

IN-VITRO INTERACTION STUDY OF CEPHRADINE WITH DIFFERENT ESSENTIAL MINERAL SALTS AND ITS INFLUENCE ON ANTIMICROBIAL ACTIVITY (MIC) OF CEPHRADINE

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Abstract

Present paper describes the interactions of Cephadrine with different essential mineral salt like Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride in an aqueous system at pH 7.4. This Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride are essential trace element. From spectrophotometric study, it has been found that Cephadrine forms 1:1 complex with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride. Spectral studies helps to detect the initial complexation between drug and mineral salts. Job's plot at 7.4 provides same type of information. The Ardon's spectrophotometric method confirmed the 1:1 complexation and the value of stability constants was calculated using Ardon's plot. An individual antimicrobial study (MIC) of Cephadrine in 1:1 mixture with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride at pH 7.4 was performed. These studies were carried out by observing the minimum inhibitory concentration (MIC) of the complexes and compared with the parent Cephadrine against both Gram negative and Gram positive microorganisms in nutrient broth medium. Study confirms interactions of the Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride and the interactions results into change the antimicrobial activity of Cephadrine.

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Result shows that the antimicrobial activity increasing trends in presence of Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate and decreasing trends in presence of Zinc Sulfate and Potassium Chloride.

Key words: Cephadrine, Job's Plot, Ardon's method, MIC, Essential trace element.

Introduction

Cephadrine is a semisynthetic cephalosporin antibiotic developed at the Squibb Institute for Medical Research¹, chemically designed as 7-[D-2-amino-(1,4-cyclohexadiene-1-yl) acetamido]-3-methyl-8-oxo-5-thia-1-azyabicyclo-octa-2-ene-2-carboxylic acid².

Ferrous sulfate USP is an important ingredient of ferrous sulfate U. S. P syrup, ferrous sulfate USP capsule and Ferrous sulfate USP tablet. Ferrous sulfate is the most widely used oral iron preparation and is considered as the drug of choice for treating uncomplicated iron-deficiency anemia. Zinc is an essential dietary mineral. Zinc deficiency is associated with impaired growth, parakeratosis (a thickened, scaly, inflamed skin) and retarded sexual maturation. Zinc sulfate is an important ingredient of Zinc sulfate syrup USP. Manganese, Ferrous, Magnesium, Zinc, Potassium and Chloride are essential trace ions³.

Gram-positive bacteria are usually sensitive, *Staphylococcus aureus*, including most penicillin-resistant⁴, but not methicillin-resistant strains, are sensitive. Cephadrine is about as resistant as cephalothin to inactivation by Staphylococcal β -lactamase^{5,6}. Most other aerobic Gram-positive cocci, such as *Staphylococcus epidermis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Streptococcus viridans*, are susceptible to cephadrine. *Streptococcus faecalis* is resistant⁷. Anaerobic Gram-positive cocci, such as the *Peptococcus* and *Peptostreptococcus spp.*, are usually cephadrine sensitive. Most strains recovered from airway associated infections are relatively sensitive (MICs 8-16 g per ml), but other strains are less so⁸. Against Gram-positive bacilli, cephadrine has a similar inhibitory action to cephalixin.

Gram-negative bacteria like *E. coli*, *Proteus mirabilis* and *Klebsiella spp.*^{9,10,11} are susceptible. Cephadrine is only moderately active against *N. gonorrhoeae*¹² but it

retains this activity against β -lactamase producing strains¹³. Cephadrine is relatively inactive against *H.influenzae*, many strains of which are completely resistant to this drug^{14,15}. The drug is moderately active against *Gardnerella vaginalis*^{16,17,18}.

Materials and method

Materials

Cephadrine, kind gifts from Medicon laboratories Ltd, Dhaka, Bangladesh. Magnesium Sulphate, Manganese Sulphate, Ferrous Sulphate, Zinc Sulphate and Potassium Chloride were from Merck Ltd, Mumbai, India.

I. Interactions (complexation) study

1. Spectral studies

Initial detection of complexation of Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride has done from the nature of spectra of pure compounds as well as their 1:1 mixtures in phosphate buffer solution of 7.4 at a fixed concentration (0.1×10^{-5} M).

2. Job's spectrophotometric method of continuous variation¹⁹

In this method, series of absorbance of Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride mixture with different molar ratios at pH 7.4 were measured by keeping the total moles constant. The absorbance of Cephadrine and Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride solutions was measured at 262 nm. The observed absorbance of the mixtures of various mole fractions was subtracted from the sum of the values for free Cephadrine and Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride. The absorbance difference (D) was then plotted against the mole fractions of drugs in the mixtures. A curve, thus, obtained showed a maximum at a point, which indicated the molar ratios of Cephadrine and Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride in the complex.

3. The Ardon's spectrophotometric methods ²⁰

In this method, concentrations of Cephadrine varied while keeping the concentrations of Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride fixed at 2×10^{-3} M. The whole experiment was performed in the buffer pH 7.4. The absorbencies were measured at 262 nm. For calculation, the Ardon's equation was used.

This equation is given below:

$$\frac{1}{(D - \epsilon_A C)} = \frac{1}{KC (\epsilon_{com} - \epsilon_A)[B]} + \frac{1}{C(\epsilon_{com} - \epsilon_A)}$$

Where,

D = Absorbance of the mixture.

B = Molar concentration of the drug.

C = Molar concentration of the mineral salt.

ϵ_{com} = Molar extinction co-efficient of the complex.

ϵ_A = Molar extinction co-efficient of the drug.

The value of ϵ_A was chosen as 1, which is an essential condition for validation of the method. The value $1 / (D - \epsilon_A C)$ were plotted versus $1/[C]$ to get the straight lines.

The concentration of all mineral salts was kept constant at 2×10^{-3} M (denoted by C in the equation) & the concentration of interacting species Cephadrine was varied (denoted by B in the equation). The 1:1 complex gave a straight line in the plots with an intercept and a slope. The stability constant of the complex was given by the relation:

$K = \text{intercept} / \text{slope}$

II. Antibacterial Studies

1. Sample collection

Organisms were employed in these studies were collected from department of microbiology, Chittagong University, Chittagong, Bangladesh.

2. Determination of gram positive and gram negative Bacteria

A smear of each organism to be stained was prepared on a clean grease free slide by taking a loopful of bacteria. The smear was air dried and then was heated by passing the slide quickly through a flame to heat fix. A drop of Ammonium oxalate crystal violate was added and kept for 1 minute. After that the slide was rinsed with tap water gently. Logul's Iodine was applied and was rinsed with tap water. After that decolorizing agent, 95% ethanol was added and kept it for 2 minutes. 1 to 2 drops Safranin was added as counter stain after washing the decolorizing agent. After 45 seconds the slide was rinsed up. By following this method the slide was prepared to differentiate the gram positive and gram negative bacteria under the microscope.

3. Estimation of MIC (Turbid metric method to determine MIC)

3.1 Turbidity Standard for preparation

To standardize the inoculum's density for a susceptibility test, a BaSO₄ turbidity standard, equivalent to a 0.5 McFarland standard was used. 0.05 ml or 50µl of 0.048 M BaCl₂ or 1.17% W/V BaCl₂.2H₂O was added to 9.95 ml of 0.18 M H₂SO₄ or 1% V/V in a screw cap test tube with constant stirring. The correct density of the turbidity standard was verified by using a spectrophotometer. The absorbance was 0.09 at 625 nm. (Normal range is 0.08 to 0.10 at 625 nm). Then the tube was capped and sealed tightly to prevent loss of evaporation.

3.2 Inoculums Preparation

Bacteria were subculture overnight on the nutrient agar medium and a loopful culture from the nutrient agar medium was inoculated into the nutrient broth. The broth was incubated at 37° C. The turbidity of the growing broth culture was adjusted with sterile saline to obtain turbidity optically comparable to the 0.5 McFarland Standard. This results in a suspension containing approximately 10⁸cfu/ml.

3.3 Different concentration antibiotic solution preparation

One gram of antibiotic was measured aseptically and was dissolved in 100 ml phosphate buffer. It was mixed vigorously. In this way $10^4 \mu\text{g/ml}$ antibiotic solution was obtained. 1 ml from this solution was mixed with 99 ml nutrient broth and made $10^2 \mu\text{g/ml}$ solutions. Again from this solution 1 ml was taken and mixed with 99 ml nutrient broth and thus a final concentration $1 \mu\text{g/ml}$ solution was obtained.

By following serial dilution method different concentration of antibiotic solution were prepared. In this test $1 \mu\text{g/ml}$, $2 \mu\text{g/ml}$, $3 \mu\text{g/ml}$, $4 \mu\text{g/ml}$, $5 \mu\text{g/ml}$, $6 \mu\text{g/ml}$, $7 \mu\text{g/ml}$, $8 \mu\text{g/ml}$, $9 \mu\text{g/ml}$, $10 \mu\text{g/ml}$ concentration was used.

0.1 ml bacterial suspension which was compared with 0.5 MacFarland solution was transferred into 9.99 ml saline water. After that, one drop of bacterial suspension containing saline water was inoculated into each different concentration antibiotic containing tubes.

This procedure was performed for both gram positive and gram negative bacteria. Then test tubes were incubated at 37°C for 48 hours. Finally turbidity was observed visually to determine MIC.

3.4 Different concentration of antibiotic and metal complex preparation

One gram of antibiotic and 1 gm specific metal salt was measured aseptically and were dissolved in 100 ml phosphate buffer. It was mixed vigorously. By following serial dilution method different concentration of antibiotic solution containing specific metal ion were prepared. In this test $1 \mu\text{g}$ antibiotic and $1 \mu\text{g}$ metal in 1ml, $2 \mu\text{g}$ antibiotic and $2 \mu\text{g}$ metal in 1ml and in such a way $10 \mu\text{g}$ antibiotics and $10 \mu\text{g}$ metal in 1ml solution was prepared and used in the test.

0.1 ml bacterial suspension which was compared with 0.5 MacFarland solutions was transferred into 9.99 ml saline water. After that, one drop of bacterial suspension containing saline water was inoculated into each different concentration antibiotic with metal ion containing tubes.

In our test we used $MgSO_4$, $MnSO_4$, $FeSO_4$, $ZnSO_4$ and KCl as the source of metal ion. This same procedure was maintained for each metal.

This procedure was performed for both gram positive and gram negative bacteria. Then test tubes were incubated at $37^\circ C$ for 48 hours. Finally turbidity was observed visually to determine MIC.

3.5 Measurement of absorbance

By using light absorbance technique, the growth of bacteria was measured at 590 nm in a spectrophotometer. Then the resistant pattern was analyzed by drawing a graph using the chart of absorbance in different concentration of antibiotic.

Results

1. Spectral study

In spectral studies, it was seen that Cephadrine gives a sharp peak at 262 nm, when the salts (Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride) mixed with Cephadrine in 1:1 ratio, the intensity of the peak of Cephadrine changes remarkably i.e. absorption characteristics are altered due to interaction but the position of the compound do not shift. Spectral result presented in figure 1.

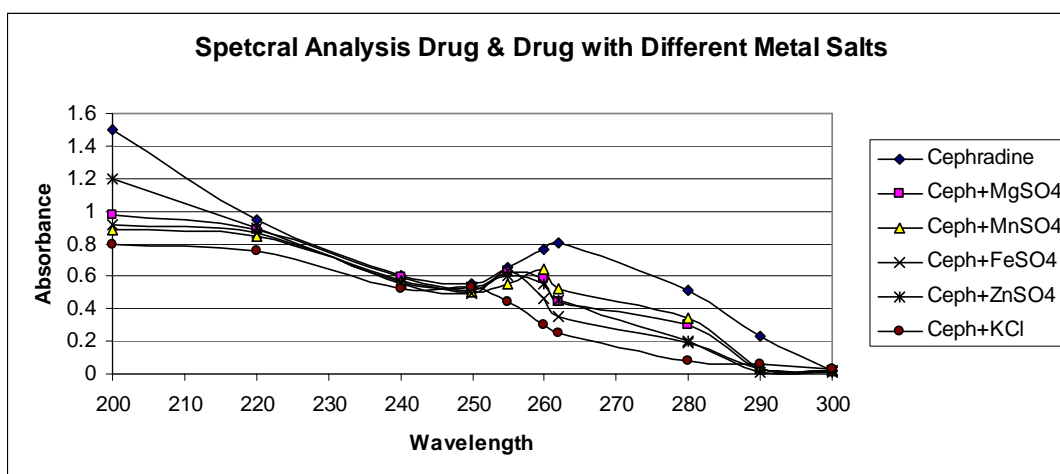


Figure 1: Combined Spectra of Drug & Drug with different Essential Metal

2. Study of job's method

The molar ratios of the complexes of Cephadrine with metal salts (Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride) were estimated by job's method of continuous variation. The observed absorbance values were measured in pH 7.4 at various concentrations (0.1×10^{-5} to 0.9×10^{-5} M) of Cephadrine with metal salts at 262 nm. The Job's plots at 7.4 were obtained by plotting absorbance differences against the mole fraction of the drug Cephadrine, which are presented in Figure 2.

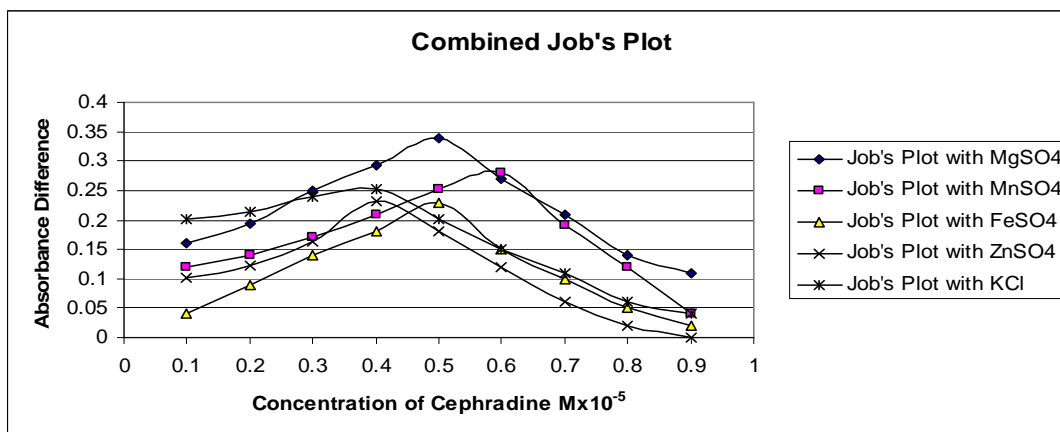


Figure 2: Job's plots for all metal salts after interaction with Cephadrine.

3. Study of Ardon's method

Ardon's plot confirmed the formation of 1:1 complex of Cephadrine with metal salts at pH 7.4, since the method is valid for only 1:1 complexes. The Ardon's plots gave straight lines intercept which are presented in Fig.4 indicating the formation of 1:1 complexes drug Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride. Results are presented in figure 3.

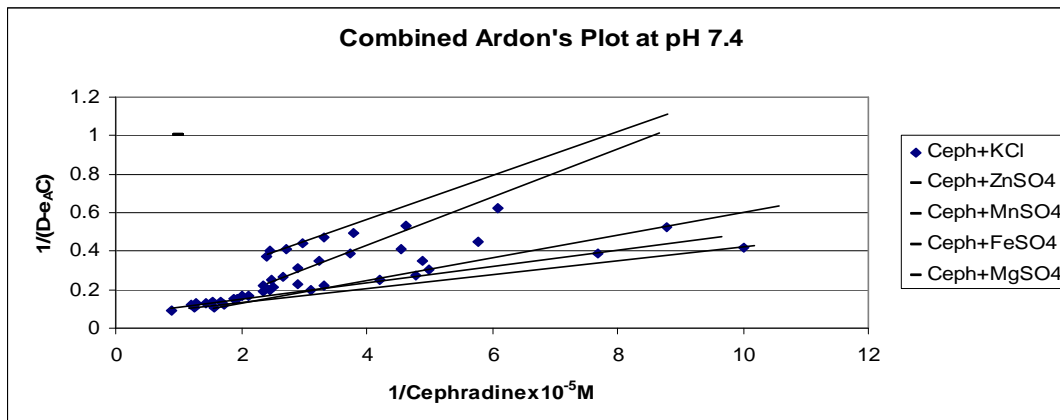


Figure 3: Represent Combined Ardon's plots at pH 7.4

4. Estimation of Stability Constant

The value of stability constant for the complexation of Cephradine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride at pH 7.4, were obtained from the spectral data using Ardon's plot. The values for stability constant were calculated from the slopes and intercepts of the straight lines from these plots. It was seen from the Ardon's equation that the values of stability constant was given as (Intercept)/(slope). The value of intercept and slope were calculated by Least Squares Method using the following equation:

$$y = mX + C$$

The values of stability constants for the drug-metal system at pH 7.4 are given in the Table 1.

Table1: Values of stability constant at pH 7.4

Drug Metal complex	System stability Constant
Cephradine + MgSO ₄	1.437
Cephradine + MnSO ₄	1.251
Cephradine + FeSO ₄	1.335
Cephradine + ZnSO ₄	0.983
Cephradine + KCl	0.795

5. Antibacterial study (MIC determination)

The MIC of Cephadrine after 1:1 interaction with the Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, was found to be decreased for most of the organism as compared to the parent drug. The decreasing trends of MICs were observed for both of the gram positive and gram negative organisms. The intensity of absorbance also decreased remarkably for both gram positive and gram negative organisms.

The MIC of Cephadrine after 1:1 interaction with the Zinc Sulfate and Potassium Chloride was found to be increased for most of the organism as compared to the parent drug. The increasing of MICs was observed for both of the gram positive and gram negative organisms. The intensity of increasing absorbance was also observed. Table: 2A & 2B shows absorbance of the growth and Figure: 5A & 5B shows the comparison.

Table 2A: Absorbance of the growth of Gram Positive Bacteria containing dilution

Conc. of Cephadrine µg/ml	Cephadrine	Cephadrine + MgSO ₄	Cephadrine + MnSO ₄	Cephadrine + FeSO ₄	Cephadrine + ZnSO ₄	Cephadrine + KCl
1	0.652	0.301	0.31	0.195	0.716	0.85
2	0.62	0.212	0.275	0.165	0.673	0.643
3	0.59	0.146	0.241	NG	0.589	0.594
4	0.527	NG	0.18	NG	0.536	0.523
5	0.505	NG	0.155	NG	0.509	0.488
6	0.425	NG	NG	NG	0.406	0.365
7	0.35	NG	NG	NG	0.295	0.239
8	NG	NG	NG	NG	0.214	0.176
9	NG	NG	NG	NG	NG	0.132
10	NG	NG	NG	NG	NG	NG

NG= No Growth was Observed visually

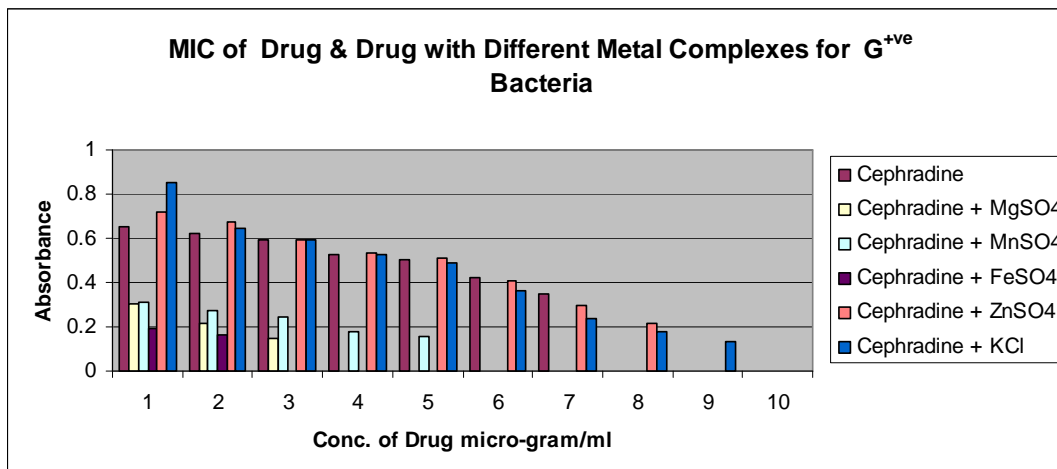


Figure: 5A shows comparison of variation of the growth of Gram Positive bacteria after using different metal with antibiotic

Table 2B: Absorbance of the growth of Gram Negative Bacteria containing dilution

Conc. of Cephadrine $\mu\text{g/ml}$	Cephadrine	Cephadrine + MgSO4	Cephadrine + MnSO4	Cephadrine + FeSO4	Cephadrine + ZnSO4	Cephadrine + KCl
1	0.714	0.325	0.401	0.21	0.975	0.85
2	0.7	0.288	0.36	0.179	0.794	0.763
3	0.659	0.169	0.281	0.145	0.625	0.701
4	0.582	NG	0.25	0.137	0.516	0.58
5	0.516	NG	NG	0.114	0.459	0.425
6	0.455	NG	NG	NG	0.346	0.347
7	0.38	NG	NG	NG	0.254	0.284
8	NG	NG	NG	NG	0.156	0.176
9	NG	NG	NG	NG	NG	0.12
10	NG	NG	NG	NG	NG	NG

NG= No Growth was Observed visually

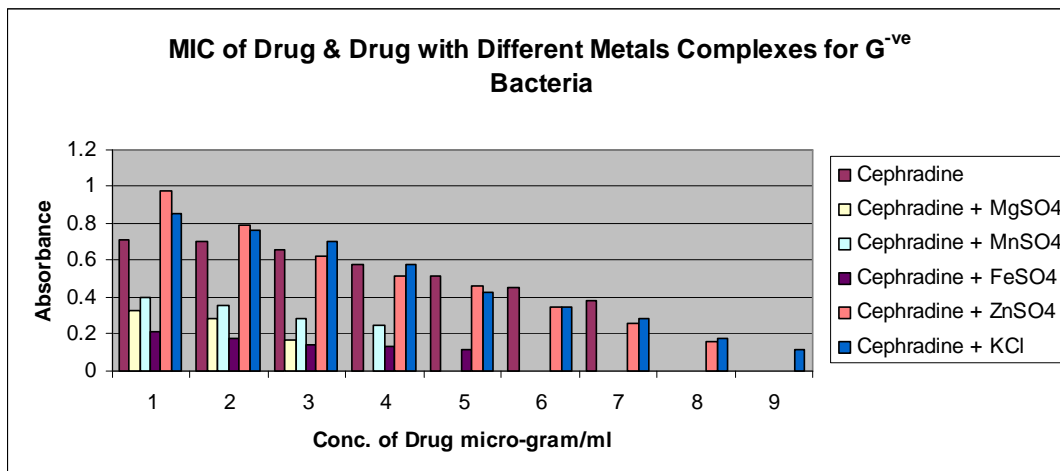


Figure 5B shows comparison of variation of the growth of Gram Negative bacteria after using different metal with antibiotic

Discussion

In the present work, the interaction of an important antibiotic, Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride has been studied in the aqueous system at pH 7.4 by a variety of physical method like inspection of spectral behavior, Job's method of continuous variation and Ardon's straight line plots by spectrophotometry. From spectral study, it has been seen that Cephadrine give a sharp peak at 262 nm. When Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride salt mixed with Cephadrine at 1:1 ratio, the intensity of the peak of Cephadrine changes remarkably, i.e absorption characteristics are altered due to interaction but the position of the compound do not shift. Job's plot has given the molar ratio of complexes of Cephadrine and with metal salts. At pH 7.4 Cephadrine forms strong 1:1 complexes with metal salts indicated as 'Λ' shaped curves. These curves may indicate strong kinetics of complexation between Cephadrine & metal salts. The Ardon's spectrophotometric plots also confirm the phenomenon of 1:1 complexation which is indicated by straight lines. The stability constant of the complex has estimated from this straight line plots using Ardon's equation.

The determined MIC of Cephadrine was performed after 1:1 interaction with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and

Potassium Chloride. Result was found to be decreased in minimum inhibitory concentration (MIC) for most of the organism as compared to the parent drug in presence of Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate. The decreasing trends in the MICs for both of the gram positive and gram negative organisms. That means the antimicrobial activity of Cephadrine increased in presence of Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate. Result also was found to be increased in minimum inhibitory concentration (MIC) for most of the organism as compared to the parent drug in presence of Zinc Sulfate and Potassium Chloride. The increase in the MICs for both of the gram positive and gram negative organisms was observed. That means the antimicrobial activity of Cephadrine decreased in presence of Zinc Sulfate and Potassium Chloride.

Conclusion

Finally it can be concluded that the antimicrobial activity is higher for 1:1 complexes of Cephadrine with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate than that of Cephadrine alone. As a result, the intake of Cephadrine with Magnesium Sulfate, Manganese Sulfate salt or Magnesium, Manganese and Ferrous complexes of Cephadrine or the concurrent therapy can increase the antimicrobial activity of Cephadrine. It is also observed that antimicrobial activity is lower for Cephadrine complexes with Zinc sulfate and Potassium chloride. So we must be careful during concurrent therapy with Magnesium Sulfate, Manganese Sulfate, Ferrous Sulfate, Zinc Sulfate and Potassium Chloride.

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