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Comparative *in vitro* Microbial Efficacy of a Fixed Dose Combination of Pantoprazole and Metronidazole with Pantoprazole and Metronidazole alone for *Helicobacter pylori*

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The present investigation was carried out to study the Minimum inhibitory Concentration (MIC) and Time kill curve of Pantoprazole (P), a proton pump inhibitor, a substituted benzimidazole: viz. 5-Difluoromethoxybenzimidazole-2-yl-3,4- dimethoxy-2-pyridylmethyl sulphoxide. It is used for inhibition of gastric acid secretion. Metronidazole (M) is a antibiotic with better broad spectrum activity against *H. pylori* infections, a gram negative organism. Pantoprazole and Metronidazole (PM) a fixed dose combination (FDC) has a wide range of susceptibility to any of these drugs individually. Chemical vector Mediated compatibility(CMVC) was obtained at the R&D center for PM and FDC of P and M in Combination of 40mg : 500mg ratio. The MIC was determined by broth micro dilution method as per guidelines of National Committee for Clinical Laboratory Standards (NCCLS). This study was aimed at evaluating microbial efficacy of PM in comparison with Pantoprazole and Metronidazole alone. Efficacy was evaluated on the basis of MIC and time kill curve analysis in *H. pylori* (HP-B_1, HP-B_2, HP-B_3 and HP-B_4) . In case of HP-B_1, HP-B_2, HP-B_3 and HP-B_4 MIC were found to be 0.5 mg/l, 0.5 mg/l, 0.25 mg/l, and 0.5 mg/l for PM respectively. In pantoprazole alone the MIC was found to be 32 mg/l, 32 mg/l, 32 mg/l and 64 mg/l respectively. For Metronidazole alone, these values were 2 mg/l, 2 mg/l, 1 mg/l, and 2 mg/l respectively. In all organisms under study, time-kill curve analysis demonstrated bacterial maximum killing at 4 hours. In conclusion, under in vitro analysis PM was found to have more bacterial inhibiting properties than pantoprazole and Metronidazole alone in vitro analysis.

Key words: Minimum inhibitory concentration, Time kill curve, PM Combination, pantoprazole and Metronidazole.

H. pylori is a predominantly extracellular , Gram negative, short, S- shaped, flagellated and motile bacterium Colonization by this Gram negative, microaerophilic bacterium is characterized by acute inflammatory reaction[1]. Nowadays , there is general consensus that *H. pylori* infection is the main etiological factor of primary gastritis in children[2] and adults[3]. Significant correlation between *H. pylori*-associated gastritis and peptic ulcer has been found, especially with duodenal ulcer[3,4]. Gastric cancer and lymphoproliferative

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gastric diseases also have been correlated with *H. pylori* infection[5].

Since that time, evidences have been monitoring that *H. pylori* has major role in causing these diseases. H. *Pylori* is a fragile bacteria that has found an ideal home in the protective mucous layer of the stomach[6]. This bacteria have long threads protruding from them that attaches to the underlined stomach cells. The mucous layer that protects the stomach cells from acid also protect *H. pylori*. The infection however is very real and it does cause the body to react. Infection fighting white blood cells move into the area and the body even develops *H. pylori* antibody in the blood[7].

H. Pylori infection probably occurs when an individual swallows the bacteria from food, fluid or perhaps from contaminated utensils. The infection is one of the most common worldwide. The rate of infection increases with , so it occur more often in old people. It also increases frequently in young people in the developing countries of the world. Since the infection tends to be more common where sanitation is poor or, living quarters are cramped. In many cases it do not produces symptoms, in other words the infection can occur without the person knowing it. The infection remains localized to the gastric area and probably is the reason for ulcers[7,8]. A peptic ulcer is a sore on the lining of the stomach or, duodenum which is a part of the small intestine the measure of peptic ulcer are caused by the H. pylori Bacterium[7,8,9].

Pantoprazole sodium is a proton pump inhibitor (PPI), a substituted benzimidazole: viz. 5-Difluoromethoxybenzimidazole-2-yl-3,4-dimethoxy-2-pyridylmethyl sulphoxide. It is used for inhibition of gastric acid secretion. It is indicated only when oral therapy cannot be given and one should shift to oral therapy as soon as practically possible. Although given as a sodium salt, doses are calculated on free-base content. Pantoprazole is extensively metabolized, mainly via hepatic cytochrome P450 (CYP) 2C19 isoenzyme [10,11].

Pantoprazole is a substituted benzimidazole which accumulates in acidic environment of patient cells after absorption, which binds to the H+/K+ AT pase, thus inhibiting the proton pump and causing potent long lasting suppression of basal and stimulatal gastric acid secretion (autylcho- line, histamine, gastrine)[10,11]. Pantoprazole becomes active in higher acidic condition and get inactive in higher pH i.e in alkaline environment.

On achieving heamostasis by treating patients with active bleeding ulcers or ulcers with major signs of recent bleeding with distilled water injection[12]. They were randomized to receive intravenous Pantoprazole. They reported that Pantoprazole was superior to ranitidine as an adjunct treatment to endoscopic injection therapy in high-risk bleeding ulcers[12,13]. pantoprazole (PPIs) to be superior to the H2 receptor antagonists[13].

Metronidazole is selectively toxic to anaerobic microorganisms *H. pylori*. After entering the cell by diffusion its nitro group is reduced by certain redox proteins operative only in anaerobic microbes to highly reactive nitro radical which exerts cytotoxicity by damaging DNA halix [14]. Metronidazole is an better broad spectrum antibiotic usual to treat the *H. pylori* infection particular gram negative infection[14-15]. Treatment usually involves a combination of antibiotic and acid suppressors proton pump inhibitor which provides protection against *H. pylori* infection[15-16].

Treatment with a combination of an antibiotic Metronidazole plus PPI pantoprazole has shown increased efficacy. Such combination therapy ensured in increased bacterial activity and or rate of killing in vitro and prevention of the emergency of drugs resistance[17].

The present study was aimed at comparing the microbial efficacy of a fixed dose combination of Pantoprazole and Metronidazole with Pantoprazole and Metronidazole alone in *H. pylori* (HP-B_1, HP-B_2, HP-B_3, and HP-B_4) strain.

MATERIAL AND METHODS

Bacterial Strains

Three biopsy specimens of *Helicobacter pylori* group (HP-B_1, HP-B_2, HP-B_3) are used which are isolated from gastric biopsy of the specimen of patient with gastric and peptic ulcer from Post Graduate Institute Chandigarh and one *Helicobacter pylori* ATCC 43504 (HP-B_4) is collected from American Type Culture Collection

through LGC Promochem India Private Limited Bangalore (india) for these experiments.

Antibiotics

Helicobacter pylori group (HP-B_1, HP-B_2, HP-B_3, and HP-B_4) were tested for response of Metronidazole, Pantoprazole and a fixed dose combination. The best combination found i*n vitro* was selected and this study was used in developing best Fixed Dose Combination (FDC) of Pantoprazole and Metronidazole using Chemical Vector Mediated Compatibility (CVMC)[8], Metronidazole and Pantoprazole used in this study were provided by manufacturer (Venus Remedies Limited, India) for study.

Medium

All the chemicals used in present study were purchased from Merck, Sigma, and Himedia etc. The culture medium Mueller-Hinton broth (MHB) supplemented with 5% defibrinated sheep blood was purchased from Himedia and used according to manufacturer's instructions for MIC and Time Kill Curve experiments. Colony counts and Susceptibility determined with Mueller-Hinton Agar (MHA) supplemented with 5% defibrinated sheep blood were purchased from Himedia and used according to manufacturer's instructions.

Preparation of inoculums

Preparation of McFarland standard:

Added 0.5 ml of 0.048M BaCl2 (1.17%w/v BaCl2. 2H2O) to 99.5ml of 0.18M H2SO4 (1%v/v) with constant stirring. The McFarland standard solution of 5ml by volume was taken in screw cap test tube for comparing the inoculum suspension.

For the preparation of inoculum, the colonies were taken directly from the overnight culture plate in 0.9% NaCl. The suspension matched the density 0.5 McFarland standard. To check the purity, the suspension was determined by photometric standardization at 625nm which showed the absorbance in between 0.121±0.004 wavelength. Antibiotic was received in powder form and dissolved in MH broth.

Susceptibility Testing

The MIC of PM, a fixed dose combination, Pantoprazole and Metronidazole for the *H. pylori* groups (HP-B_1, HP-B_2, HP-B_3, and HP-B_4) were determined in cation-supplemented MH broth supplemented with 5% defibrinated sheep blood by the microdilution technique [18,19]. Overnight MH broth supplemented with 5% defibrinated sheep blood cultures were used to prepare inocula of 10^5 CFU/ml. The MIC was defined as the lowest concentration (0.03125mg/l) and highest dilution (256 mg/l) of antimicrobial agent that prevented turbidity after 24 hrs under microaerobic conditions (10% CO2, 10% O2, 80% N2) in Mueller– Hinton broth, supplemented with 5% defibrinated sheep blood of incubation at 37°C.

Time kill Curve

For each *H. pylori* groups (HP-B_1, HP-B_2, HP-B_3, and HP-B_4), time-kill curve studies were performed in MH broth in glass flasks with an inoculum of 5×10^6 to 1×10^7 CFU/ml in the presence of a single Pantoprazole or Metronidazole and a fixed dose combination[20]. A flask of inoculated MH broth supplemented with 5% defibrinated sheep blood with no antibiotic served as a control. The surviving bacteria were counted after 0, 2, 4, 6, 8, 10, and 12hrs under microaerobic conditions (10% CO_2, 10% O_2, 80% N_2) of incubation at 37°C, by subculturing 50-µl serial dilutions (in 0.9% sodium chloride) onto MH Agar plates supplemented with 5% defibrinated sheep blood with a spiral plater.

RESULTS

In the present study we evaluated *in vitro* antimicrobial activity of Pantoprazole, Metronidazole and their combination in some clinical microorganisms. The results showed that, the combination of both drugs increased the antimicrobial activity as compared to alone in *H. pylori* groups (HP-B_1, HP-B_2, HP-B_3, and HP-B_4).

Susceptibility studies: MIC of *H. pylori* groups (HP-B_1, HP-B_2, HP-B_3, and HP-B_4) under study resulted in significant reduction in PM, a fixed dose combination when compared with Pantoprazole, and Metronidazole alone (Table 1).

In case of three gastric biopsy specimen of *Helicobacter pylori* groups (HP-B_1, HP-B_2, HP-B_3) and one *Helicobacter pylori* ATCC 43504 (HP-B_4), they were found to be 0.5 mg/l, 0.5 mg/l, 0.25 mg/l, and 0.5 mg/l for PM respectively. In Pantoprazole alone the MIC were found to be 32 mg/l, 32 mg/l, 32 mg/l, and 64 mg/l respectively. For Metronidazole alone, these values were 2 mg/l, 2 mg/l, 1 mg/l, and 2 mg/l respectively.

Table 1. MIC of PM Combinations in ratio 1:12.5 in comparison with Pantoprazole and Metronidazole in *H. pylori*

Helicobacter pylori (In code form)	Mean Values of MIC (mg/l) Pantoprazole (P)	Metronidazole (C)	PM Combinations in ratio 1:12.5
HP-B_1	32	2	0.5
HP-B_2	32	2	0.5
HP-B_3	34	1	0.25
HP-B_4	64	2	0.5

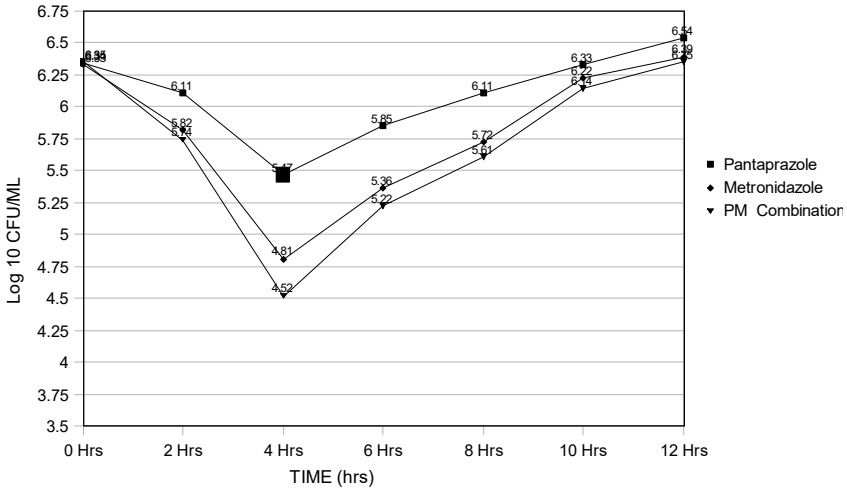

Fig. 1. Time kill curve of PM Combinations in ratio 1:12.5 in comparison with Pantoprazole and Metronidazole in HP-B_1

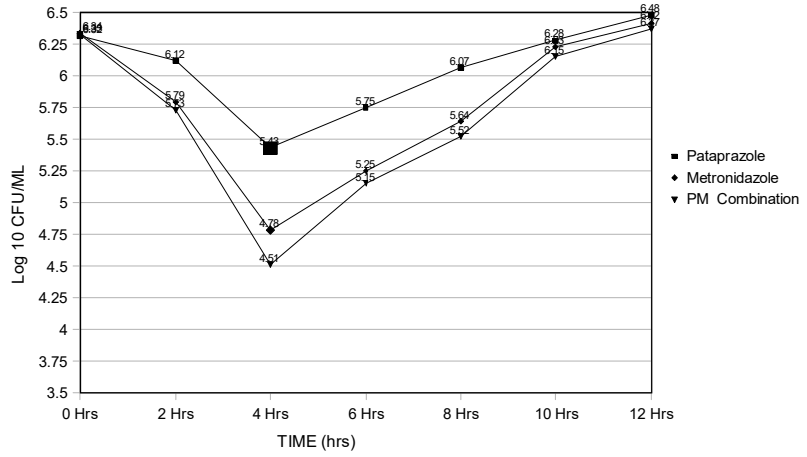

Fig. 2. Time kill curve of PM Combinations in ratio 1:12.5 in comparison with Pantoprazole and Metronidazole in HP-B_2 .

Time-kill curve analysis: Bactericidal effect, with 2×the MIC of PM, Pantoprazole and Metronidazole achieved the earliest killing at 4 hours. Bacterial killing rate in PM was distinctly higher than Pantoprazole and Metronidazole alone in all the *H. pylori* group under study.

In *H. pylori* (HP-B_1), time-kill curve analysis demonstrated bacterial killing from 6.35 to 4.52 $\log_{10}$ CFU /ml by 4 hours for PM, killing from 6.34 to 5.47 $\log_{10}$ CFU /ml and killing from 6.33 to 4.81 $\log_{10}$ CFU /ml for Pantoprazole and Metronidazole (Fig-1). Pantoprazole has killing of 5.43 $\log_{10}$ CFU /ml, 5.45 $\log_{10}$ CFU /ml, and 5.49 $\log_{10}$ CFU /ml after 4 hours in HP-B_2, HP-B_3, and HP-B_4 respectively. The Metronidazole has killing of 4.78 $\log_{10}$ CFU /ml, 4.72 $\log_{10}$ CFU /ml, and 4.83 $\log_{10}$ CFU /ml after 4 hours in HP-B_2, HP-B_3, and HP-B_4 respectively. When PM was tested with these organisms *H. pylori* killing of 4.51 $\log_{10}$ CFU /ml, 4.46 $\log_{10}$ CFU /ml, and 4.54 $\log_{10}$ CFU /ml after 4 hours was reported (Fig-2, 3, 4) .

DISCUSSION

Pantoprazole sodium is a substituted benzimidazole

viz. 5-Difluoromethoxybenzimidazole-2-yl-3,4- dimethoxy-2-pyridylmethyl sulphoxide that is potent inhibitors of gastric acid secretion which exert their effects by inactivation of (H+/K+) ATPase in the parietal cell canaliculus after proton-dependent activation of the producing compounds[11]. The pantoprazole also has low therapeutic potential and bacteriocidal activity at neutral pH using short incubation periods against *H. pylori*[21].

Metronidazole is one of the most successful drugs used in combination to eradicate *H. pylori*. However, distribution of its MIC against *H. pylori* in vitro has not been regularly show[22]. It is generally accepted that metronidazole MICs of more than 2mg/l are resistant for *H. pylori* strains[23]. These Antibiotics is currently accepted as the most effective drug in *H. pylori* infection, with a 80.2 % eradication rate[24]. Moreover, the eradication rate increased with the use of antibiotics metronidazole and combinations including PPIs pantoprazole.

The *in vitro* study, low eradication rates, PPI- based effective drug of metronidazole in two regimen (Pantoprazole plus Metronidazole) have been reported in *H. pylori* infection[25]. Metronidazole is a compound that have potent bacterial activities against gram negative organism *H. pylori*, particularly clinically relevant pathogen[26]. The susceptibility data from our study demonstrated that PM, a fixed dose combination has higher MIC value than Metronidazole and Pantoprazole, suggesting higher bactericidal activity in PM in isolated gastric biopsy and ATCC 43504 *H. pylori* groups (HP-B_1, HP-B_2, HP-B_3, and HP-B_4). This was confirmed by time-kill analysis, which demonstrated that PM has better bactericidal activity than Metronidazole and Pantoprazole, even at a concentration of 2x the MIC after 12 hours. Indeed, in all organisms under study, PM, demonstrated similar pattern of bactericidal activities when compared with alone.

In summary, the results MIC and time kill studies are concordant for the three gastric biopsy (HP-B_1, HP-B_2, HP-B_3) and one ATCC 43504 (HP-B_4) of *H. pylori* strains. Pantoprazole plus Metronidazole has shown better bactericidal effect than Pantoprazole and Metronidazole alone in organisms under study.

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REFERENCES

1. Williams, C.L. *Hlicobacter pylori*: bacteriology and Laboratory Diagnosis. *J Infec.,*1997; **34**: 1-5.
2. Blecker, U. *H. pylori* disease in childhood. *Eur J pediat*., 1996; **155**: 733-735.
3. Blaser, M. J., *H. pylori*; Its role in disease. *Clin Infe Dis*., 1992; **15**: 386-393.
4. Rautelin, H., Kosunen, T.U. *H .pylori* and associated gastroduodenal diseases. *APMIC*., 1991; **99**: 677-695.
5. Wotherspoon, A.C., Ortiz - Hidalgo, C., Falzon, M.R. *H .pylori* associated gastritis and primary B – cell gastric lymphoma. *Lancet*., 1991; 338:1175-1176.
6. Josenhan, S., Labigne, C. A., Suerbaum, S. Comparative ultrastructural and functional studies of *Helicobacter pylori* and *Helicobacter mustelae* flagellin mutants: both flagellin

subunits, FlaA and FlaB, are necessary for full motility in *Helicobacter* species. *J.Bacteriol.,* 1995; **177**: 3010-3020.

7. Lam, S.K., Ching, C.K. Dose treatment of *H.pylori* with antibiotics alone heal duodenal ulcer? A randomised double blind placebo controlled study. *Gut*.; 1997; **41**:43-8.
8. Adamek, R.J., Szymanski, C., Pfaffenbach, B., Opferkuch, W., Ricken, D., Wegener, M. Tripel-Therapie mit Pantaprozole, Clarithtomycin and Metronidazol zur Helung der *H.pylori* infection. *DMW*., 1995; **120**: 358-60.
9. Greig, A., Hanslo, D., Lastivica, A., Louw, J. A. In vitro antibiotic sensitivity of *H. pylori. S Afr Med J*., 1993; **83**:784.
10. Anderson, E.T., Young, L.S., Hewitt, W.L. Antimicrobial synergism in the therapy of gram-negative rod bacteremia. *Chemotherapy*., 1978; **24**: 45-54.
11. Klastersky, J., Zinner, S.H. synergistic Combinations of antibiotics in gram-negative bacillary infections. *Diseases.*, 1982; **4:** 294-301.
12. Hsu, P.I., Lo, G.H., Lo, C.C., Lin, C.K., Chan, H.H., Wu, C.J., Shie, C.B., Tsai, P.M., Wu, D.C., Wang, W.M., Lai, K.H. Intravenous Pantoprazole versus ranitidine for prevention of rebleeding after endoscopic hemostasis of bleeding peptic ulcers. *World J Gastroenterol.,* 2004; **10**(24): 3666-9.
13. Huggins, R.M., Scates, A.C., Latour, J.K. Intravenous proton-pump inhibitors versus H2-antagonists for treatment of GI bleeding, *Ann Pharmacother*., 2003; **37**(3):433-7.
14. Tripathi, K.D. Antiamoebic and other antiprotozoal drugs. *Medical Pharmacology* 5th edn. India: 2003; chapter. 58; pp 750-52.
15. Pavicic, M.J.A.M., Namavar, F., Verboom, T., Winkelhoff, A.J. Van., Graaff, J.D. In vitro susceptibility of Helicobacter pylori to several antimicrobial combinations. *Antimicrob. Agents Chemother.,* 1993; **37**:1184-1186.
16. Rautelin, H., Seppala, K., Renkonen, O.V., Vainio, U., Kosunen. T.U. Role of metronidazole resistance in therapy of Helicobacter pylori infections. *Antimicrob. Agents Chemother.,* 1992; **36**: 163-166.
17. Menbonca, S., Ecclissato, C., Sartori, M.S., Goboy, A.P.O., Guerzoni, R.A., Degger, M., Pedrazzoli, J. Prevalence of Helicobacter pylori resistance to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone in Brazil. *Helicobacter.,* 2000; **5**: 79-83.
18. Amsterdam, D. Susceptibility testing of antibiotics in liquid media, In V. Lorian (ed.), Antibiotics in laboratory medicine,The Williams & Wilkins Co., Baltimore, Md. 4th ed., 1996; pp. 52-111.
19. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., 1997; Approved standard M7-A4. *National Committee for Clinical Laboratory Standards*.
20 Pearson, R.D., Steigbigel, R.T., Davis, H.T., Chapmann, S.W., Method for reliable determination of minimal lethal antibiotic concentrations. *Antimicrob. Agents Chemother*., 1998; **18**: 699-708.
21. Diez Enciso, M. servicio de Microbiologia, Hospital "La paz", Madrid, Spain. *paseo de la Castellana.,*1999; **261**: 28046
22. Huaxiang, X., Conor, T.K., Beattle, S., Colm, A. O. morain. Standardization of disk diffusion test and its clinical significance for susceptibility testing of metronidazole against *Helicobacter pylori. Antimicrobial Agents and Chemotherapy.,* 1994; pp. 2357-2361.
23. Glupczynski, Y., Burrette, A., Koster,E.D., Nyst,J.F., Deltener, M., Cadranel, S., Bourdeaux,L., Vos,D.Van. Metronidazole resistance in *Helicobacter pylori*. *Lancet.,* 1990; 335:976-977.
24. Xia, H. X., Daw,M.A., Sant, S., Beattia, S., Keane, C.T., Morain, C.A.O. Clinical efficacy of triple therapy in Helicobacter pylori-associated duodenal ulcer. *Eur. J. Gastrointestinal.,* 1993; Hepatol. 5:141-144.
25. Goodwin, C. S., Blake, P., Blincow. E. The minimum inhibitory and bactericibal concentrations of antibiotics and antiulcer agents against Campylobacter pyloridis. *J. Antimicrob. Chemother.,* 1986; 17:309-314.
26. Graham, D.Y. Metronidazole for treatment of *H. pylori* infection. *Eur. J. Gastroenterol Hepatol*., 1995; 7(suppl 1): 55-8.