Chapter 4

Stability & Human bioavailability of novel rifampicin and isoniazid FDC

“Every great advance in science has issued from a new audacity of imagination”

.........John Dewey
Stability and human bioavailability study of novel rifampicin and isoniazid FDC

4.1 Introduction

TB is a major health problem in the developing countries like India, which has the maximum pool of TB patients. As of now the only available treatment lies in the effective utilization of few available anti-TB drugs, especially rifampicin and isoniazid. However, the emergence of resistant strains has come as a major ‘bottleneck’ in containing the spread of TB and its treatment. Combination of drugs can effectively counter this problem that led to the concept of FDCs. At the same time, it is very important to ensure that the bioavailability of the drugs combined in the FDCs is not compromised. This is particularly true for rifampicin where there are ample reports of reduced bioavailability from FDCs (Agrawal et.al., 2002). Rifampicin is a critical component in the therapeutic armamentarium for tuberculosis, and more recently for treating opportunistic infections associated with the acquired immune-deficiency syndrome (AIDS). The problem of reduced bioavailability of rifampicin from FDC products of anti-tuberculosis drugs is a matter of global concern. An integral part of the strategy to fight the disease is use of quality anti-TB drugs. The deficiency in delivery of proper dose of rifampicin has serious implications as it is known that doses of rifampicin less than 9 mg/kg body weight can result in therapeutic failure (Long et.al., 1979) and hence can be the cause of development of drug resistance. The problems associated with quality of FDC products are in the current focus.

Over the years, two serious problems have been reported with rifampicin and isoniazid FDCs that includes (Laserson et.al., 2001; Shishoo et.al., 2001; Immanuel et.al., 2003; Singh and Mohan, 2003; Bhutani et.al., 2004; Luyen et.al., 2005):

- The impaired and variable bioavailability of rifampicin from FDC formulations with isoniazid
- Poor stability of rifampicin containing FDCs

The use of substandard FDC ultimately results in drug resistant TB and treatment failure (Panchagnula et.al., 1999; IUTALD/ WHO, 1994). In both cases, the problem has been ascribed to the decomposition of rifampicin in the presence of isoniazid to form
isonicotinyl hydrazone (Singh et al., 2000; Shishoo et al., 2001). In this backdrop, both WHO and IUATLD recommend the use of FDCs proven bioavailability of rifampicin (IUTALD/WHO, 1999; Panchagnula et al., 2003).

It is thus expected that bioavailability concerns associated with rifampicin could be overcome by developing a system that attains segregated delivery of the two drugs, with rifampicin released immediately in the stomach and isoniazid in the small intestine (through development of an enteric-release system), thus targeting them to their respective absorption windows (Mariappan and Singh, 2003). This strategy would also preclude physical interaction of these drugs within the dosage form during storage. In view of that, in the present study a novel formulation was designed and developed to incorporate the following components of anti-TB FDC in a capsule:

- **Rifampicin**: Total dose of rifampicin was subdivided into two components
  - (i) Immediate release pellets of rifampicin- Loading dose of rifampicin
  - (ii) Gastroretentive floating pellets of rifampicin- Maintenance dose of rifampicin

- **Isoniazid**: Delayed release pellets of isoniazid

The present chapter will cover the stability studies of the developed novel anti-TB FDC at room temperature and accelerated conditions. The oral bioavailability of novel rifampicin and isoniazid FDC, using a commercially available rifampicin and isoniazid FDC tablet as reference will also be covered in this chapter.

### 4.2 Stability studies of rifampicin and isoniazid FDC

#### 4.2.1 Methods

The weighed amount of rifampicin pellets, a rifampicin tablet and weighed amount of isoniazid was filled an empty hard gelatine capsule. The capsules prepared were subjected for stability studies at room temperature and at accelerated stability condition. The capsules were stored in a tightly closed HDPE container and aluminium (Alu.) packs. The stability studies were carried out at room temperature and accelerated relative humidity conditions as per ICH guidelines. The accelerated relative humidity conditions were 40°C ± 2°C/75% RH ± 5% RH (ICH, 2003). The stability samples were withdrawn and analysed at 1, 2, 3 and 6 M for drug content, water content and release profile. The assay, water content and dissolution studies of the floating rifampicin tablet...
were carried as per the methods described earlier. To ensure the equivalence in release profile of the stability samples with that of initial samples, the fit factor, $f_2$, was calculated. The formula used for calculating $f_2$ values is shown in Eq. (27):

$$f_2 = 50 \log \left\{ \left[ 1 + \frac{1}{n} \sum_{i=1}^{n} (R_i - T_i) \right]^{0.5} \times 100 \right\} \quad -(27)$$

4.3 Bioavailability studies of rifampicin and isoniazid FDC in human volunteers

4.3.1 Materials

R-Cinex tablets (Lupin Pharmaceuticals Limited, Pune, India) were procured from the market and were used as a reference product. Novel rifampicin isoniazid FDC capsules were prepared as previously described and packed in aluminum bags, sealed and labelled with full composition, batch number. Papaverine hydrochloride and pyrazinamide was kindly gifted by Biologicals E. Ltd, India and Macleods Pharmaceuticals, India. Dichloromethane, methanol, potassium dihydrogen orthophosphate and acetonitrile were obtained from Qualigens (Delhi, India). All reagents were of analytical or high performance liquid chromatography (HPLC) grade and are enlisted in Annexure 1.

4.3.2 Methods

4.3.2.1 Clinical protocol

An open label, balanced, randomised, three-treatment, three-sequence, three period, crossover, single centre bioavailability study of single oral dose of fixed dose combination of rifampicin and isoniazid in twelve healthy, adult, male, human subjects under fasting conditions was carried out. The study was performed at the B. V. Patel PERD Centre, Ahmedabad. The study protocol was approved by the Insititutional Ethics Committee of B. V. Patel PERD Centre. The study was conducted in accordance with the Declaration of Helsinki ethical principles (WMA, October 2008).

Subjects underwent a screening 14 days prior to the day of first dosing. Volunteers gave a written informed consent after receiving a detailed explanation of the investigational nature of the study. They were non-smokers, and were judged healthy on the basis of medical history, physical examination, electrocardiogram and investigation of biochemical, immunological, parasitological and haematological parameters in blood and urine.
Upon entering into the study, the subjects were housed in the clinical facility of the trial site for 10-12 h, prior to dosing till 24 h post dose in each of the three periods. All the subjects were fasted overnight, at least 12 h, before scheduled time for the dose administration. A standardised meal was given at 4 h and 12 h post dose in each period. During housing, meal plans for all the periods was same.

*Prior and concomitant therapy*

All the subjects were abstained from intake of medication from two weeks prior and during the study. They were asked to abstain from beverages containing alcohol or quinine between 24 h before and 48 h after drug dosing per experimental period. Drinking of water was allowed up to 2 h before drug administration. The subjects fasted for at least 10 h before administration of the medication.

*Procedure*

Before drug administration, an intravenous canula was placed in an antecubital vein and kept patent by use of a saline solution. The drug was administered with a glass of water (about 240 ml). From 2 h after dosing, intake of water was allowed. A standard lunch and dinner were served at 4 and 10 h post-dosing, respectively.

*Blood sampling*

Venous blood samples (6 ml) were taken before and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12 and 24 h after drug intake. Exact times of blood sampling were noted in the case report Form. Blood samples were collected in prelabelled heparinised tubes and centrifuged for 7 min., at 4000±100 rpm, at a temperature below 4°C within 2 h after collection for collecting the plasma. Separated plasma was aspirated with a disposable pipette and transferred to plastic plasma vials containing ascorbic acid. The plasma vials were sealed and labelled with the mentioning project number, subject number, period, sampling time point, and sample number and stored at -20°C for interim storage and at -80°C until assay of rifampicin and isoniazid by HPLC.

*Randomisation*

The study was conducted in a randomized crossover design. Subjects were randomly assigned to receive a single dose of 450mg rifampicin and 300 mg isoniazid. A washout period of 1 week separated both drug intakes. Subjects entering the study were allocated a number from 1 to 12 and were administered medication as per the randomization schedule.
4.3.3 Determination of rifampicin in plasma

Rifampicin concentrations were determined by a validated HPLC method: 0.5 ml plasma was spiked with 50 µl aqueous ascorbic acid solution (10 µg/ml) and 9 µg internal standard, papaverine HCl in methanol. Samples were then buffered with 0.5 ml of 0.005M K2HPO4 containing 1 µg/ml ascorbic acid (pH 7), and extracted with 6 ml of dichloromethane. The organic layer was transferred to conical centrifuge tubes and evaporated until dry, under a gentle nitrogen stream. The residue was redissolved in 100 µl mobile phase containing ascorbic acid (50 mg/l), and a 50 µl aliquot was injected onto a 5 µm particle size, reverse phase, C-18, Qualisil column (250 X 3.9 mm).

Chromatographic conditions

The HPLC equipment consisted of a solvent pump (Jasco PU 980, Tokyo, Japan) set at a constant flow rate of 1.00 ml/min, a UV detector (Jasco UV 875, Jasco, Tokyo, Japan) set at 320nm wavelength, a C-18 reversed phase precolumn and Base deactivated (BDS) column Kromasil (LCGC, USA) and an automatic integration system (Borwin, Japan). The mobile phase was based on the composition described by Pargal and Rani (2001). It consisted of a filtered and degassed mixture of 45% acetonitrile and 55% of 0.01M KH2PO4 (pH 6.5). The method was validated as per ICH guidelines and was found to be specific, accurate, linear in the concentration range of 20 to 0.5 µg/ml, limit of quantitation was 0.5 µg/ml and limit of detection was 0.1 µg/ml (Pund, 2010).

4.3.4 Determination of isoniazid in plasma

Isoniazid concentrations were determined by a validated HPLC method: 200µl of pyrazinamide (Internal standard) solution in acetonitrile was added to 20 µl plasma and centrifuged for 10 min at 10,000 rpm. The supernatant was collected and dichloromethane was added to it. This mixture was then centrifuged for 10 min at 10,000 rpm and 100 µl of aqueous phase was collected. 50 µl of the sample was then diluted with an equal amount of mobile phase and injected onto 5 µm particle size, reverse phase, C-18 BDS column (250 X 3.9 mm).

Chromatographic conditions

The HPLC equipment consisted of a solvent pump (Jasco PU 980, Jasco, Tokyo, Japan) set at a constant flow rate of 1.00 ml/min, a variable wavelength detector (Jasco UV 875, Jasco, Tokyo, Japan) set at 264 nm wavelength, a C-18 reversed
phase precolumn and Base deactivated (BDS) column Kromasil (LCGC, USA) and an automatic integration system (Borwin, Japan). Mobile phase consisted of a filtered and degassed mixture of 3.5% acetonitrile and 97.5% of 0.01M KH₂PO₄ buffer. The method was validated as per ICH guidelines and was found to be specific, accurate, linear in the concentration range of 20 to 0.5 µg/ml, limit of quantitation was 0.5 µg/ml and limit of detection was 0.1 µg/ml (Pund, 2010).

4.3.5 Statistical analysis

4.3.5.1 Pharmacokinetic Analysis

Maximal plasma concentration (C_{max}) and time to reach the peak concentration (T_{max}) were obtained directly by the visual inspection of each subject's plasma concentration-time profile. The slope of the terminal log-linear portion of the concentration-time profile was determined by least-squares regression analysis and used as the elimination rate constant (K_{el}). The elimination half-life was obtained from the formula,

\[ t_{1/2} = \frac{\ln(2)}{K_{el}} \]  \hspace{1cm} (28)

Where ‘ln’ is the natural logarithm.

The Area Under Curve (AUC) from time zero to the last quantifiable point (C_t) was calculated using the trapezoidal rule. The area under the plasma concentration-time from 0 to infinity (AUC_{0-\infty}) was calculated as the sum of the AUC_{0-t} plus the ratio of the last measurable concentration to the elimination rate constant.

For any AUC computation, concentration at time point ‘t’ (C_t) values below limit of quantification (LOQ) was set to zero. (C_t) values below (LOQ) were to be ignored in the linear regression analysis. Statistical analysis for evaluating bioequivalence were carried out on logarithmically (natural) transformed pharmacokinetic parameters of rifampicin and isoniazid (C_{max} and AUC_{0-t}). The parameter T_{max} was analyzed on untransformed data.

4.3.5.2 Descriptive statistics

Descriptive statistics of C_{max} and AUC_{0-t} for test (novel rifampicin isoniazid FDC) and reference (marketed rifampicin isoniazid FDC) products were calculated. The individual
values of these endpoints together with descriptive statistics and ratios B/A were tabulated for test and reference formulations of rifampicin and isoniazid.

### 4.3.5.3 Analysis of variance

The ln-transformed pharmacokinetic parameters (C\text{max}, AUC\text{0-\text{t}}, and AUC\text{0-\infty}) were analyzed using an ANOVA model with the main effects of sequence, subject nested within sequence, period and formulation.

### 4.3.5.4 90% Confidence interval

For the pharmacokinetic parameters C\text{max} and AUC\text{0-\text{t}}, 90% confidence intervals for the ratios of test and reference product averages were calculated using the ANOVA of the ln-transformed data. Consistent with the two one sided test for bioequivalence, 90% confidence interval for the ratio of both the products averages were calculated by first calculating the 90% confidence interval for the differences in the averages of the ln-transformed data and then taking the antilogarithms of the obtained confidence limits.

### 4.3.5.5 Bioequivalence criteria

Bioequivalence was evaluated using average bioequivalence approach; this is based on the ratio of average ln-transformed responses. The 90% confidence interval for ratio of average ln-transformed C\text{max}, AUC\text{0-\text{t}} of rifampicin and isoniazid was the basis for concluding the equivalence of product A and B. The 90% CI should hence lie within the bioequivalence limit (80.00-125.00).

### 4.4 Results and discussion

#### 4.4.1 Stability studies of rifampicin and isoniazid FDC

The rifampicin and isoniazid FDC capsules were subjected to stability studies at room temperature and at the accelerated stability conditions (40°C ± 2°C/75% RH ± 5%) for the duration of 6 M. The samples were withdrawn at 1 M, 2 M, 3 M and 6 M and analysed for their water content, drug content and the dissolution profile. The water content and drug content in rifampicin isoniazid FDC on stability are shown in Table 30. The drug content of the rifampicin and isoniazid from the FDC on stability was found to be in the range between 98.99-100.65% and 98.55-100.05%, respectively. While, the water content of rifampicin –isoniazid FDC ranged between 4.05- 4.52% w/w. According to the ICH guidelines on stability testing, if ‘significant change’ occurs at any
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time during 6 M of testing at the accelerated storage conditions of 40°C ± 2°C/75% RH ± 5%. The ‘significant change’ for a solid drug product is defined as: a 5% change in assay from its initial value; or failure to meet the acceptance criteria for appearance, physical attributes and dissolution test (ICH, 2003). Hence, it can be concluded that no significant change was observed in FDC after 6 M with respect to its water content and drug content.

The release profile of isoniazid from the stability samples of novel FDC at room temperature and accelerated stability conditions are shown in Fig 3. It can be concluded from the graph that the gastric acid resistance property of the isoniazid delayed release pellets had no affect on the stability. Even after 6 M stability the amount release at the end of 120 min. in 0.1N HCl was found to be ~10%, which was similar to that of initial sample of isoniazid delayed release pellets. While ~85% of isoniazid was released in phosphate buffer pH 6.8 within 1 h, even after 6 M stability.

Table 30. Assay and water content of stability samples of rifampicin- isoniazid pellets

<table>
<thead>
<tr>
<th>40°C ± 2°C/75% RH ± 5%</th>
<th>At room temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDPE</td>
</tr>
<tr>
<td><strong>Assay of Rifampicin (%)</strong></td>
<td></td>
</tr>
<tr>
<td>1M</td>
<td>99.35</td>
</tr>
<tr>
<td>2M</td>
<td>100.08</td>
</tr>
<tr>
<td>3M</td>
<td>100.45</td>
</tr>
<tr>
<td>6M</td>
<td>99.87</td>
</tr>
<tr>
<td><strong>Assay of Isoniazid (%)</strong></td>
<td></td>
</tr>
<tr>
<td>1M</td>
<td>99.43</td>
</tr>
<tr>
<td>2M</td>
<td>99.98</td>
</tr>
<tr>
<td>3M</td>
<td>98.55</td>
</tr>
<tr>
<td>6M</td>
<td>100.02</td>
</tr>
<tr>
<td><strong>Water Content (%w/w)</strong></td>
<td></td>
</tr>
<tr>
<td>1M</td>
<td>4.21</td>
</tr>
<tr>
<td>2M</td>
<td>4.34</td>
</tr>
<tr>
<td>3M</td>
<td>4.39</td>
</tr>
<tr>
<td>6M</td>
<td>4.18</td>
</tr>
</tbody>
</table>
**Fig 33.** Dissolution profile of initial, 3 M and 6 M stability sample of isoniazid from rifampicin-isoniazid FDC

The release profile of rifampicin from the stability samples of rifampicin and isoniazid FDC capsules at room temperature and accelerated stability conditions are shown in Fig 34. It can be seen in the graphs that the amount of rifampicin released from the initial samples was 85±2.79% at the end of 520 min., while the FDC samples after 6 M showed a release of 85.2 ± 1.78% of rifampicin.

**Fig 34.** Dissolution profile of initial, 3 M and 6 M stability sample of rifampicin from rifampicin-isoniazid FDC

However, rifampicin follows a pH dependent degradation pattern. In the acidic medium, rifampicin hydrolyzes to 3-Formyl rifamycin SV (3-FRSV) which gets precipitated in acidic conditions. The 3-FRSV formed shows high *in vitro* antimicrobial activity but is inactive *in vivo* (Savale, 2003). Therefore, formation of 3-FRSV in the acidic environment of stomach is an important factor affecting bioavailability of rifampicin and cannot be overlooked (Shishoo *et al.*, 1999).
Shishoo et al., 1999, evaluated various marketed FDCs of rifampicin isoniazid and found that there is a significant increase in formation of 3-FRSV in presence of isoniazid. This indicates that the presence of isoniazid catalyzes degradation of rifampicin to 3-FRSV in 0.1 N HCl. Also, with the increase in time the formation of 3-FRSV increases. Hence, it was essential to estimate the amount of 3-FRSV formed from the novel FDC of rifampicin and isoniazid. The amount of 3-FRSV formed from novel FDC was compared with the market sample of rifampicin and isoniazid FDC.

The amount of 3-FRSV formed in rifampicin-isoniazid FDC on stability is shown in Fig 35. A comparison of amount of 3-FRSV formed from market FDC and novel FDC of rifampicin and isoniazid is shown in Table 34.

Fig 35. Formation of 3-FRSV in dissolution samples of initial, 3 M and 6 M stability sample of rifampicin-isoniazid FDC

The amount of 3-FRSV formed at the end of 520 min. was found to be around 12.9% as against 25.76% of 3-FRSV formed from the market FDC of rifampicin and isoniazid. The amount of 3-FRSV formed from novel anti-TB FDC was almost 40.61% less than that of the amount formed from the marketed sample. While after 6 M, the amount of 3-FRSV formed increased from 12.9% to 15.3% only.

This is in conjunction with the earlier study done by Shishoo et al., 1999, carried out to evaluate the dissolution pattern of the various market FDC of rifampicin isoniazid and found that a maximum of 21% of 3-FRSV was formed in 45 min. from the market sample of rifampicin –isoniazid FDC during dissolution studies in 0.1N HCl.
### Table 3. Amount of 3-FRSV formed in 0.1N HCl from the market FDC and novel FDC on stability

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Market FDC</th>
<th>Novel FDC (Initial)</th>
<th>Novel FDC (3M)</th>
<th>Novel FDC (6M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5.98</td>
<td>1.99</td>
<td>1.87</td>
<td>2.6</td>
</tr>
<tr>
<td>30</td>
<td>7.58</td>
<td>3.6</td>
<td>2.87</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>12.36</td>
<td>5.1</td>
<td>4.8</td>
<td>7.5</td>
</tr>
<tr>
<td>120</td>
<td>14.54</td>
<td>5.9</td>
<td>6.7</td>
<td>9.3</td>
</tr>
<tr>
<td>180</td>
<td>15.43</td>
<td>7.5</td>
<td>8.38</td>
<td>10.5</td>
</tr>
<tr>
<td>240</td>
<td>17.09</td>
<td>9.2</td>
<td>9.73</td>
<td>10.7</td>
</tr>
<tr>
<td>300</td>
<td>19.93</td>
<td>10</td>
<td>11.4</td>
<td>12.6</td>
</tr>
<tr>
<td>360</td>
<td>21.36</td>
<td>11.7</td>
<td>13.4</td>
<td>14.4</td>
</tr>
<tr>
<td>480</td>
<td>23.32</td>
<td>12.6</td>
<td>14.7</td>
<td>14.9</td>
</tr>
<tr>
<td>520</td>
<td>25.76</td>
<td>12.9</td>
<td>15.1</td>
<td>15.3</td>
</tr>
</tbody>
</table>

In contrast, the amount of 3-FRSV formed during dissolution studies of the developed novel rifampicin-isoniazid FDC in 60 min. was found to be around 5.1% from initial sample and 7.5% after 6 M stability. The developed novel rifampicin isoniazid FDC after 6 M showed only an increase of 2.4% of 3-FRSV at the end of 520 min. (Table 31), indicating that the developed FDC formulation is stable.

Earlier studies have confirmed the enhanced degradation of rifampicin in presence of isoniazid (Shishoo et.al, 1999; Singh et.al., 2000). An elegant mechanism to explain increased degradation of rifampicin in acidic conditions in presence of isoniazid has been suggested. The reaction mechanism can be represented as below-

\[
\text{Rifampicin} + \text{H}_2\text{O} \rightleftharpoons 3\text{-FRSV} + 1\text{-amino 4-methyl piperazine}
\]

\[
\text{Isoniazid} + 3\text{-FRSV} \rightleftharpoons \text{Isonicotinyl hydrazone} + \text{H}_2\text{O}
\]

It has been found that subsequent to hydrolysis of rifampicin to 3-FRSV, isoniazid reacts with 3-FRSV to form isonicotinyl hydrazone of 3-FRSV in a reversible manner where the forward reaction is faster second order reaction while the backward reaction is a slower first order reaction. The overall reaction is favoured towards formation of hydrazone and hence, an overall increase in degradation of rifampicin to 3-FRSV is observed. At the same time, hydrazone are known to hydrolyse in the acidic medium resulting in regeneration of isoniazid and 3-FRSV. This indicates that isoniazid remains
unaffected and plays a role of a “catalyst” in the degradation of rifampicin in acidic conditions to 3-FRSV (Savale, 2003; Singh, et.al., 2006). And hence a higher degradation or formation of 3-FRSV was found in the case of market samples, wherein both rifampicin and isoniazid are released together in the acidic medium and facilitate the formation of hydrazone via formation of 3-FRSV.

While in case of novel FDC, 41% reduction in the formation of 3-FRSV can be attributed to the segregated release of rifampicin and isoniazid. In acidic medium, only rifampicin is released and not the isoniazid. The isoniazid is not released in acidic medium since it has been coated with enteric polymers, which provide the protection to isoniazid from being released in acidic medium. As a result of the absence of isoniazid in the acidic medium, the degradation of rifampicin is not favoured in the forward direction and hence there is a reduced formation of 3-FRSV in acidic medium. Here it is worthwhile to note that, the amount and profile of formation of 3-FRSV from the novel FDC of rifampicin isoniazid is not affected at accelerated stability conditions, even after 6 M (as shown in Fig 35 and Table 31).

It can be hence concluded that even after 6 M, rifampicin and isoniazid are found to be stable in the developed novel FDC. Also, minimal decomposition of rifampicin in vitro, thus provides a proof of the concept that formulating FDC rifampicin and isoniazid with segregated site of drug delivery results in improved stability of rifampicin in the FDC.

Additionally, to assess change in release profile statistically, similarity factor ($f_2$) was also calculated. To statistically confirm the similarity between the release profiles, $f_2$, factor was calculated. The $f_2$ factor for rifampicin release profile was found to be $>85$, while, for isoniazid $f_2 > 90$. The $f_2$ value above, 50 is indicative of similarity between the two release profiles compared.

Thus, it can be concluded that the release of rifampicin and isoniazid from the novel developed rifampicin isoniazid FDC is similar and is not significantly affected by the higher humidity and temperature conditions and are stable at accelerated stability conditions.

Based on non-significant change in uptake of water and drug content and $f_2$ values indicates that novel FDC of rifampicin and isoniazid is stable till 6 M at room
temperature and at accelerated stability conditions. Such formulations would rule out the possibility of failure in the performance of formulations due to stability-related problems during distribution and handling and especially in region IV countries. Region IV countries experience high temperature and humidity and are mainly categorised as TB high-burden countries like India, South Africa etc.

4.4.2 Bioavailability studies
Using the proposed sensitive and specific HPLC method, plasma levels of rifampicin and isoniazid were monitored after administration of rifampicin and isoniazid FDC formulation to human volunteers. Typical chromatograms showing peaks for rifampicin and isoniazid in human plasma samples of a volunteer collected at various time points after administration of rifampicin and isoniazid FDC are shown in Fig 36 and 37, respectively.

**Fig 36.** A typical chromatogram of rifampicin analysed in human plasma along with papaverine hydrochloride (internal standard)

![Chromatogram of rifampicin](image)

**Fig 37.** A typical chromatogram of isoniazid analysed in human plasma along with pyrazinamide (internal standard)

![Chromatogram of isoniazid](image)
Comparative plasma concentration-time profiles of rifampicin, after administration of rifampicin and isoniazid FDC, novel FDC and marketed FDC formulation are shown in Fig 38. Comparative plasma profiles of rifampicin after administration of rifampicin and isoniazid FDC, novel FDC reveals, an increase in plasma levels of rifampicin from the novel FDC formulation in comparison to marketed FDC formulation (Fig 38).

**Fig 37.** Mean plasma rifampicin concentration (µg/ml) versus time profile of isoniazid for novel FDC and market FDC of rifampicin and isoniazid in healthy male subjects under fasting conditions

![Graph showing mean plasma rifampicin concentration (µg/ml) versus time profile of isoniazid](image)

Similarly, comparative plasma concentration-time profiles of isoniazid, after administration of rifampicin and isoniazid FDC, novel FDC and marketed FDC formulation are shown in Fig 39. Plasma profile of isoniazid from novel rifampicin isoniazid FDC formulation clearly shows a lag time of 2 h followed by a rapid absorption of isoniazid. Whereas, in case of marketed FDC formulation an immediate absorption of isoniazid can be seen (Fig 39).
Fig 39. Mean plasma isoniazid concentration (µg/ml) versus time profile of isoniazid for novel FDC and market FDC of rifampicin and isoniazid in healthy male subjects under fasting conditions

Various pharmacokinetic parameters, mainly $C_{\text{max}}$, $K_{\text{el}}$, $T_{\text{max}}$, $T_{1/2}$ and $\text{AUC}_{0-\infty}$ for rifampicin and isoniazid obtained after administration of novel rifampicin isoniazid FDC formulation of rifampicin isoniazid FDC were determined by subjecting the respective plasma concentration-time data to noncompartmental analysis and are summarized in Table 32 and 33.

Bioequivalency is the most important quality control tool as a surrogate for the therapeutic efficacy. The rate and extent measures become surrogate indicators of therapeutic outcome to assess the drug product performance. The maximum plasma concentration ($C_{\text{max}}$) and the time of its occurrence ($T_{\text{max}}$) are thought to be reasonable measures for rate of absorption. The determination of the area under the concentration–time curves (AUCs) is the method most commonly used by regulatory agencies to assess the extent of drug absorption after single-dose administration of oral products (Panchagnula et al., 2006).

The average $C_{\text{max}}$ value (representing the rate of absorption) for rifampicin after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed FDC formulation, was found to be 10.27 µg/ml and 8.64 µg/ml, respectively (as shown in Table 32). Thus, it can be concluded that an increase of about 18.87% was observed in $C_{\text{max}}$ after administration of novel rifampicin-isoniazid FDC in comparison to the marketed FDC.
Table 32. Summary of pharmacokinetic parameters of rifampicin plasma profile, after administration of the market FDC vs novel FDC formulations

<table>
<thead>
<tr>
<th>Formulation A (Market FDC)</th>
<th>Formulation B (Novel FDC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
</tr>
<tr>
<td>Mean</td>
<td>8.64</td>
</tr>
<tr>
<td>SD</td>
<td>3.85</td>
</tr>
<tr>
<td>SEM</td>
<td>1.22</td>
</tr>
<tr>
<td>Min</td>
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</tr>
<tr>
<td>Max</td>
<td>14.79</td>
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<tr>
<td>% CV</td>
<td>44.59</td>
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<table>
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<th>Formulation A (Market)</th>
<th>Formulation B (Novel FDC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
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</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>1.10</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µg.h/ml)</td>
<td>29.54</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (µg.h/ml)</td>
<td>29.54</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>3.27</td>
</tr>
<tr>
<td>K&lt;sub&gt;el&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<tr>
<td>Mean</td>
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<tr>
<td>SD</td>
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<tr>
<td>SEM</td>
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<tr>
<td>Min</td>
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<tr>
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Table 33. Summary of pharmacokinetic parameters of isoniazid plasma profile, following administration of the market FDC vs novel FDC formulations
The mean AUC \(0-\infty\) values (representing the extent of absorption) for rifampicin after administration of rifampicin and isoniazid FDC formulation, novel FDC as well as marketed FDC formulation, were found to be 92.58 µg.h/ml and 46.64 µg.h/ml respectively (Table 32). Thus, almost a 2 fold increase was observed in the AUC of rifampicin after administration of novel anti-TB FDC.

The average values of \(K_{el}\) for rifampicin were found to be 1.09 h\(^{-1}\) and 1.05 h\(^{-1}\) after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed FDC formulation, respectively. Similarly, mean \(t_{1/2}\) for rifampicin after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed FDC formulation, were found to be 3.00 h and 0.77 h, respectively (Table 32). As evidenced by the respective \(t_{1/2}\) values, availability of rifampicin from novel anti-TB FDC is prolonged. This is in-line with the formulation design of rifampicin formulation wherein, immediate release of rifampicin is followed by prolonged and sustained release of rifampicin.

Here it is worthwhile to mention that, for the success of the treatment of tuberculosis, good bioavailability leading to adequate plasma concentrations of rifampicin is an absolute prerequisite. It has been postulated that peak plasma rifampicin concentrations should be of the order of 10-15 µg/ml for good therapeutic response. Lower plasma levels of rifampicin results in the reduced rate of sputum conversion (Immanuel et.al., 2003). This further causes incomplete treatment, which may eventually leads to development of drug resistant TB (Bloomberg et.al., 2002).

Over the years, it has been confirmed that the bioavailability of rifampicin is significantly impaired when it is administered along with isoniazid as a FDC, in comparison with administration of formulation containing only rifampicin (Shishoo et.al., 2001; Immanuel, et.al., 2003). An almost 30% fall in bioavailability of rifampicin has been reported on administration of rifampicin isoniazid FDC. The drop of bioavailability of rifampicin from FDC products has been attributed to a facile drug-drug reaction between rifampicin and isoniazid in the acidic medium of stomach, whereby significant loss of drug occurs before absorption. The proposed mechanism demonstrates that rifampicin is first hydrolysed under acid conditions to 3-formylrifamycin, which reacts further with isoniazid to form isonicotinyl hydrazone (HYD). The HYD converts back to isoniazid and 3-FRSV, resulting in the recovery of
isoniazid, but does cause the loss of rifampicin. This explains why the bioavailability problem is confined to rifampicin alone and not to isoniazid (Shishoo et. al., 2001; Singh et. al., 2001; Singh et. al., 2006). This is reflected in the poor bioavailability from the rifampicin isoniazid FDC formulation. This reaction has been ascribed to be responsible for the reduced bioavailability of rifampicin from FDC products (Immanuel et. al., 2003; Shishoo et. al., 2001). The deficiency in delivery of proper dose of rifampicin has serious implications as it is known that doses of rifampicin less than 9 mg/kg body weight can result in therapeutic failure (Long et. al., 1979) and hence can result in the development of drug resistance.

As a result, the IUATLD and the WHO issued a joint statement advising tuberculosis control programme managers intending to use FDC drugs to purchase only products with demonstrated rifampicin bioavailability (IUATLD/WHO, 1999).

Thus, a 2 fold increase in AUC and 18.87% increase in $C_{\text{max}}$ of rifampicin on oral administration of novel developed FDC is an indicator of better therapeutic efficacy of rifampicin from novel anti-TB FDC.

While for isoniazid the average $C_{\text{max}}$ values (representing the rate of absorption) after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed FDC formulation was found to be 6.04 µg/ml and 5.74 µg/ml (Table 3). The average $T_{\text{max}}$ values for rifampicin after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed FDC formulation were 3.00 h and 2.30 h, respectively. While corresponding values of the average $T_{\text{max}}$ for isoniazid after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed FDC formulation were 2.80 h and 1.10 h, respectively (Table 3). Delay in $T_{\text{max}}$ value indicates a delay in absorption of isoniazid from novel anti-TB FDC. This confirms the hypothesis that the delayed release isoniazid pellets releases isoniazid at the site of maximum absorption i.e. in intestine.

The average values of $K_{\text{el}}$ for isoniazid were 0.28 h$^{-1}$ and 0.31 h$^{-1}$ after administration of rifampicin and isoniazid FDC formulation, novel FDC and formulation, respectively. While, mean $t_{1/2}$ for isoniazid after administration of rifampicin and isoniazid FDC
formulation, novel FDC and marketed FDC formulation, were 3.27 h and 3.01 h, respectively (Table 33).

The mean AUC$_{0-\infty}$ values (representing the extent of absorption) for isoniazid after administration of rifampicin and isoniazid FDC formulation, novel FDC as well as marketed FDC formulation, are 33.93 µg.h/ml and 29.54 µg.h/ml respectively (Table 33). Thus, it can be concluded that the extent of absorption is not affected by the delay in absorption of isoniazid from novel anti-TB FDC. This may be attributed to the fact that the HYD formed due to interaction between rifampicin and isoniazid gets converted back to isoniazid and 3-formylrifamycin, resulting in recovery of isoniazid, but eventually causing the loss of rifampicin. Thus, not affecting the extent of absorption i.e. bioavailability of isoniazid (Shishoo et al., 2001; Singh et al., 2001; Singh et al., 2006).

**Statistical analysis of the human bioavailability data**

Bioavailability parameters (C$_{\text{max}}$ and AUC$_{0-\infty}$) for both rifampicin and isoniazid from novel FDC formulation of rifampicin and isoniazid FDC were compared with that of market FDC formulation of rifampicin and isoniazid FDC by applying ANOVA test. A summary statistics of rifampicin and isoniazid on administration of rifampicin isoniazid FDC to healthy male subjects under fasting conditions are given Table 34.

The current USFDA criteria for average bioequivalence of the dosage forms requires that the mean pharmacokinetic parameters (C$_{\text{max}}$ and AUC$_{0-\infty}$) for novel rifampicin and isoniazid FDC) should be within 80-125% of the marketed FDC formulation) using the 90% CI.

In the present study for rifampicin, it was observed that the mean pharmacokinetic parameters (C$_{\text{max}}$ and AUC$_{0-\infty}$) did not fall within 90% CI (Table 34). Thus, the results clearly demonstrate that the two product compared are not bioequivalent. The average C$_{\text{max}}$ and AUC$_{0-\infty}$ indicates that there is significant improvement in bioavailability of rifampicin from reference formulation as compared to that of novel FDC formulation of rifampicin and isoniazid, which is an indicator of its better therapeutic effectiveness. This is in confirmation to earlier observation made by Shishoo et al., 2001 and Singh et al., 2001, that segregated release of rifampicin and isoniazid in different parts of GIT tract, will prevent the two drugs coming into contact with each other in the stomach and thereby improving the bioavailability of rifampicin.
In case of isoniazid, it was observed that the mean pharmacokinetic parameters ($C_{\text{max}}$ and AUC$_{0-\infty}$) for novel rifampicin isoniazid FDC dosage form were well within 80-125%, indicating that the two products were bioequivalent. This is in line with the fact stated in earlier independent findings by Shishoo et al., 2001 and Singh et al., 2001, that the bioavailability problem is confined to rifampicin alone and not isoniazid. This has been attributed to the fact that HYD formed due to a facile interaction between rifampicin and isoniazid, is converted back to isoniazid and 3-formylrifamycin, resulting in recovery of isoniazid.

On the other hand, $T_{\text{max}}$ value of 2.80 ± 0.17 clearly indicates a delay of approximately 3h in absorption of isoniazid (Table 34). This is in agreement with the formulation design, wherein the isoniazid is formulated in a delayed release formulation. Thus the bioavailability study provides the direct confirmation to the concept that segregated delivery of rifampicin and isoniazid from anti-TB FDC should result in improved bioavailability of rifampicin from FDC’s.
Table 34. Summary statistics of rifampicin and isoniazid on administration of rifampicin isoniazid FDC to healthy male subjects under fasting conditions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RIFAMPICIN</th>
<th>ISONIAZID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>$AUC_{\infty}$ (µg.hr/ml)</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novel FDC (B)</td>
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<td>92.58</td>
</tr>
<tr>
<td>Market FDC (A)</td>
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<tr>
<td>Least Square Mean (LSM)</td>
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<tr>
<td>Novel FDC</td>
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<td>39.7671</td>
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<tr>
<td>LSM Ratio</td>
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</tr>
<tr>
<td>B/A %</td>
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<td>90 % Confidence Interval</td>
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<tr>
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</tr>
<tr>
<td>Upper Limit</td>
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<td>384.5</td>
</tr>
<tr>
<td>Point estimate</td>
<td>1.18</td>
<td>1.985</td>
</tr>
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</table>
4.5 Conclusions
The novel rifampicin isoniazid FDC capsules was prepared and evaluated for:
   a. Stability studies
   b. Human bioavailability studies
Based on the study the following conclusions could be drawn:

- The stability study of rifampicin-isoniazid FDC capsules was carried out at room temperature and at the accelerated stability conditions. The drug content within pharmacopoeial limits and constant water uptake indicates non significant influence of higher humidity and temperature on the novel anti-TB FDC.

- The samples of 6 M of novel FDC showed a release of 85.2 ± 1.78% of rifampicin and formation of 6.7 ± 1.12% in 60 of 3-FRSV along with 85% of release of isoniazid. The amount of 3-FRSV formed from the novel anti-TB FDC was almost 37.22% less than that of the amount formed from the marketed sample. The similarity factor, $f_2$ factor, of all the release profiles for the stability samples with respect to their initial values was > 50, indicative that release of rifampicin and isoniazid from the novel developed rifampicin isoniazid FDC is similar and is not affected by the higher humidity and temperature conditions. Minimal decomposition in vitro, thus provide a proof of concept that formulating FDC rifampicin and isoniazid with segregated site of drug delivery results in improved stability of rifampicin in the FDC.

- The novel rifampicin-isoniazid was evaluated for bioavailability of rifampicin and isoniazid in comparison to the marketed rifampicin-isoniazid FDC sample. The 90% CI for the pharmacokinetic parameter $C_{\text{max}}$ and $\text{AUC}_{0-\infty}$ of rifampicin was well outside the bioequivalence criteria. This indicates that the two products compared are not bioequivalent. However, the average $C_{\text{max}}$ values for rifampicin after administration of rifampicin and isoniazid FDC formulation, novel FDC and marketed formulation, were 10.27µg/ml and 8.64 µg/ml, corresponding to an increase of about 18.87%. Thus, a 2 fold increase in AUC and 18.87% increase in $C_{\text{max}}$ of rifampicin on oral administration of novel developed FDC is an indicator of better therapeutic efficacy of rifampicin from novel anti-TB FDC.

- Also, as evidenced by the respective $t_{1/2}$ values, novel rifampicin and isoniazid FDC offers an immediate release of rifampicin followed by prolonged and the sustained release of rifampicin. This is in agreement with the formulation design of rifampicin
formulation wherein, immediate release of rifampicin is followed by prolonged and sustained release of rifampicin.

- The 90% CI for the pharmacokinetic parameter $C_{\text{max}}$ and $AUC_{0-\infty}$ of isoniazid was well within the bioequivalence criteria. This indicates that the two products compared are bioequivalent. However, $T_{\text{max}}$ value of $2.80 \pm 0.17$ clearly indicates a delay of approximately 3h in absorption of isoniazid. This indicates that the extent of absorption is not affected by the delay in absorption of isoniazid from novel anti-TB FDC. This is in agreement with the formulation design, wherein the isoniazid is formulated in a delayed release formulation.

- The bioavailability study provides the confirmation to the concept that segregated delivery of rifampicin and isoniazid from anti-TB FDC will result in improvement of bioavailability of rifampicin from FDC’s without affecting the bioavailability of isoniazid. Such formulations would rule out any possibility of performance failure of the formulations due to stability-related problems during distribution and handling and especially in the climatic region IV countries.

- Finally, it can be concluded that the developed novel FDC formulation is a stable formulation with improved bioavailability. Thus, this formulation could have better therapeutic efficacy.
References


Stability & Human bioavailability of novel rifampicin and isoniazid FDC


