta-lactamases.[45] cefsulodin tested against 180

clinical isolates of Ps. aeruginosa inhibited 90% of strains at a concentration of 16 µg/ml or less, whereas only 10% of the strains were inhibited by the same concentration of carbenicillin, 16 µg/ml is a concentration that can be achieved in the serum by normal dosage of this antitiotic[45].

Table 3.1 Relationship of hydrolysis by Beta lactamase, possession of a permeability barrier and MIC to out come of treatment.

Antibiotic	Strain	B.lactamase hydrolysis	Barrier	MIC ug/ml	Zone-size
Ampicillin	E.coli	-	+	1-2	Large
	E.coli	+	+	128	None
	H.influenzae	-	-	0.25	Large
	H.influenzae	+	-	2	Slight reduction
Cephaloridine	E.coli	-	-	1-2	Large
	E.coli	+	-	4-8	Slight reduction.
	H.influenzae	-	+	4-8	small.
	H.influenzae	+	+	8-16	Small.
	B.fragilis	-	-	0.5	Large.
	B.fragilis	-	+	16	Moderate
	B.fragilis	+	+	128	None.

Ref. :- Williams, J.D. (1978). The correlation of in vitro susceptibility tests with vivo results of antibiotic treatment. scand J. infect. Dis. (Suppl), 13, 64.

Table 3.2 - Activity of orally administered cephalosporins against Gram - positive cocci.

Organism	MIC (µg/ml) for 50% of Strain		
	Cephalexin	Cepharakine	Cefaclor
Staph aureus	1.5	0.1	1.5
Str.Pyogenes	0.5	0.5	0.5
Str.Pneumoniae	0.1	0.5	0.5
Str.faecalis	100	50	40 50

Ref:- Shadomy, S. and Wagner, G.(1977) Antimicrob. Agents. Chemother, 12, 609.

Table 3.3 - Activity of some parenterally administered caphalosporins against Gram-positive cocci.

Organism	MIC (µg/ml) for 50% of strain tested			
	Cephalothin	Cephaloridine	Cefazolin	Cefamandole
Staph aureus				
*Blac -	0.1	0.19	0.3	0.1
** Blac +	0.3	1.6	-	0.4
Staph.epidermidis	0.1	-	0.2	0.1
Strept.pyogenes	0.19	0.19	0.19	0.03
Strept.pneumoniae	0.32	0.2	0.4	0.4
Strept.faecalis	50	8	12.5	25

* Non Beta-lactamase - producing strains

** Beta-lactamase - producing strains.

Table - 3.4 - Activity of cephalosporins against Ha emophilus influenzae

Cephalosporin	MIC (µg/ml) for 50% of strains	
	Beta-lactamase producers	Non b-lactamase producers
Cephalothin	6.0	0.4
Cephaloridin	16.0	4.0
Cafazolin	12.0	-
Cephalexin	25.0	4.0
Cepharadine	48.0	10.0
Cefamandole	0.75	0.2
Cefuroxime	0.5	0.5
Cefoxitin	3.0	2.0
Cefotaxime	0.1	0.0004

Table 3.5 - Activity of some parenterally administered cephalosporins against aerobic Gram-negative bacilli 9, 10, 13, 15.

Organisms	MIC (µg/ml) for 50% of strains tested						
	Cepna-ithin	Cepha-lori-din	Cefaz-olin	Cefam-andol	Cefu-rox-ime	Cefox-itin	Cefo-tax-ime
E.coli	50	2	12.5	6.2	6.2	12.5	0.07
Kelbsiella	3.1	3	3.1	0.4	1.6	1.6	0.08
Enterobacter	R	R	40	3.1	6.2	50	30
Citrobacter	24	-	R	0.2	3.1	6.2	2.4
Serratia	R	R	R	R	50	50	0.85
Proteus mirabilis	6.2	2	6.2	1.6	1.6	3.1	0.03
Proeus(indole)	R	R	40	3.1	25	6.2	0.75
Providencia	R	R	40	12.1	25	6.2	0.12
Salmonella spp.	6.3	-	1.7	3.1	-	1.6	0.12
typhi	0.8	-	0.75	0.19	-	-	0.06
ParatyphiA	5.0	-	2.0	1.0	-	-	-
Shigella	25	-	-	1.6	-	6.3	-

R = > 64 µg/ml

Table 3.6 - Activity of orally administered cephalosporins against aerobic Gram-negative rods 14, 16, 17, 18.

Organism	MIC (µg/ml) for 50% strains		
	Cephalexin	Cephradine	Cefaclor
E.coli	4	8	12
Klebsiella	-	6	6
Enterobacter	32	50	4
Citrobacter	-	-	-
Serratia	R	-	-
Proteus mirabilis	4	20	4
Proteus (indole$^+$)	50	R	32
Salmonella (spp)			
typhi	2.5		0.3
paratyphi A	10.0		1.0
Shigella			12

R = > 64 ug/ml

It is seen that the cephaloridine is highly active against penicillinase producing staph. aureus and quite active against some Gram-negative organisms, including E.coli and H. influenzae. However, it is susceptible to Gram-negative Beta-lactamases and is thus less active against acipicillin resistant E.coli, E cloacae and Pseud. aeruginosa.

Cetoxitin is stable to lactamases and is active against the ampicillin resistant E.coli. Its inactivity aganist Ent.cloacae and Pseudomonas may be due to poor

penitration of the bacterial cell.

Cefuroxime is similar to cefoxitin against staph. aureus and E.coli but is much more active against H. influenzae. It is moderatly active against Ent.cloacae but inactive against Pseudomonas.

Cefotiam, an aminothiazole with no oxime function, is only moderately stable to Beta-lactamases but has the high intrinsic activity seen with the aminothiazolyl cephalosporins. It overall activity thus falls between that of cefoxitin and the aminothiazolyl oximes below it. Cefotaxime is less active than cephaloridine against staph. aureus but is very active against all the Gram-negative bacteria except pseudomonas against which it shows only moderate activity. However, its metabolite, desacetyl-cefotaxime, is noticeably less active than the parent compound against staph. aureus, H.influenzae and Pseudomonas. Ceftizoxim & is similar to cefotaxime, but is metabolically stable.

Ceftazidime is somewhat less active in vivo against staph. aureus than the other aminothiazolyl oximes shown here. However as we shall see presently, its performance against this organism 'in vivo' is acceptable. Its activity against Gram-negative bacteria is generally similar to that of the other aminothiazolyl oximes but it is more active against resistant organisms, such as pseud.aeruginasa.

METHODS OF ASSAY -

A. Chemical Methods - When an antibiotic is available in pure form, and its structure is least partially known, the amount of it can be determined by chemical methods based on its reactive groups. For instance, the lactam ring of penicillin can be hydrolyzed with alkali to sodium penicilloate and the amount of base consumed can then be determined by backtitrating or the penicilloic acid formed can be broken down further with acid to yield dimethylcysteine, which can be determined by the ninhydrine reaction for amino acids.

The most specific methods, however, are those based on antibiotic power. These, of course, can be used with impure preparations at every stage from the first crude extract onward. The procedures adopted can be given as[19].

B) All-or-none Growth Method:-

Tubes of nutrient both are seeded with a standard small inoculum of a susceptible organism and serial dilutions of the antibiotic added. The tubes are incubated and examined. The lowest concentration (Minimum Inhibitory concetration, (MIC) necessary to inhibit growth completely is taken as end point, and the amount of antibiotic determined by comparison with similar dilutions of a known standard substance. For penicillin, M.aureus, incubated 16 hours at 37°C, is used. An advantage of this method is that the volumes used can be quite small[20].

c) Turbidimetric method:-

The tubes of broth are seeded with a small inoculum, but the time course of growth is followed with a turbidi meter or colorimeter. In this curve using penicillin, the higher concentrations cause auto lysis after four hours. A Plot of the turbidity or optical density at a fixed time against concentration is a curve, the same plotted against long concentration usually gives a straight line, from which the concentration needed for 50 per cent inhibition is easily read off.

D) Diffusion Methods :-

These methods are the ones most particularly associated with antibiotics. A plate of solid medium, inoculated in bulk with test organisms, is treated in spots with the solution and then incubated. As the substance diffuses out into the agar, it inhibits growth throughout a zone surrounding the place of application. The radius of the zone is a measure of the amount or concentration of the antibiotic. If the antibiotic applied is small in amount, the radius of the inhibited zone is proportional to its amount. This is true of a small drop of solution, and also, roughly, with small filter paper circles, soaked in the soulution. However, if a relatively large volume of solution is used, the radius of the zone is proportional to the concentration, or more nearly to the logarithm of the concentration, of the antibiotic in it. This is the case with the classical "Oxford Cup" method[21] in which small glass cylinders, their ends warmed in the flame, are placed on the agar and filled with 1 to 2 ml of the test liquid.

When this method is used with penicillin, Bal. subtilis is used, since bulk inoculation of agar has to be done at 48^0c, a temperature that may damage a coccus.[22] The original Oxord unit of penicillin was established by the cup assay. It was defined as the amount of penicillin which, under the standard conditions, using "Staph. " aureus, gave an inhibition zone of diameter 24 mm.

EXPERIMENTAL TECHNIQUES :-

To study the biological activity of prepared Beta lactam Cephalosporins the methods used can be summerised as.

A)**For sensitivity test** - To test wheather the antibiotic is sensitive to inhibit growth of bacteria were tested by Agar diffusion method. (Pour plate method)

B) **For MIC values** :- If the antibliotic was capable of inhibition of becterial growth, then the Minimum Inhibitory Concentrations (MIC values) were obtained by "Ten fold dilution method ".

A) **Sensitivety test** :- The steps involved can be summerised as follow -

1) Sterilization :- It is the complete distruction of all living organisms by physical or chemical agents.

Plugged glassware and petri dishes must be sterilized before used to destroy all living organisms adhering to the inner surfaces. Likewise, culture media must be sterilized prior to use to destroy all contaminating organisms presents. When once sterilized, glassware may be [illegible] condition indefinitely if protected from outside contamination. The same applies to culture media if, [illegible]

kept in a sterile condition indefinitely if protected from outside contomination. The same applies to culture media if, in addition, evaporation can be prevented.

Three types of sterilizers are generally used in bacteriology 1) the hot - air sterilizer, 2) the Arnold sterilizer, and 3) the autoclave.

The sterilization method adopted can be given as. The petriplates and glass wares were washed thoroughly and covered with papers and placed in an oven at high temperature till the paper becomes black.

The nutrient agar used for plate culture and slant cullture is sterilized by placing them glass test tubes having cotton stopper in pressure cooker, after one whistle the burners were simmed off and further heating for 20 minutes.

2) **Method for inoculation of media** :- The pure culture can be prepared by a) Agar deep culture, b) Agar slant culture, c) Broth culture.

b) Agar slant culture :- This technique is used for preparation of pure culture for "sensitivity test" as well as "MIC test" sterilize the wire loop in the flame. Allow the loop to cool for a few moments. Remove the cotton stopper from the culture, by grasping it with small finger of the right hand and flame the neck of the tube. Hold the tube slanted, not upright, to minimize aerial contomination. Remove a loopful of the growth with the loop, Again flame the neck of the culture,replace the cotton stopper, and set the

tube aside in test tube block. Remove the cotton stopper from the agar slant to be inoculated by grasping it with the small finger of the right hand. Flame the neck of the tube. Spread the inoculam over the surface of the agar slant by making streaks back and forth a few millimeters apart. Withdraw the loop from the tube. Again flame the neck of the tube, and replace the cotton stopper. Flame the loop before setting it down on the table. Mark the tube with the name of the organism and the date. Incubate the culture in slant position in incubator at 35^{0}0c for 24 hours to allow proper growth of organism for the experiment.

The transfer of culture from Agar slant culture to sensitirity test in petriplates can be done as.

Microorganisms from culture were transfer to tube containing 1 ml sterile saline solution (Nacl solution) in the similar way as explained in Agar slant culture taking precaution that it does not get contaminated with outside atmosphere.

The sensitivity tests were done by transfer of culture in petri plates. Two methods are generally followed.
a) Streak plate method and 2) pour plate method.
b) <u>Pure plate method</u> :- Take a sterile Agar solution tube of 20 ml volume remove the cotton stopper and flame the neck of the tube, hold the tube in up right position in between the flames of two bumers and mix the sterile saline solution (1 ml) tube having culture of specific organism, again flame the neck of tube and replace the cotton stopper.

stir the tube holding in between two palms of hands so as to make uniform suspension of organism in Agar Medium. Place the tube aside. To avoid coagulation of Agar medium in tube it should be transfered to sterile petri plate while it is in liquid form. Take a sterile petriplate and raise the lid of petriplate just high enough to transfer Agar medium from tube. By holding petriplate in between the flames of two bumers, Transfer the Agar medium from tube and cover the lid of petri plate. Place the petri plate on the table having uniform surface and allow to solidify the Agar medium along with culture .

Take a metal borer having the diameter of 2 mm and sterile it by flame and allow to cool take a petriplate in one hand and raise the lid. make a 4 wells with the help of borer in such a way that each well should occupy corner of regular square, cover the lid of petri plate and place it aside.

3) **Preparation of Antibiotic solution to study " sensitivity & MIC value** :- The solution of antibiotics can be prepared in buffer. These are the salts of weak acids which have the power of preventing pronounced changes in the reaction of solutions on the addition of acid and alkall.

Bacteriological peptones contain proteoses, peptones, peptides and amino acids all of which are buffers. They posses both acidic and basic properties. The acidity of solution up to certain concentration prevents further multiplication of the organisms.

In absence of buffer, the activity of the organisms may case after a few hours. Clark and Lubs[23] proposed a series of buffer standards covering the range from p[H] 1.2 to 10.

Taking advantage of this fact the buffer solution is used for preparation of Antibiotic solution.

A known wt of Antibiotic is dissolved in a known volume of buffer solution & stoppered with cotton stopper.

Take a sterile mocropipet or a dropper & take small quantity of above solution by holding the tube & dropper in flame of two burners. Place the micropipette in such a way that its mouth should be between two burners to avoid contamination.

Take a petriplate having wells in it, lift the lid of plate and allow one drop of above to fill the single well. In similar manner, allow four wells to fill by solutions of 4 different antibiotics, cover the lid and Mark each well by its antibiotic on the bottom withsketch pen or Marker.

Place the petriplates in an incubator at 35*0*c for 24 hours to have proper growth of organisms and their inhibition by antibiotics.

Observe the plates after 24 hours. There appears an inhibition zones on culture media which looks like a transperent while other surface is full of growth of microorganism measure the diameter of the inhibition zone

which is "Sensitivity test" of perticular organism for particular antibiotics.

MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES :

After sensitivity test, depending upon size of "Inhibition Zone" it is possible to select perticular concentration of that antibiotic for further study of M.I.C. value. It is calculated by "Ten fold dilution method" which can be summerised as follow:

The stepsinvolved can be given as :

1) **Sterilization** : The sterilization of micropipet , test tubes, loop etc. can be done before start of experiment as given in "Sensivity test".

The sterilization of Agar Neutrient is also done in the similor way.

2) Inoculation of media : The inoculation of media as well as transfer of microorganisms were done in the similar way as that of "sensitivity test".

3) MIC values :- MIC values can be determined as-place 9 ml of the Neutrient Agar in each test tube for one organism ten test tubes were required which forms Ten different concentrations of antibiotics under study. Stoper the tubes with cotton stopper lightly and sterilize them in presure cooker as explained in first step.

The solution of known quantity (5 ml) of antibiotic is prepared in 1 ml of Buffer solution which gives the concentration as 5000 meg / ml

The test tubes of Neutrient Agars after sterilization were numbered from 1 to 10.

The saline solution of microorganism is taken in micropipette and one drop of it is aded to above test tubes. One ml. of above antibiotic solution is added to test tube number 1 and it is stirred well. One ml of Agar Neutrient from test tube no. 1 is transfered to test tube no. 2 and test tube no. 1 is transfered to test tube no. 2 and test tube no. 1 is corked tightly and placed apart the similar procedure was repeated for test tubes no. 2-10 Now one ml of Agar from test tube no 10 is taken out and placed apart. All the test tubes are stimed well and placed in an incubator at 37^0 for over night.

The concentration of antibiotic in each tube can be given as in Table No. 3.7..

Test tube No.	1	2	3	4	5	6	7
Concentration of Antibiotic in mcg/ml	$5x10^2$	$5c10^1$	$5x10^0$	$5x10^{-1}$	$5x10^{-2}$	$5x10^{-3}$	$5x10^{-4}$

Test Tubes no.	8	9	10
Concentration of Antibiotic in mcg/ml.	$5x10^{-5}$	$5x10^{-6}$	$5x10^{-7}$

All the tes tubes were observed after over night. The growth of microorganisms occurs in the form of turbidity in tes tubes. If the antibiotic is effective against

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perticular organism for specific concentration then from above that concentration. There is no growth of micro-organisms and the neutrient agar appears as clear(No turbidity). The concentration at which no turbidity appears in test tube is the 'Minimum Inhibitody Concentration' (MIC) of that antibiotic for that microorganims.

Observations :-

The c ephalosporin derivatives thus prepared were further tested for their antibacterial activity and MIC values as explain above. The antibacterial activity of the compounds prepared, at concentration 500 µg/ml is given in Table No. 3.8

Table No. 3.8

Antibacterial Activity of Cephalosporin Derivatives

Microorganims	compound				
	3a	3B	3C	3D	3E
S.Aureus	+	-	-	-	+
E.coli	-	-	+	-	-
s.typhi	+	-	-	-	+
K.pneumonae	-	-	+	-	+
P.Aeruginosa	+	-	-	-	-

NOTE :- +ve sign indicates that the compound is active
-ve sign indicates that the compound is inactive.

Determination of MIC values :- The MIC values were also observed for each compound for each microorganism

by Tenfold dilution method and can be summerised in Tabular form as in Table No. 3.9

Table No. 3.9

MIC values

Microorganism	MIC values in mcg/ml				
	3a	3b	3c	3d	3e
S-aureus	500	*	*	*	500
E.Coli	*	*	50	*	*
S.typhi	500	*	*	*	50
K.Pheumoneae	*	*	50	*	500
P. aeruginasa	500	*	*	*	*

NOTE: * - above 500 mcg/ml

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