Comparative in vitro Microbial Efficacy of a Fixed Dose Combination of Pantoprazole and Metronidazole with Pantoprazole 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-pyridymethyl sulphonide. It is used for inhibition of gastric acid secretion. Metronidazole (M) is a antibiotic with better broad spectrum activity against Helicobacter 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 Helicobacter pylori (HP-B1, HP-B2, HP-B3 and HP-B4). In case of HP-B1, HP-B2, HP-B3 and HP-B4, 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. Nowadays, there is general consensus that H. pylori infection is the main etiological factor of primary gastritis in children and adults. Significant correlation between H. pylori-associated gastritis and peptic ulcer has been found, especially with duodenal ulcer. Gastric cancer and lymphoproliferative
gastric diseases also have been correlated with \textit{H. pylori} infection\textsuperscript{v}.

Since that time, evidences have been monitoring that \textit{H. pylori} has major role in causing these diseases. \textit{H. Pylori} is a fragile bacteria that has found an ideal home in the protective mucous layer of the stomach\textsuperscript{v}. 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 \textit{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 \textit{H. pylori} antibody in the blood\textsuperscript{v}.

\textit{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 \textit{H. Pylori} is a fragile bacteria that has found an ideal home in the protective mucous layer of the stomach\textsuperscript{v}. 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 \textit{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 \textit{H. pylori} antibody in the blood\textsuperscript{v}.

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\textit{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\textsuperscript{v}. 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\textsuperscript{v}.\textsuperscript{v}\textsuperscript{v}.

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 \textsuperscript{v}\textsuperscript{v}.\textsuperscript{v}\textsuperscript{v}.

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

On achieving haemostasis by treating patients with active bleeding ulcers or ulcers with major signs of recent bleeding with distilled water injection\textsuperscript{v}\textsuperscript{v}. 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\textsuperscript{v}\textsuperscript{v}\textsuperscript{v}. pantoprazole (PPIs) to be superior to the H2 receptor antagonists\textsuperscript{v}\textsuperscript{v}.\textsuperscript{v}\textsuperscript{v}.

Metronidazole is selectively toxic to anaerobic microorganisms \textit{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 \textit{H. pylori} infection particular gram negative infection\textsuperscript{v}\textsuperscript{v}\textsuperscript{v}.\textsuperscript{v}. Treatment usually involves a combination of antibiotic and acid suppressors proton pump inhibitor which provides protection against \textit{H. pylori} infection\textsuperscript{v}\textsuperscript{v}\textsuperscript{v}.\textsuperscript{v}.\textsuperscript{v}\textsuperscript{v}.

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

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 \textit{H. pylori} ( HP-B\textsubscript{1}, HP-B\textsubscript{2}, HP-B\textsubscript{3}, and HP-B\textsubscript{4} ) strain.

**MATERIAL AND METHODS**

**Bacterial Strains**

Three biopsy specimens of \textit{Helicobacter pylori} group (HP-B\textsubscript{1}, HP-B\textsubscript{2}, HP-B\textsubscript{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 \textit{Helicobacter pylori} ATCC 43504 (HP-B\textsubscript{4} ) is collected from American Type Culture Collection
through LGC Promochem India Private Limited Bangalore (India) for these experiments.

**Antibiotics**

*Helicobacter pylori* group (HP-B₁, HP-B₂, HP-B₃, and HP-B₄) were tested for response of Metronidazole, Pantoprazole and a fixed dose combination. The best combination found *in 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). 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 0.18MH₂SO₄ (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-B₁, HP-B₂, HP-B₃, and HP-B₄) 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 10⁵ CFU/ml. The MIC was defined as the lowest concentration (0.03125mg/l) and highest dilution (256mg/l) of antimicrobial agent that prevented turbidity after 24hrs 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-B₁, HP-B₂, HP-B₃, and HP-B₄), time-kill curve studies were performed in MH broth in glass flasks with an inoculum of 5 × 10⁶ to 1 × 10⁷ CFU/ml in the presence of a single Pantoprazole or Metronidazole and a fixed dose combination. 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% CO₂, 10% O₂, 80% N₂) 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-B₁, HP-B₂, HP-B₃, and HP-B₄).

Susceptibility studies: MIC of *H. pylori* groups (HP-B₁, HP-B₂, HP-B₃, and HP-B₄) 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-B₁, HP-B₂, HP-B₃, and HP-B₄) and one *Helicobacter pylori* ATCC 43504 (HP-B₅), 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*

<table>
<thead>
<tr>
<th>Helicobacter pylori (In code form)</th>
<th>Mean Values of MIC (mg/l)</th>
<th>PM Combinations in ratio 1:12.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>HP-B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>HP-B&lt;sub&gt;3&lt;/sub&gt;</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>HP-B&lt;sub&gt;4&lt;/sub&gt;</td>
<td>64</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 1. Time kill curve of PM Combinations in ratio 1:12.5 in comparison with Pantoprazole and Metronidazole in HP-B<sub>1</sub>.

Fig. 2. Time kill curve of PM Combinations in ratio 1:12.5 in comparison with Pantoprazole and Metronidazole in HP-B<sub>4</sub>.
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-B1), 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-B1, HP-B2, and HP-B3, 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-B1, HP-B2, and HP-B3, 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-pyrindimethyl 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 compounds11. The pantoprazole also has low therapeutic potential and bacteriocidal activity at neutral pH using short incubation periods against H. pylori11.

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 show22. It is generally accepted that metronidazole MICs of more than 2mg/l are resistant for H. pylori strains23. These Antibiotics is currently accepted as the most effective drug in H. pylori infection, with a 80.2 % eradication rate25. 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 infection25. Metronidazole is a compound that have potent bacterial activities against gram negative organism H. pylori, particularly clinically relevant pathogen29. 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-B1, HP-B2, HP-B3, and HP-B4). 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-B1, HP-B2, HP-B3) and one ATCC 43504 (HP-B4) 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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