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BACTERIOCIDAL STUDY OF MIXED LIGAND COMPLEXES OF PYRAZINE CARBOXAMIDE AND ISONICOTINIC ACID HYDRAZIDE

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ABSTRACT

Pyrazine carboxamide and isonicotinic acid hydrazide are commonly known as Pyrazinamide (PZA) and Isoniazide (INH) respectively. They are antitubercular drugs effective against *Mycobacterium tuberculosis* of human type resistant, but is ineffective against the bonine and typical forms of tubercle bacilli. The drug shows antituberculosis activity *in vitro* at an acidic pH and is effective only against tubercle bacilli within macrophages. It is observed that when Pyrazinamide administered alone was almost as effective as isoniazid for the first two months but when both the drugs were given together, the effect was appreciably greater than with either drug alone. Further, it was compared with the effect of mixed ligand complexes of Pyrazinamide. Isoniazid and metals, the antitubercular study revealed an effective increase in potency of INH and PZA when chelated with Mn^{2+}, Cd^{2+} and Ca^{2+}.

Keywords: Mixed ligand, Antitubercular activity, pyrazinamide and Isoniazid.

INTRODUCTION

Tuberculosis (TB) is a disease known to man from the earliest recorded history. It is the single largest killer among infectious diseases. INH, and PZA are used in the treatment of tuberculosis.

Isoniazid is appreciably more effective *in vitro* for the inhibition of multiplication of virulent *Mycobacteria* depending upon the nature of the culture medium. No growth will occur in concentration from 0.015 to 0.25 µg/ml. The activity of Pyrazinamide appears to be pH dependent with good *in vitro* activity at pH 5.5, but the compound is nearly active at neutral pH. The mixed ligand complexes have prepared and studied.

EXPERIMENTAL

Techniques for detection and measurement of Chemotherapeutic activity

The antimicrobial agents currently used for the therapy depends on their capacity to inhibit the multiplication of, or to kill the invading microorganisms under the conditions which exists *in vivo*. It would be desirable to have a test which could be more simply and rapidly performed, and which would only a very small amount of drug.

The *in vitro* bacteriostatic test fulfils these requirements. A major disadvantage of this test, however, is that the overwhelming majority of compounds, found to produce a significant degree of bacteriostasis *in vitro.* Hundred or thousands of compounds may be tested *in vitro* before an active *in vivo* agent is discovered.

Technique for detection *in vitro*

In setting up *in vitro* bacteriostatic test, several important factors must be considered. These include, the nature of the culture medium, the nature of the test microorganisms, the amount of inoculum, and the time and temperature of incubation. There are mainly two types of techniques used for detection *in vitro* depending upon the nature of media

1. In liquid media
2. In solid media

Mostly, detection can take place in liquid media. The liquid media as processes are carried out in the following steps

1. Preparation of glassware (In sterilized form)
2. Choice of culture medium
3. Choice of test organism
4. Preparation of drug dilution
5. Time and temperature of incubation and controls

Chemotherapeutic effects of mixed-ligand complexes

(A) On acid fast bacilli

With the belief that metal complexes play an important role in biological activity of drugs, the present study was carried out. As the pyrazinamide possess chelating sites initially, the systematic study of mixed ligand complexes of pyrazinamide and isoniazid with metals were synthesized with special interest to determine the bactericidal effects on Myco-tuberculosis.

MATERIAL AND METHODS

Material used for this study are as follows

1. Plastic petri plates, 90 mm
2. Synthesized metal complexes of pyrazinamide.
3. Middle brook 7H11 Agar base
4. Autoclave
5. Incubator

Methodology

Preparation of Growth media

10.5 gms 7H11 media was dissolved in 200ml of distilled water and stir properly then 2.5ml glycerol was added. The volume was made up to 450 ml and media was autoclaved for 15 minutes at 121°C. After autoclaving, the media was kept at 56°C for two hours and 20 ml was poured into each plate under sterile conditions.

(b) Preparation of solutions of mixed ligand complexes

A stock solution of 1µg/ml was prepared for each compound to facilitate the further dilutions. Autoclaved distilled water was used as solvent.

(c) Preparation of serial dilution

Serial dilutions were prepared as to obtain the desired conc. of 0.1 µg/ml; 0.01µg/ml in autoclaved distilled water.

(d) Spreading of bacteria and incubation

Bacterial suspension of *Mycobacterium tuberculosis* H37 RV was added to serially diluted compounds so that, the final concentration became 0.1µg, 0.01 µg and then plates were spread with the help of a spreader.

(B) On Gram-positive and gram negative bacteria

An acute illness is often due to bacterial infection. In bacterial disease, the primary aim should be to find out the causative organism whenever practicable, collection of suitable material, choice of the right media for culture, necessary biochemical tests, sometimes pathogenicity test. Even depending upon the duration of illness, or difficulty in cultivating the particular organism, some indirect evidences are sometimes sought for.

Gram stain[12-13] was first introduced by Gram in 1884 to differentiate bacteria by their staining reactions. In this, smears are strained with gention violet or methyl violet treated with Grams or Lugol's iodine solution and decolourised with a solvent like alcohol or acetone, washed and counter strained with saffranin, neutral red or Ziehl's fuschin stained by this method. Bacteria included into two main groups Gram positive and Gram negative. Bacteria retaining the violet colour and termed Gram positive and other which are decolourised by alcohol and take and gain the reddish counter stain only are called Gram negative.

In last two decades, stained it has been shown that mixed ligand complexes of the drug proved more potent than the pure drug[14]. It became evident that a clinical history is of immense help in the selection of right material and media for culture and in the choice of different serological or other laboratory test.

In the present study, screened for the activity of metal complexes of pyrazinamide on Gram positive and Gram negative bacteria viz Table 1 and 2.

Methodology

The isolation and identification of organism from an infected specimen necessitates certain stepwise procedures of which the most important are

1. Collection of material

Escherichia coli is incriminated as a pathogen outside the gut and particularly in the urinary tract. *E. coli* associated with two main clinical syndromes (a) Acute gastroenteritis, (b) Infection of the urinary tract.

Staphylococcus is the cause of wide range of different kinds of major and minor pyogenic (pus formation).

(2) Preparation of suitable medium

Nutrient agar media was used for cultivation of *Staphylococcus* bacteria from pus, sputum and throat swab. Mac-Conkey agar media was used for cultivation of *E. coli* bacteria for urine.

For the preparation of solid media, nutrient broth, or Mac-Conkey's broths 100 ml was taken and mixed with 25 gram of agar and heated in a steam sterilizer for 1 hour. When it was fully dissolved, poured into the sterile petridishes under aseptic conditions.

(3) Sterilization of Culture media

Media is allowed to set on the working table with the petri dish lid slightly lifted, so as to allow the steam to escote, then the lid was set to put in position and then plates were dried for an hour to remove the moisture.

(4) Culture of bacteria

The process of inseminating bacteria in culture media is called inoculation. It may be done from pathological material or from another culture. The former is known as "primary culture" and the latter as "subculture". A platinum loop or stainless steel loop is used for inoculating media. Hold the wire loop, the free end is bent in the form of a loop of 2-4 mm, internal diameter in the right hand and flame it, pick up the tube or bottle in left hand and remove the cap or plug with the crook third and fourth finger of the right hand. Remove the inoculum by scrapping growth from the surface of solid medium or taking up a loopful of liquid medium. Flame the mouth of the tube, replace the cap or plug and return the tube or bottle to its place. For seeding the plate, lift the bottom of petridish containing Mac-Conkey's medium from its lid with the left hand and hold it round the side with the thumb and middle finger. Put the petri dish in an incubator with the help of wire loop, peptone water and keep in an incubator for 4 hours at 37°C.

(5) Identification of bacteria

For identification of bacteria following methods were employed

(a) Morphological character (Microscopic study)
(b) Gram stain
(c) Motility
(d) Biochemical test
- Sugar test
- Indole test
- Coagulation test
- Catalous test

(6) Sensitivity test

The drugs isoniazid and pyrazinamide with its metal complexes were screened *in vivo* for sensitivity test. The methods used in this study was the disc diffusion test due to Bauer[15].

This test consisted of impregnating small disc of standard filter paper with given amount of chosen range of drugs. These are placed on plates of culture medium, previously spread uniformly with an inoculum of the bacterial isolate to be tested. After incubation, the degree of sensitivity is determined by measuring the easily visible areas of inhibition of growth produced by the diffusion of the drug from the disc into the surrounding medium. Punch disc of 6mm diameter was prepared using Whatman filter paper No. 1. This disc was taken in screw-capped bottle and sterilized by dry heat at 140°C for 60 minutes.

Table - 1 : Effect of mixed ligand complexes of PZA-INH on *Mycobacterium tuberculosis*

Temp.	37°	Concentration	0.01 µg
Time	21 days	MIC of PZA	0.10 µg
		MIC of INH	0.10 µg

S. No.	Compound	CFU Obtained
1.	Pure pyrazinamide (PZA) Control	30 colonies
2.	Pure isoniazid (INH) Control	40 colonies
3.	INH-PZA-Zn^{2+}	40 colonies
4.	INH-PZA-Cu^{2+}	25 colonies
5.	INH-PZA-Co^{2+}	20 colonies
6.	INH-PZA-Cd^{2+}	05 colonies
7.	INH-PZA-Mg^{2+}	19 colonies
8.	INH-PZA-Ca^{2+}	06 colonies
9.	INH-PZA-Mn^{2+}	04 colonies
10.	INH-PZA-Fe^{3+}	40 colonies
11.	INH-PZA-Al^{3+}	32 colonies

(PZA - Pyrazinamide, INH - Isoniazid, CFU - Colony formation Unit)

(7) Preparation of drug and mixed ligand complex solution

Mixed ligand complexes and isonex, pyrazinamide solution was prepared of 0.1mg for sensitivity test.

Table - 2 : Sensitivity test of mixed-ligand complexes of pyrazinamide and isoniazid

S. No.	Mixed-ligand complexes	Standard Strain Staphylococci	Strain isoalted from patients
1.	PZA-INH-Zn^{2+}	0 mm	0 mm
2.	PZA-INH-Cu^{2+}	0 mm	14 mm
3.	PZA-INH-Co^{2+}	0 mm	0 mm
4.	PZA-INH-Cd^{2+}	0 mm	10 mm
5.	PZA-INH-Mg^{2+}	0 mm	12 mm
6.	PZA-INH-Mn^{2+}	0 mm	8 mm
7.	PZA-INH-Fe^{3+}	0 mm	12 mm
8.	PZA-INH-Al^{3-}	0 mm	10 mm

RESULTS AND DISCUSSION

The bacteriocidal screening *in vitro* of the various mixed-ligand complexes of pyrazinamide and isoniazid against gram positive and gram negative bacteria shows the higher biocidal activity may also be due to the combined bioactive effect of metal and the ligand. The higher concentration of the ligand in chelate helps for the anti growth of bacteria species due to the exchange of trace metal of the metaloenzymes with metal ions of the chelate under test.

The antitubercular study revealed an effective increase in potency of isoniazid and pyrazinamide when chelated with Mn^{2+}, Cd^{2-}, Ca^{2+}. In these cases pronounced decrease in the number of colonies as compared to pure isoniazid and pyrazinamide. The values of log K is also supported for these activity. This is due to low dissociation constant (High stability constant) and shows a strong metal-ligand-ligand bond.

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