EFFECT OF PIPER NIGRUM ON IN-VITRO RELEASE OF RIFAMPICIN MICROSPHERES

PINGALE PRASHANT L.*, RAVINDRA R.P.
SVKM’s NMIMS, School of Pharmacy & Technology Management, Shirpur-425 405 Dist: Dhule Maharashtra, INDIA.
Email: prashant.pingale@gmail.com
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INTRODUCTION

Tuberculosis therapy remains a challenge even today, mainly due to the problems of bacterial resistance, side effects and low patient compliance. A bioenhancer is an agent capable of enhancing bioavailability and bioefficacy of a particular drug with which it is combined, without any typical pharmacological activity of its own at the dose used. Microspheres are one of the multiparticulate drug delivery systems prepared to obtain prolonged drug delivery, to improve its bioavailability or stability and to target drug to specific sites. Rifampicin is an important component of fixed dose combination used in first line therapy of tuberculosis. In the present study, we attempted an innovative approach to overcome the above-mentioned problems of tuberculosis therapy by combining both these approaches of microspheres and bioenhancers. Small amount of herbal extract of Piper nigrum were incorporated as a bioenhancer in variable amount for each dose of rifampicin. The drug loading efficiency and in-vitro release behavior of loaded microspheres were found to be satisfactory. Prolonged release of the drug from the microspheres was demonstrated in a simulated intestinal fluid. In-vitro release of rifampicin from the microspheres containing variable fractions of bioenhancer showed significant increase in release profile i.e. 80.24 and 86.16% in formulation containing bioenhancer against 44.54% for the formulation without bioenhancer prepared by complex coacervation method.

Keywords: Tuberculosis, Microspheres, Drug release, Bioenhancer, Rifampicin, Piper nigrum

Tuberculosis is an infectious disease caused by the Mycobacterium tuberculosis. The disease was called "consumption" in the past because of the way it would consume from within anyone who became infected. TB is a major cause of illness and death worldwide, especially in Africa and Asia. Each year the disease kills almost 2 million people. The disease is also prevalent among people with HIV/AIDS. Currently available treatment regimens are prolonged, placing unmanageable demands on indigent populations from the perspectives of both supervision and adherence. The result is the burgeoning tide of drug resistance. Repeated inadequate courses of therapy in patients with relapsing TB generate incremental increase in the degree of drug-resistance[1].

In 2011, there were 8.7 million new cases of active tuberculosis worldwide (13% of which involved co-infection with the human immunodeficiency virus [HIV]) and 1.4 million deaths, including 430,000 deaths among HIV-infected patients1 representing a slight decrease from peak numbers in the mid-2000s. It has been estimated that there were 310,000 incident cases of multidrug-resistant tuberculosis, caused by organisms resistant to at least isoniazid and rifampin, among patients who were reported to have tuberculosis in 2011. More than 60% of these patients were in China, India, the Russian Federation, Pakistan, and South Africa[2].

Rifampicin works by killing the bacteria that are causing the infection. It does this by targeting and inactivating a bacterial enzyme called RNA-polymerase. The bacteria use RNA-polymerase to make essential proteins and to copy their own genetic information (DNA). Without this enzyme the bacteria cannot reproduce and they die [3,4,5].

Rifampicin inhibits DNA-dependent RNA polymerase activity in susceptible Mycobacterium tuberculosis organisms. Specifically, it interacts with bacterial RNA polymerase, but does not inhibit the mammalian enzyme[4].

C.K. Atal, the Director of the institute scrutinized a list of ancient Indian Ayurvedic formulations used in the treatment of a wide range of diseases. He observed that a majority of Ayurvedic formulations contained either Trikatu or else one of the ingredients of Trikatu, namely Piper longum (P. longum) (210 formulations out of 370 reviewed) which is used in a large variety of diseases. He formed the working hypothesis that Trikatu increased the efficacy of formulations. Trikatu has three ingredients: black pepper (Piper nigrum), long pepper (P. longum) and ginger (Zingiber officinale). Based on this hypothesis, these ingredients were studied, which found that one of the ingredients, ‘P. longum,’ ‘Piper’ increased the bioavailability of many drugs[6].

Piperine, the active principal present in P. nigrum or P. longum was isolated and its bioavailability enhancing action was established. Further research on several classes of drugs including anti-tuberculare, anti-leprosy, antibiotics, non-steroidal anti-inflammatory drugs, CVS and CNS drugs showed similar results. Piperine was found to increase bioavailability of different drugs ranging from 30% to 200%. Subsequent research has shown that it increases curcumin bioavailability by almost ten-fold with the use of piperine[7,8]. The release of isoniazid was increased almost double (from 45 to 90%) in microspheres if piperine were used as bioenhancer in the preparation[9].

The drug delivery system offers several potential advantages: drug release rates can be modified to the needs of a specific application; it provides a constant rate of delivery or pulsatile release. It provides protection of drugs, especially proteins, which are otherwise rapidly destroyed by the body. The controlled release systems of microspheres can increase patient comfort and compliance by replacing frequent (e.g., daily) doses with infrequent (once per month or less) injection.

Hence the effective formulation strategy for the optimization of the pharmacokinetic characteristics of anti-tubercular agent would be to use bioenhancers in combination therapy to improve the release performance and ultimately maximize their effectiveness along with the microspheres as a drug delivery system.

In this study, we have attempted a collective approach consisting of microspheres and bioenhancer in order to overcome the problems associated with the anti-TB drug therapy.
The objectives of this study were to
- Develop sustained release microspheres of rifampicin using various methods;
- Study the effect of various concentrations of bioenhancer (extract of *Piper nigrum*) on *in-vitro* drug release from microspheres; and
- Evaluate the effect of various processing variables on characteristics of microspheres.

**MATERIALS AND METHODS**

Rifampicin was obtained as a gift sample from Lupin Pharmaceuticals Ltd., Aurangabad, Maharashtra. *Piper nigrum* (Black pepper) was purchased from local market and authenticated from Dept. of Botany, Dr.P.R.Ghogrey College of Science, Dhule, Maharashtra. All other chemicals/polymers were of analytical grade.

**Extraction and isolation of *Piper nigrum* used as bioenhancers[10,11,12]:**

Macroscopy of *Black pepper*: The entire fruit was almost globular in shape, with 4-6.5 mm of diameter, brownish to black in color. The surface was found to be uneven. The seeds were almost brown or black in color, aromatic with a pungent taste (Figure 1).

**Figure 1: *Piper nigrum* seeds and powder**

*Phytochemical evaluation of *Black pepper*[12]:* The phytochemical evaluation of fully mature, dried fruits of *Piper nigrum* Linn, family *Piperaceae* was carried out as per the provisions of *Ayurvedic Pharmacopoeia* for various parameters, the results of which are mentioned in Table 1.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameter</th>
<th>Standard (%) w/w</th>
<th>Observed value (%) w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total ash</td>
<td>Not more than 5.5</td>
<td>4.10</td>
</tr>
<tr>
<td>2</td>
<td>Water soluble ash</td>
<td>Not more than 4.5</td>
<td>3.76</td>
</tr>
<tr>
<td>3</td>
<td>Acid insoluble ash</td>
<td>Not more than 1.0</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>Loss on drying</td>
<td>Not more than 4</td>
<td>3.05</td>
</tr>
<tr>
<td>5</td>
<td>Water soluble extract</td>
<td>Not less than 15</td>
<td>23.11</td>
</tr>
<tr>
<td>6</td>
<td>Ethanol soluble extract</td>
<td>Not less than 8</td>
<td>10.50</td>
</tr>
</tbody>
</table>

**Evaluation of *piperine* (as an active constituent) from *Black pepper* seeds:** It was determined by liquid chromatography as given in *Indian Pharmacopoeia*-2010. The amount of piperine was found to be 2.89 %w/w (IP-2010 limit not less than 2.50 %w/w).

**Isolation of *piperine* from *Black pepper*:** Black pepper (100 g) was ground to a fine powder and extracted with 95% ethanol (500 ml) in a Soxhlet extractor for 2 hours. The solution was filtered and concentrated in vacuum on a water bath at 60°C. 50 ml of 10% alcoholic KOH solution was added to it and after a while, the clear liquid decanted from the insoluble residue. The alcohol solution was left overnight. Yellow needle shaped crystals of piperine were obtained. The melting point of the crystals was found to be 124-126°C.

**Evaluation of *piperine* hydro-alcoholic extract:** The bioenhancers extract were evaluated against standards laid by IP-2010, and *Ayurvedic Pharmacopoeia* of India-2007 [12,13] as given in Table 2.

**Table 2: Evaluation of Herbal Extract from *Black pepper***

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Test</th>
<th>Standard value</th>
<th>Observed value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LOD (% w/w)</td>
<td>&lt; 5.0</td>
<td>2.10</td>
</tr>
<tr>
<td>2</td>
<td>Total Ash (% w/w)</td>
<td>&lt; 20.0</td>
<td>7.61</td>
</tr>
<tr>
<td>3</td>
<td>Acid Insoluble Ash (% w/w)</td>
<td>&lt; 3.0</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>Residual solvent analysis</td>
<td>&lt; 3000 ppm</td>
<td>&lt; 2291 ppm</td>
</tr>
</tbody>
</table>

**PREPARATION OF RIFAMPICIN MICROSPHERES:** Microspheres may be produced by several methods such as emulsion crosslinking method, multiple emulsion method, coacervation method, solvent evaporation method, spray-drying method etc. In this study, complex coacervation method was used to prepare the microspheres[14,15,16,17,18,19].

The mixture of chitosan/gelatin B used as coating material. Chitosan and gelatin were dissolved in dilute acetic acid solution (1% v/v) together at concentrations of 1-4% w/v and adjusted to a certain solution pH 5.0. Rifampicin (150 mg) was dissolved in the above polymeric solution. The drug in polymeric solution was emulsified in 200 ml of liquid paraffin (1:1 mixture of light and heavy liquid paraffin) containing 1 ml Tween 80 (2% w/v). The emulsification time was allowed for 10 min under mechanical stirring (Remi Motors, India) at a variable rpm (1400-1600 rpm). Then 50 ml Na-TTP (2% w/v) with pH in the range 4-5 was added drop wise. Stirring was continued for 30 min. (cross linking time optimized 15-45 min.) to obtain cross-linked microspheres. Microspheres were collected by centrifugation and washed with double distilled water several times, then with acetone to remove water and dried at room temperature under vacuum. The prepared microspheres were stored in desiccator for further studies. The drug loaded microspheres with different polymer compositions (1:1; 1:2; 1:3 and 1:4) were prepared and studied for various parameters as shown in Table 3.

**Optimization parameters:** Polymer-copolymer ratio and concentration, drug-polymer ratio, concentration of cross-linking agent and time required for crosslinking were considered in the optimization of the formulation[20].

**Table 3: Composition of RIF Microspheres by Complex Coacervation Method**

<table>
<thead>
<tr>
<th>Batch code</th>
<th>RIF (mg)</th>
<th>Chitosan: Gelatin B</th>
<th>Polymer Concentration (% w/v)</th>
<th>Na-TTP (% w/v)</th>
<th>RPM (±100)</th>
<th>Cross-linking time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form.1</td>
<td>150</td>
<td>1</td>
<td>1:1</td>
<td>1</td>
<td>1400</td>
<td>15</td>
</tr>
<tr>
<td>Form.2</td>
<td>150</td>
<td>2</td>
<td>1:2</td>
<td>2</td>
<td>1500</td>
<td>30</td>
</tr>
<tr>
<td>Form.3</td>
<td>150</td>
<td>3</td>
<td>2:1</td>
<td>1</td>
<td>1600</td>
<td>45</td>
</tr>
<tr>
<td>Form.4</td>
<td>150</td>
<td>4</td>
<td>1:1</td>
<td>1</td>
<td>1400</td>
<td>30</td>
</tr>
<tr>
<td>Form.5</td>
<td>150</td>
<td>4</td>
<td>1:2</td>
<td>2</td>
<td>1500</td>
<td>30</td>
</tr>
<tr>
<td>Form.6</td>
<td>150</td>
<td>4</td>
<td>2:1</td>
<td>1</td>
<td>1600</td>
<td>45</td>
</tr>
</tbody>
</table>

**Addition of bioenhancer extract in optimized batch of rifampicin microsphere:** To the optimized batch of rifampicin microsphere prepared by complex coacervation method, the extract of black pepper was added. The detailed formulae are shown in Table 4.
Evaluation of rifampicin microspheres:

Fourier Transform Infra-Red (FT-IR) analysis: Drug, physical mixture of drug, polymer and herbal extract and formulation were analyzed using Fourier Transform Infra-Red (FT-IR) spectroscopy. The purpose of the study is to check the compatibility of polymers and other excipients with the drug. 1 to 2 mg of drug, physical mixture (drug + polymer + herbal extract) and drug microsphere samples were weighed and mixed perfectly with potassium bromide (0.3-0.4 g) to a uniform mixture. A small quantity of the powder was compressed into a thin semi-transparent pellet by applying pressure.

Differential Scanning Calorimeter (DSC) study: DSC analyses were carried out with a Mettler TA4000 Star® software apparatus (Mettler Toledo, Switzerland) equipped with a DSC 25 cell. Samples of about 5-10 mg were weighed (Mettler M3 microbalance) in pierced aluminium pans and scanned under static air over a temperature range of 30° to 250°C at a heating rate of 10°C/min. Calibration of temperature and heat flow were performed with standard Indium samples.

Particle size: Particle size of the formulation has significant influence on the dissolution rate. An increase in the total effective surface area of formulation in contact with GI fluids causes an increase in its dissolution rate. The smaller the particle size, the greater the effective surface area exhibited by a given mass of drug formulation and the higher the dissolution rate. The microspheres were characterized for size by motie microscope (DMB series). Particle size of both - plain drug microspheres as well as microspheres with bioenhancer was measured using Motic microscope at 40 X magnification. In all measurements, at least 100 particles in each of 3 different fields were examined.

Percentage yield: The yield of microspheres was determined by comparing the whole weight of microspheres obtained against the combined weight of the polymer, drug and bioenhancers used for formulation. The percentage yield of the microsphere was determined using following formula: The percentage yield of prepared microspheres was determined by using the formula:

\[
\text{Percentage yield} = \frac{\text{Wt of microspheres prepared}}{\text{Total wt of drug, polymer used for formulation}} \times 100
\]

Percentage encapsulation efficiency: The drug content of the microspheres was determined spectrophotometrically (UV spectrophotometer Perkin Elmer, USA Lambda 25 model). Microspheres (10 mg) loaded with drug were dissolved in 10 ml of isotonic phosphate buffer pH 6.8 under sonication for 20 min. The solutions were filtered through 0.22 µm Millipore filters and the absorbance of the drug at a specific wavelength. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at a specific wavelength. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at 327 nm. The percent drug entrapment was calculated using following formula:

\[
\text{Percentage encapsulation efficiency} = \frac{\text{Wt. of drug present in microsphere}}{\text{Total wt. of drug, polymer used for formulation}} \times 100
\]

Percentage bioadhesion: In-vitro bioadhesion was determined for microspheres (in triplicate) by falling liquid film method.

Microspheres (50 mg) were placed on albino rat small intestine (area 2 cm²) and kept for 20-30 minutes in a humidity temperature controlled cabinet (Thermolab, India), maintained at 75 (±5)% relative humidity and temperature of 25 (±2)°C to allow hydration of the microspheres. This was followed by thorough washing of the mucosal layer with isotonic phosphate buffer pH 6.8, and then dried at 70°C in a hot air oven. Percent bioadhesion was determined by the following formula:

\[
\text{Percentage yield} = \frac{\text{Wt of adhered microsphere}}{\text{Wt. of applied microspheres}} \times 100
\]

In-vitro drug release: Dissolution studies were carried out using USP XXXI rotating basket method. The release profiles of rifampicin from microspheres were studied in simulated gastric fluid (SGF pH 1.2) and simulated intestinal fluid (SIF pH 6.8). The drug-loaded microspheres (equivalent to 20 mg of rifampicin) filled in empty capsule shells were put into the basket (50 rpm) and placed in 500 ml of the dissolution medium, thermostated at 37°C. Samples of 2 ml each were withdrawn at regular time intervals, filtered, diluted suitably, analyzed using double beam UV spectrophotometer at 475 nm and an equal volume of fresh medium was immediately added to maintain the dissolution volume. Dissolution studies were carried out up to 12 h. The drug release experiments were conducted in triplicate.

RESULTS AND DISCUSSION:

Fourier Transform Infra-Red (FT-IR) analysis: Pure drug RIF, placebo microspheres, RIF microspheres (2-5 mg) prepared with and without bioenhancer were weighed and mixed perfectly with potassium bromide (0.1 to 0.2 g) to form a uniform mixture. Potassium bromide pellet method was employed and background correction was applied under identical conditions. The IR spectrum of the pellet were recorded using FTIR (Perkin Elmer, USA, Spectrum RX1 Model) taking air as the reference and compared with each other to identify drug-excipient interaction, if any.

Each spectrum was derived from 16 single averaged scans collected in the region 400-4000 cm⁻¹ at a spectral resolution of 2 cm⁻¹. FT-IR spectra of pure drug and rifampicin microsphere are shown in Figure 2a and 2b respectively.

Differential Scanning Calorimeter (DSC) study: Rifampicin powder sample (2-8 mg) was weighed into an aluminum pan and analyzed as sealed with pinholes and an empty aluminum pan was used as a reference. To determine the thermodynamic relationship of two forms, heat-cool-heat cycle was also used. The DSC
endotherm showed a sharp melting endotherm for rifampicin at 190°C.

**Table 5: Particle Size, % Yield, % Drug Entrapment, % Bioadhesion and % Drug Release of Rifampicin Microspheres by Complex Coacervation Method**

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Mean particle size (µm)</th>
<th>Yield (%)</th>
<th>Drug Entrapment (%)</th>
<th>Bioadhesion (%) ± SD</th>
<th>Drug release at (12th hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF1</td>
<td>126-131</td>
<td>36.75 ± 1.89</td>
<td>41.45 ± 1.77</td>
<td>40.19 ± 1.21</td>
<td>46.81 ± 1.92</td>
</tr>
<tr>
<td>CRF2</td>
<td>123-128</td>
<td>49.77 ± 1.89</td>
<td>56.71 ± 2.12</td>
<td>53.89 ± 1.79</td>
<td>59.67 ± 1.86</td>
</tr>
<tr>
<td>CRF3</td>
<td>115-122</td>
<td>62.63 ± 1.87</td>
<td>68.34 ± 1.85</td>
<td>67.56 ± 1.19</td>
<td>73.23 ± 1.19</td>
</tr>
<tr>
<td>CRF4</td>
<td>110-116</td>
<td>69.20 ± 1.43</td>
<td>76.50 ± 1.17</td>
<td>80.50 ± 2.04</td>
<td>86.16 ± 1.07</td>
</tr>
</tbody>
</table>

**Percentage bioadhesion:** The bioadhesion of the microspheres in the optimized formulations showed a significant change with the presence and quantity of bioenhancer. The bioadhesion study was performed (in triplicate) using a previously reported method.15 The percentage bioadhesion was found to be 40.19 to 80.50% as shown in Table 5. The bioadhesive property of microspheres resulted in prolonged retention in the small intestine. It has been observed that, the microspheres containing comparably higher amount of bioenhancer showed significant increase in bioadhesion about 86%. It has also been observed that, the percentage bioadhesion increases as the amount of bioenhancer increases as shown in Table 5. The bioadhesive property of these particles resulted in prolonged retention in the small intestine.

**In-vitro drug release:** The in-vitro release study of rifampicin microspheres prepared by complex coacervation method in simulated gastric fluid (SGF), pH 1.2 and simulated intestinal fluid (SIF), pH 6.8, is shown in figure 5. Approximately, 8-10% of the drug was released in the SGF, pH 1.2 over a period of 2 h and about 35-80% in SIF, pH 6.8 up to 12 h. It has been found that the microspheres containing bioenhancer show greater increase in drug release as compared to microspheres without bioenhancer. In Figure 5, CRF1 formulation without bioenhancer and CRF2, CRF3 and CRF4 are the formulations with bioenhancers prepared by complex coacervation method whereas the fraction of bioenhancer were used in 5, 10 and 15 mg respectively in 3rd, 4th and 5th formulations in both the methods as shown in Figure 5.
CONCLUSION

In this study, microspheres of rifampicin were prepared by complex coacervation method using chitosan and gelatin as a polymer and Na-TPP as a cross-linking agent. Effect of variables like drug-polymer ratio, cross linker concentration and the cross-linking time on in-vitro release of rifampicin was examined. The mean particle size of the microspheres increased with an increase in the concentration of polymer. The increase in the concentration of Na-TPP and chitosan-gelatin caused the increase in the entrapment efficiency and the extent of drug release. The cross-linking time shorter than 20 minutes resulted in higher entrapment efficiencies. The microspheres were spherical and well formed. The mean diameter, entrapment efficiency and bioadhesion of the optimized microspheres were found to be 103.112 μm, 85.87 ± 1.75% and 87.42 ±1.51% respectively. The release profiles of rifampicin from microspheres were examined in simulated gastric fluid (SGF pH 1.2) and simulated intestinal fluid (SIF pH 7.4). About 10% of the rifampicin was released in the SGF in first 2 hours and released quickly about 85% in 10 hrs in SIF. The concentration of polymers and the presence of cross-linking agent had a great affect on the release of rifampicin. The most important finding of this study relates to the very significant enhancement in drug release (46.81 to 86.16%), due to co-administration of 15 mg bioenhancer along with each dose of rifampicin microspheres.

REFERENCES