PUBLICATIONS, PATENTS AND PRESENTATIONS
Publications


Patent filed


Presentations

1. DST Sponsored Indo–Taiwan International Workshop Theme: "Drug Development For Cancer And Infectious Diseases: From Basic To Industry" held at ISF College of Pharmacy, Moga, Punjab, India from December 14 & 15, 2011.
   - Harinder Singh, Rohit Bhandari, Sahil Jindal, Indu Pal Kaur. Entrapment of rifampicin in SLNs to avoid its degradation and interaction with isoniazid at acidic pH and evaluating its safety.

2. International Conference NanoSciTech-2012 on “Frontiers in Nanoscience, Nanotechnology and Applications” during February 16-18, 2012 at the Panjab University, Chandigarh
Publications, Patents and Presentations

3. VIth Chandigarh Science Congress on Synergy-Together Towards Tomorrow 26-28 February, 2012 at Panjab University, Chandigarh.

- Harinder Singh, Sahil Jindal, Rohit Bhandari, Indu Pal Kaur, Acute toxicity of Rifampicin and Isoniazid loaded Solid lipid nanoparticle formulations in wistar rats, as per OECD guideline 425.


*Best prize in oral presentation

Encapsulation of Rifampicin in a solid lipid nanoparticulate system to limit its degradation and interaction with Isoniazid at acidic pH

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ABSTRACT

Rifampicin (RIF), a vital constituent of antitubercular therapy, hydrolyzes at the acidic pH of the stomach. The degradation is further enhanced by its interaction with Isoniazid (INH). Extent of RIF decomposition, in the presence and absence of INH, was determined at pH 1.2 (pH of empty stomach) at 37 °C for 4 h (maximum stomach residence time). Both the drugs decomposed at gastric pH (26.5% and 1.43% for RIF and INH respectively).

Considering that solid lipid nanoparticles (SLNs) avert drug–drug interaction and also drug degradation, we incorporated RIF into SLNs. Latter reduced its degradation to ~9% (from 26.50% when present alone) and to ~20% (from 48.81% when INH was also present).

Subsequent to this, we also incorporated INH into SLNs and the percent degradation of RIF in this combination (RIF SLNs + INH SLNs) further reduced to 12.35%. Furthermore, the degradation of INH in combination with RIF also reduced significantly from 13.2% to 2.7% when both the drugs were encapsulated individually within SLNs.

Therefore, the need to develop combinations of antitubercular drugs (ATDs) with caution and also establishes the usefulness of nanoparticulate technology to avoid drug–drug interaction.

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1. Introduction

Rifampicin (RIF) dosages of less than 9 mg/kg of body weight/day are inadequate for treatment of pulmonary tuberculosis leading to failure of therapy and emergence of drug resistance. Currently, the prescribed dose of RIF is 10 mg/kg (600 mg RIF for an average patient of 60 kg), which means a narrow margin of only 10% in actual delivered dose and the minimum necessary for therapeutic action. Despite good lipid solubility (logP 3.72; (Anonymous, 1991; Acocella, 1989; Anonn, 1991; Doshi et al., 1986; Ellard et al., 1986; Fox, 1990; Mouton et al., 1979)), in 1994, World Health Organization (WHO) and International Union against Tuberculosis and Lung Disease (IUATLD) cautioned that antitubercular fixed dose combination (FDC) formulations should be used only if the bioavailability of RIF has been demonstrated convincingly (IUATLD/WHO, 1994). RIF has been reported to undergo rapid decomposition in the presence of INH under acid conditions (Anonymous, 2003; Shishoo et al., 2001). This means there exist a strong possibility of the dose of RIF falling below the minimum required level after the administration of FDCs containing the two drugs. It has been reported in an in vitro study that RIF in the presence of INH undergoes significant decomposition under acidic conditions (existing in the stomach), as compared to RIF alone (Shishoo et al., 2001). It is first hydrolyzed to 3-formylrifamycin (Gallo and Radaelli, 1976; Frankerd et al., 1992); the hydrolysis being significantly accelerated in the presence of INH (Shishoo et al., 1999; Singh et al., 2000) to form isonicotinyl hydrazine (HYD). HYD converts back to INH and 3-formylrifamycin, resulting in significant recovery of INH with an eventual loss of RIF. This explains why the bioavailability problem is confined to RIF and does not invariably extend to INH. This pH dependent decomposition of RIF follows a bell-shaped curve (Sankar et al., 2003) and the decomposition of RIF under acid conditions varies from 8.5% to 50% in the time range corresponding to the normal gastric residence time in humans (Singh et al., 2000).

In three independent studies (Immanuel et al., 2003; Luyen et al., 2005; Shishoo et al., 2001), where FDCs were tested against RIF alone formulations, almost 30% fall in the bioavailability of RIF was observed. Binary mixtures of INH and RIF are indicated to exhibit thermal stabilities lower than each drug alone (Freire et al., 2009).

In the present study, we propose the use of solid lipid nanoparticle (SLN) based carrier system for encapsulating RIF so as to minimize its acidic pH and INH induced degradation. SLNs
constitute an attractive colloidal drug carrier system consisting of spherical solid lipid particles in the nano range, which are dispersed in water or in aqueous surfactant solution (Chen et al., 2001; Fundaro et al., 2000; Reddy and Venkateshwarlu, 2004; Wang et al., 2002). SLNs are reported to prevent degradation of insulin in the gastrointestinal tract upon oral administration (Sarmiento et al., 2007) and similar protection to RIF was expected presently.

2. Materials and methods

2.1. Materials

RIF and INH were obtained as a gift sample from Panacea Biotech, Ludhiana, Punjab, India. Compritol® ATO 888 was obtained as a gift sample from Gattefosse, Germany. All other chemicals or solvents were of analytical or HPLC grade.

2.2. Methods

2.2.1. Analytical method

Analysis was carried out on a reversed-phase HPLC using C-18 symmetry shield column (25 cm, 4.6 mm, 5 mm). Various compositions of mobile phase were tried in isocratic and gradient mode. A mobile phase consisting of a gradient of methanol and 0.1 M phosphate buffer at pH 6.8 was selected as described in Table 1. The flow rate was maintained at 1 mL/min and the analytical wavelength was set to 254 nm. The method was validated for linearity, precision, accuracy, ruggedness, and specificity. For determination of linearity, a stock solution of RIF and INH was prepared by dissolving the drugs in methanol, at a concentration of 1 mg/mL. From these stock solutions, dilutions were made in the range of 5–600 μg/mL and 5–500 μg/mL for RIF and INH, respectively. The solutions were injected after filtering through 0.2 μm nylon membrane, keeping the injection volume constant (20 μL). The repeatability was determined by replicate injections of a solution of the two drugs at three concentrations: 5, 100, and 500 μg/mL. For intermediate precision, 300 μg/mL of RIF and INH solution was prepared six times separately, and the peak areas were recorded.

2.2.2. Solubility of rifampicin

Standard curves of RIF were prepared in distilled water, methanol, and methanol:chloroform (1:2). Saturation solubility of RIF in water was obtained at room temperature (30°C). To determine the effect of SLNs on the solubility of RIF, solubility was also observed in blank SLNs prepared by the microemulsification method as described below.

2.2.3. Preparation and characterization of solid lipid nanoparticles

The solid lipid nanoparticles were prepared by modification of the microemulsification method as reported earlier (Kakkar et al., 2011; Bhandari and Kaur, 2013a). Briefly the lipidic phase (containing lipid-13% and polysorbate 80) and the aqueous phase (soy lecithin and water) were heated —10°C above the lipid melt temperature of 70°C. The drug was added to the lipid–surfactant mixture containing RIF under magnetic stirring at 600 rpm. The aqueous phase to lipid–surfactant mixture containing RIF under magnetic stirring at 1500 rpm. Stirring was continued for 2–10 min at 70°C, thus formed spontaneously, was kept, and heated at a predefined rate of 10°C/min over the temperature range of 20 and 300°C in nitrogen atmosphere. Thermal analyses of DSC thermograms were conducted using TA Instruments Universal Analysis 2000 software (version: 4.5A). The s-recorded and plots between heat flow (w/g) and temperature (°C) were obtained. Indium standard was used to calibrate the DSC.
2.2.9. Degradation studies

A stock solution of 1 M HCl was prepared and diluted to obtain 0.1 M HCl (pH 1.2). Accurately weighed quantities of 1.8 mg of RIF and 1.2 mg of INH (this 3/2 ratio of the two drugs was taken to maintain equivalence with the drug ratio in the marketed formulations) each were added separately to 5 mL of 0.1 N HCl. The solution was mixed well and a sample was withdrawn immediately, suitably diluted with methanol:chloroform (1:2) and analyzed by HPLC. Several particles in the range of 40-100 nm could also be observed under TEM as the particle size distribution varied from 20 to 150 nm. Very small sized SLNs were visible under TEM. Both the SLNs were found to be spherical in shape. The shape and surface morphology of prepared SLNs was studied by TEM. Both the SLNs were found to be spherical in shape (Fig. 2). The solubility of RIF in triple distilled water and blank SLNs was found to be 0.663 ± 0.05 and 4.40 ± 0.35 mg/mL respectively at room temperature (30 °C).

3. Results and discussion

3.1. Analytical method

Out of the various mobile phase combinations, a gradient method consisting of methanol:phosphate buffer pH 6.8 was selected based on the fact that good separation and peak purity was obtained for this combination (Bhandari and Kaur, 2012). Retention time for INH and RIF was 3.1 min and 11.5 min respectively (Fig. 1). The response of the method was linear in the range of 5-600 μg/mL and 5-500 μg/mL for RIF and INH, respectively. A total of six standard curves were prepared on consecutive days and the mean data are given in Table 3. The results of the recovery and precision experiments are given in Tables 4 and 5. The percent relative standard deviation (RSD) for all six injections in the repeatability and intermediate precision experiments was found to be well below the limit of 1% and 2%, respectively. The resolutions obtained during experiments were satisfactory for both the drugs.

3.2. Solubility of RIF

Linearity of standard curves was found to be 0.9997, 0.9998 and 0.9999 in triple distilled water, methanol and methanol:chloroform (1:2) respectively. The solubility of RIF in triple distilled water and blank SLNs was found to be 0.663 ± 0.05 and 4.40 ± 0.35 mg/mL respectively at room temperature (30 °C).

3.3. Preparation and characterization of solid lipid nanoparticles

RIF and INH loaded SLNs showed an average particle size below 150 nm (Table 6). Both the formulations had a near neutral zeta potential and showed an entrapment efficiency of more than 65%.

3.4. Transmission electron microscopy

The shape and surface morphology of prepared SLNs was studied by TEM. Both the SLNs were found to be spherical in shape (Fig. 2). Several particles in the range of 40-100 nm could also be observed under TEM as the particle size distribution varied from 20 to 150 nm. Very small sized SLNs were visible under TEM.

3.5. Differential scanning calorimetry (DSC) studies

DSC thermograms of pure drugs (RIF and INH), excipients and SLNs were performed (Fig. 3). The main component Compritol® showed an endothermic peak around 72 °C which corresponds to its melting point. Free RIF showed an endothermic peak around 188 °C corresponding to its melting point, while soya lecithin exhibited sharp endothermic peaks at 150 °C. RIF SLNs showed a very small endothermic peak at 188 °C representing the free drug in the sample and a small sharp endothermic peak around 70 °C was corresponding to the Compritol®. Similarly INH SLNs showed a peak corresponding to Compritol® but no peak of INH was observed indicating its successful encapsulation in SLNs. Reduction of peak height and disappearance of peaks pointed toward a positive interaction. The decrease in enthalpy change pointed toward a reduction in crystallinity indicating successful entrapment of the respective drugs in SLNs which in turn ensure a decreased drug expulsion and a better controlled release from the SLNs with a minimal burst effect (Fig. 3).
Table 6

<table>
<thead>
<tr>
<th>SLN formulation</th>
<th>Mean particle size ± SD (nm)</th>
<th>Total drug content ± SD (%)</th>
<th>Entrapment efficiency ± SD (%)</th>
<th>Polydispersity index (PDI) ± SD</th>
<th>Zeta potential ± t</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIF SLNs</td>
<td>141.2 ± 13.5</td>
<td>92.5 ± 2.2</td>
<td>65.3 ± 3.1</td>
<td>0.320 ± 0.032</td>
<td>-3.5 ± t</td>
</tr>
<tr>
<td>INH SLNs</td>
<td>120.0 ± 0.7</td>
<td>94.2 ± 0.7</td>
<td>69.3 ± 0.6</td>
<td>0.281 ± 0.019</td>
<td>-0.101 ± t</td>
</tr>
</tbody>
</table>

3.6. In vitro release

RIF SLNs showed a release of 70% in phosphate buffer pH 6.8 after 9 days while INH SLNs showed a release of 62% in phosphate buffer pH 6.8 after 24 h (Fig. 4). This is due to the hydrophobic nature of RIF and controlled release due to SLNs. INH, being hydrophobic nature, showed a much faster release.

3.7. Degradation studies

SLNs are reported to be a suitable carrier system for pharmaceuticals with various benefits including the controlled release of incorporated compounds (Müller et al., 1995; Mühlen and Mehnert, 1998). They are also recommended for protection of labile drugs against chemical degradation (Jer and Gohla, 2001; Müller et al., 1995). Entrapment of calcitriol in isobutylcyanoacrylate nanocapsules has been reported to protect the drug against the action of proteases (Lowe and Terenius, 1994). Administration of insulin through the oral route prevents its degradation in the gastrointestinal tract (Sarmento et al., 2007).

As the gastric residence time of the formulations may vary from 15 min to as long as 4 h depending upon the absence/presence of food, the degradation studies were conducted, presently for 4 h as reported in the literature (Doshi et al., 1986; Mouton et al., 1988) that due to its interaction with INH, RIF undergoes rapid degradation in acidic media. Degradation of RIF was thus studied in the presence of INH at acidic pH 1.2 (pH of empty stomach). Studies including RIF entrapped inside SLNs and its combination with free INH and INH SLNs (Table 2) maintained at acidic pH also performed to determine the protective effect of SLNs. Extensively, the degradation of free RIF increased in the presence of INH. At 4 h, free RIF decomposed by 26.5% which increased to 41.5% in the presence of free INH, while free INH decomposed approx. by 13% when present in combination with RIF. How RIF entrapped inside SLNs degraded only by 19.5% in the presence of free INH while it degraded to only 12% in the presence of SLNs. INH entrapped in SLNs degraded by only 3% in the presence of free RIF. Figs. 5 and 6 show comparison of different combinations (Table 3) with respect to percent degradation. Extent of degradation of RIF and INH in various combinations at different time points is given in Tables 7 and 8 respectively.

It was observed that free RIF at pH 1.2 underwent significant degradation after 4 h. From Fig. 5 we can see that highest degradation of free RIF was observed in combination with free INH degradation was significantly prevented by SLNs by almost 31% as compared to free RIF. Free RIF degradation almost doubled in
The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P< 0.05) except those marked similarly.

### Table 7

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>RIF SOLN</th>
<th>RIF SLNS</th>
<th>Free RIF + free INH</th>
<th>RIF SLN + INH free</th>
<th>Free RIF + INH SLNS</th>
<th>RIF SLNS + INH SLNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>2.53 ± 2.42</td>
<td>1.18 ± 0.84</td>
<td>4.59 ± 1.55</td>
<td>3.68 ± 0.66</td>
<td>3.62 ± 1.45</td>
<td>1.65 ± 0.95</td>
</tr>
<tr>
<td>1</td>
<td>10.95 ± 2.91</td>
<td>3.93 ± 1.77*</td>
<td>19.17 ± 2.62</td>
<td>8.40 ± 1.80*</td>
<td>11.59 ± 1.53*</td>
<td>5.00 ± 1.22</td>
</tr>
<tr>
<td>2</td>
<td>17.64 ± 2.05*</td>
<td>6.25 ± 1.21*</td>
<td>34.56 ± 3.32</td>
<td>12.33 ± 1.58*</td>
<td>19.38 ± 2.15*</td>
<td>7.09 ± 0.87*</td>
</tr>
<tr>
<td>4</td>
<td>26.50 ± 1.69*</td>
<td>8.68 ± 1.42*</td>
<td>48.81 ± 2.50</td>
<td>19.49 ± 2.01</td>
<td>29.62 ± 1.75*</td>
<td>12.33 ± 2.49*</td>
</tr>
</tbody>
</table>

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P< 0.05) except those marked with #, *, $, ?, & +.

### Table 8

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>INH SOLN</th>
<th>INH SLNS</th>
<th>INH free + RIF free</th>
<th>INH SLNS + RIF free</th>
<th>INH free + RIF SLN</th>
<th>INH SLNS + RIF SLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.18 ± 1.05</td>
<td>0.09 ± 1.25*</td>
<td>3.66 ± 2.11*</td>
<td>1.95 ± 0.85*</td>
<td>0.66 ± 0.25*</td>
<td>0.85 ± 0.33</td>
</tr>
<tr>
<td>1</td>
<td>0.58 ± 0.81</td>
<td>0.38 ± 1.56</td>
<td>8.05 ± 1.08</td>
<td>4.11 ± 1.21*</td>
<td>1.41 ± 0.64*</td>
<td>1.48 ± 0.78</td>
</tr>
<tr>
<td>2</td>
<td>0.92 ± 1.87</td>
<td>0.65 ± 0.95</td>
<td>11.25 ± 1.22</td>
<td>4.83 ± 0.65*</td>
<td>1.98 ± 0.78*</td>
<td>1.99 ± 0.65</td>
</tr>
<tr>
<td>4</td>
<td>1.43 ± 0.55</td>
<td>0.94 ± 0.78*</td>
<td>13.21 ± 1.45</td>
<td>5.65 ± 0.88*</td>
<td>3.05 ± 0.68*</td>
<td>2.69 ± 0.91</td>
</tr>
</tbody>
</table>

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P< 0.05) except those marked with #, *, $, ?, & +.
Conflict of interest

The authors report no conflict of interest.

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