Chapter Six

Summary and Conclusion
6. SUMMARY AND CONCLUSION

6.1. SUMMARY

The aim of the present work was the development of oral particulate system of rifampicin and isoniazid for intestinal tuberculosis. The major objectives were:

➢ To develop a site specific particulate delivery system of antimycobacterial drugs, this would overcome the bioavailability problems, reduce the dose dependent side effects of the drugs and also minimize the dosing frequency of the formulations.
➢ To evaluate the system for intestinal antitubercular activity.
➢ To evaluate the optimized particulate delivery system.
➢ To evaluate the possibility of drug-polymer (excipients) interference and interaction by interference and interaction studies.
➢ To perform the in vivo studies of the formulation on animals.
➢ To determine the physicochemical stability and shelf life of the formulations.

Particulate technology has been greatly explored for the site specific delivery of drugs. It has been utilized for oral delivery of drugs and bioactives, mainly for the purpose of by passing the first pass hepatic metabolism and sustaining the drug levels. Extensive literature survey showed the potential of such a delivery system to achieve the desired purpose.

Reported data on drug permeability suggested that rifampicin was well absorbed from the stomach and isoniazid from the intestine. A wide range of both enteric as well as non-enteric biodegradable polymers was screened for the study. Sodium alginate, alkaline extracted ispaghula husk (AEISP), Eudragit RSPO and ethylcellulose were selected for the preparation of microparticles.

Isoniazid and rifampicin were obtained as gift sample from Cipla Ltd., Mumbai. Physicochemical characterization and identification of drugs were performed according to following studies- appearance, color, odor, melting point, solubility, pH, fourier transform-infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), ultraviolet (UV) spectral analysis. A stability indicating high performance thin layer chromatographic (HPTLC) method was developed for isoniazid and rifampicin. To investigate the possibility of interference between drugs and polymers, a preliminary interference study was carried out. The study revealed that none of the polymer showed absorbance at the λ max of the two drugs.
Drug polymer interaction study was performed by FTIR and DSC methods. Comparison of FTIR spectra showed almost similar peaks indicating the insignificant interaction between the drugs and polymers. Comparison of DSC thermogram showed almost similar peaks (decreased intensity) for the drug and drug-polymer combination, indicating the insignificant interaction between the drugs and polymers.

Isoniazid microparticles were prepared by emulsification-internal ionic gelation method. A four-factor, three-level Box-Behnken design was used for the optimization procedure. The optimized formulation was evaluated by determining entrapment efficiency; surface morphology and particle size analysis; drug polymer interaction study in the formulation; study of mucoadhesive property of optimized microparticles; swelling behaviour of microparticles; *in vitro* drug release study and *in vivo* study in Wistar rats.

The drug content of the microparticles was determined spectrophotometrically at $\lambda_{\text{max}}$ 263 nm. The entrapment efficiency of optimized formulation was calculated as 83.43 %. The shape and surface morphology of the drug-loaded as well as blank microparticles were investigated using a scanning electron microscope (SEM). The optimized formulation showed spherical and rough surface microparticles. The sizes of the microparticles were determined by an optical microscope fitted with an ocular micrometer. The optimized formulation showed an average of 51.53 µm particle size. The drug polymer interaction study of microparticles was carried out by differential scanning calorimetry (DSC), Fourier transform-infrared spectroscopy (FTIR) and X-ray diffraction analysis (XRD). The DSC thermogram of isoniazid showed a sharp melting endotherm at 178 °C. The intensity of the peak was slightly diminished in the physical mixture, which can be due to the dilution factor. The endothermic peak of isoniazid in drug loaded microparticles was not distinctive indicating that the drug was no longer present in the crystalline form. The FTIR spectrum of isoniazid showed a strong C=O stretching at 1667 cm$^{-1}$ and N-H stretching at 3304 cm$^{-1}$. Most of the characteristic peaks were present in the formulation but some peaks were not appearing may be due to dilution factor. The drug-loaded microparticles diffractogram showed completely diminished signal intensity as compared to pure isoniazid, suggesting that the drug was not in crystalline form.

The results of the wash off test indicated that the optimized formulation exhibited fairly good mucoadhesive (87±0.57 % up to 10 h) properties as compared to non-mucoadhesive ethylcellulose microparticles. The maximum water uptake was obtained at 10 h
in simulated intestinal fluid, pH 7.4 (swelling index, 749.80±4.10), after which erosion and break down of microparticles occurred. The result suggest that the dried gel microparticles would swell slightly in the stomach and, when they are transferred to upper intestine, the microparticles would start to swell more and behave as matrix for controlled release of incorporated drug, but they are eroded in the lower intestine.

It is normally seen that when microparticles of hydrophilic polymers are immersed in water, they swell and form a gel like diffusion layer that may produce hindrance for outward transport of the drug and hence providing a controlled release effect. But at acidic pH, the alginate microparticles shrink due to compactness of the gel network. Sodium alginate-AEISP microparticles swelled more in simulated intestinal fluid (pH 7.4) as compared to simulated gastric fluid (pH 1.2). The release would be dependent on diffusion of isoniazid through the sodium alginate-AEISP polymer matrix in simulated gastric fluid. Slow erosion of barium-sodium alginate-AEISP complex could occur through slight degradation of polymers backbone into smaller molecular weight component. The results of the dissolution study indicated that the amount of drug release significantly decrease with an increase in the concentration of polymers (p < 0.05). It can be attributed to increase in the densities of the polymer matrix resulting in larger microparticles and this in turn increase the diffusional path length. As the concentration of cross-linking agent increased the drug release from the microparticles were also delayed (p < 0.05). The optimized formulation gave the sustained release of the drug up to 12 h in simulated intestinal fluid (pH 7.4).

In order to find out the mechanism of drug release, the in vitro dissolution data were applied to various kinetics models. The best fit with highest regression coefficient values ($R^2$) was predicted by zero order (0.998), Higuchi model (0.933) and Hixson-Crowell (0.944) model. This indicates that the release of isoniazid from microparticles is diffusion controlled. The experimental data were further applied to the Korsemeyer and Peppas equation, which characterized the transport mechanism. The value of release exponent (n) for the proposed model was 0.3915, indicating a Fickian transport mechanism controlled by swelling and relaxation of polymer. The dried gel microparticles swelled slightly in simulated gastric fluid. However they swelled more in simulated intestinal fluid (pH 7.4). So the release of drug from microparticles took place by diffusion through the swelling of matrix and relaxation of the polymers in simulated gastric fluid. However at pH 7.4, the release was due to diffusion of drug from the swelled matrix.
The uptake of the pertechnetate anions by the microparticles was determined using an auto gamma counter. Nearly the uptake value was more than 95%. Various concentration of stannous chloride dihydrate were used for the formation of complex of $^{99m}\text{Tc}$–pertechnetate–microparticles. Among these the 60 µg concentration of stannous chloride dihydrate was the suitable showing up to 96.1% labeling efficiency. The pH of the reaction varied from 1.2-8.0. Maximum labeling efficiency was observed at pH 7.5. Radiochemical impurity that is likely to exist in the form of unconjugated technetium was found up to 7.1% of free $^{99m}\text{Tc}$ - pertechnetate determined by ITLC. The stability study was carried out to confirm that the $^{99m}\text{Tc}$ remained bound to the formulation for the duration of the study i.e. during transit time of formulation through GI tract. The stability of $^{99m}\text{Tc}$ labeled microparticles was tested in the saline and in the serum at 12 h. Only 3.9% to 7.9% (in saline) and 4.3% to 10.9% (in serum) degradation was seen during the study.

Biodistribution studies in the Wistar rats showed that the major accumulation of the activity in terms of % administered dose per gram of organ was in the intestine (12.3±0.023) followed by liver (9.5±0.05) and lung (9.1±0.39).

During the gamma scintigraphy study the presence of microparticles could be marked in the intestinal lumen 0.5 h after the oral administration. Microparticles could also be detected in the intestine after 12 h however the percent radioactivity had significantly decreased (t1/2 of $^{99m}\text{Tc}$ = 4–5 h). Presence of microparticles in the GIT could not be assessed after 12 h of administration due to negligible radioactivity.

The different pharmacokinetic parameters of isoniazid loaded microparticles were calculated by determining the concentration of drug in blood plasma. The pharmacokinetic parameters of isoniazid loaded microparticles were compared with the pharmacokinetic parameters of free isoniazid. There was an increased drug concentration achieved in plasma within 6 h having C$_{\text{max}}$ (3.626±0.35 µg/mL) in case of isoniazid loaded microparticles as compared to free isoniazid (C$_{\text{max}}$ 2.366±0.13 µg/mL in 1 h). The drug levels at different time intervals in plasma were higher in animals resulting in significant improvement in AUC$_{0-\infty}$. The AUC$_{0-\infty}$ was found to be higher for isoniazid microparticles resulting in a significant increase ($p < 0.001$) in relative bioavailability of encapsulated drugs in comparison with free drugs.
Rifampicin microparticles were prepared by quasi-emulsion solvent diffusion method. A four-factor, three-level Box-Behnken design was used for the optimization procedure. The optimized formulation was evaluated by determining entrapment efficiency; surface morphology and measurement of micromeritic properties of microparticles; in vitro buoyancy studies; drug polymer interaction study in the formulation; in vitro drug release study and in vivo study.

The drug content of the microparticles was determined spectrophotometrically at $\lambda_{\text{max}}$ 475 nm. The entrapment efficiency of optimized formulation was calculated as 86.65 %. The shape and surface morphology of the drug-loaded as well as blank microparticles were investigated by using scanning electron microscope (SEM). The optimized formulation showed spherical and rough surface microparticles. The sizes of the microparticles were determined by an optical microscope fitted with an ocular micrometer. The optimized formulation showed an average of 40.46 $\mu$m particle size. Bulk density, compressibility index, Hausner’s ratio and angle of repose of the optimized formulation were also calculated. More than 80 % of the particles kept floating for at least 10 h according to buoyancy study. Floating of microparticles for 10 h was considered satisfactory performance. The drug polymer interactions study of microparticles was carried out by differential scanning calorimetry (DSC), fourier transform-infrared spectroscopy (FT-IR) and X-ray diffraction analysis (XRD). The intensity of the melting endotherm (190 °C) was slightly diminished in the physical mixture of drug and polymers which may be due to the dilution factor. The endothermic peak of rifampicin in drug loaded microparticles was not distinctive indicating that the drug was no longer present in the crystalline form. The FTIR spectrum of drug loaded microparticles showed almost similar characteristic peaks indicating the insignificant interaction between the drugs and polymers. The drug-loaded microparticles diffractogram showed decrease in the intensity of the signals as compared to the pure rifampicin suggesting that the overall crystallinity of the drug-loaded microparticles was decreased.

The rate of drug release from the rifampicin microparticles could be controlled by adjusting the concentrations of the polymers and drug dispersing agents. EuRSPO and ethylcellulose were selected as the retarding agent to control the drug release rate. Increasing the amount of polymers resulted in a marked decreased in drug release ($p < 0.05$). The release rate of rifampicin from microparticles was increased with increasing the amount of talc in the
formulation ($p < 0.05$). The optimized formulation gave the sustained release of the drug up to 12 h in simulated gastric fluid (pH 1.2).

In order to find out the mechanism of drug release, the *in vitro* dissolution data were applied to various kinetics models. The best fit with highest regression coefficient values ($R^2$) was predicted by zero order (0.968), Higuchi model (0.956) and Hixson-Crowel (0.936). This indicates that the release of rifampicin from microparticles was concentration independent. The experimental data were further applied to the Korsemeyer and Peppas equation, which characterized the transport mechanism. The value of release exponent ($n$) for the proposed model was 0.3056, indicating a Fickian transport mechanism.

The uptake of the pertechnetate anions by the microparticles was determined using an auto gamma counter. Nearly the uptake value was more than 95 %. Various concentration of stannous chloride dihydrate were used for the formation of complex of $^{99m}\text{Tc}$ - pertechnetate- microparticles. Among these the 60 µg concentration of stannous chloride dihydrate was suitable showing up to 97.69 % labeling efficiency. The pH of the reaction varied from 1.2-8.0. Maximum labeling efficiency was observed at pH 6.8. Radiochemical impurity that is likely to exist in the form of unconjugated technetium was found up to 6.79 % of free $^{99m}\text{Tc}$ - pertechnetate determined by ITLC. The stability study was carried out to confirm that the $^{99m}\text{Tc}$ remained bound to the formulation for the duration of the study i.e. during transit time of formulation through GI tract. The stability of $^{99m}\text{Tc}$ labeled microparticles was tested in the saline and in the serum at 12 h. only 2.31 % to 7.59 % (in saline) and 3.11 % to 9.86 % (in serum) degradation was seen during the study.

Biodistribution studies in the Wistar rats showed that the major accumulation of the activity in terms of % administered dose per gram of organ were in the stomach (14.7±0.12) followed by liver (10.7±0.08) and lung (9.8±0.54).

Examination of the sequential gamma scintigraphic images during the study clearly indicated that the rifampicin microparticles, remained buoyant and uniformly distributed in the gastric contents for the study period of 6 h. Prolonged GRT of microparticles was achieved in all rats for over 6 h, which remained buoyant in the stomach for the entire test period. After swallowing, the microparticles adopted a floating position on top of the stomach content. This might be due to the presence of porous low density polymers and hollow cavity inside the microparticles. Measurable number of counts of $^{99m}\text{Tc}$-tagged...
microparticles during 6 h study period suggested very good gastro-retentive property, because the administered microparticles remained floating and distributed properly in the stomach contents during the study period of 6 h. Gamma scintigraphy was performed for 6 h i.e. the half-life of the $^{99m}$Tc being 6 h. Microparticles could also be detected in the intestine after 12 h, however the percent radioactivity had significantly decreased ($t_{1/2}$ of $^{99m}$Tc = 4–5 h).

The different pharmacokinetic parameters of rifampicin loaded microparticles were calculated by determining the concentration of drug in blood plasma. The pharmacokinetic parameters of rifampicin loaded microparticles were compared with the pharmacokinetic parameters of free rifampicin. There was an increased drug concentration achieved in plasma within 4 h having $C_{\text{max}}$ (1.89±0.02 µg/mL) in case of rifampicin loaded microparticles as compared to free rifampicin ($C_{\text{max}}$ 1.21±0.1802 µg/mL in 1 h). The drug levels at different time intervals in plasma were higher in animals resulting in significant improvement in AUC$_{0-\infty}$. The AUC$_{0-\infty}$ was found to be higher for rifampicin microparticles resulting in a significant increase ($p < 0.001$) in relative bioavailability of encapsulated drugs in comparison with free drugs.

Results obtained from in vitro antimycobacterial study revealed that the developed antitubercular formulation was successful in inhibiting the growth of mycobacterium in simulated gastrointestinal tract environment. In this way we can say that the developed antitubercular formulation can be used for the treatment of abdominal/intestinal tuberculosis. Results obtained from in vitro antibacterial studies revealed, prolong antibacterial efficacy of developed formulations which support the prolonged release effect of optimized formulation.

Optimized formulation was found to be stable. Degradation of the isoniazid was faster as compared to rifampicin. So the shelf life can be predicted by the isoniazid shelf life. At higher temperature ($K = 2.0727 \times 10^{-4}$ days$^{-1}$ at 60 °C) leading to more than 1.92 % degradation by the end of 90 days of isoniazid. Degradation rate constant at 25 °C was 1.7073 $\times 10^{-4}$ days$^{-1}$ predicting a shelf life 1.69 year for the formulation.

The oral prolonged release dosage form was prepared by incorporating an equivalent amount of isoniazid and rifampicin microparticles in a hard gelatin capsule shell as in the marketed FDC product. In vitro dissolution studies of marketed formulations comprising of single drug and combination of isoniazid and rifampicin suggested the presence of some interference between the two drugs in simulated gastric fluid (pH 1.2). Only minor
differences were seen in the extent of drug release of both the drug in simulated intestinal fluid (pH 7.4). However, this drug delivery system was successful in preventing this interference to a great extent by reducing the release of isoniazid in the gastric environment from microparticles. Rifampicin was released to the maximum extent in the gastric environment while isoniazid was released to the maximum extent in the intestinal environment.

6.2. CONCLUSION

The present work was an attempt to develop a site specific oral sustained release particulate delivery system of isoniazid and rifampicin for the treatment of intestinal tuberculosis with the aim to reduce the interaction between the two drugs.

The following conclusions were drawn from the results obtained:

- Physicochemical characterization of the drug was performed which indicated the authenticity of the active ingredient.
- Compatibility studies showed no interaction between drug and polymers.
- Development and optimization of the particulate system was performed with the help of Box-Behnken statistical design.
- Optimized formulation was characterized for various parameters.
- Interaction study of the formulation was performed by using FTIR, DSC and XRD method, which indicated some insignificant interactions.
- Particulate delivery system for both the drug showed in vitro drug release up to more than 12 h.
- Results obtained from in vitro antimycobacterial study revealed that the developed antitubercular formulation was successful in inhibiting the growth of mycobacterium in simulated gastrointestinal tract environment.
- Results obtained from in vitro antibacterial studies revealed, prolong antibacterial efficacy of developed formulations which support the prolonged release effect of optimized formulation.
- Biodistribution studies in the Wistar rats showed that the major accumulation of the activity in terms of % administered dose per gram of organ were in the stomach (rifampicin delivery system) and in intestine (isoniazid delivery system).
- Gamma scintigraphy studies showed presence of rifampicin particulates in the stomach up to 12 h and for isoniazid particulates in the intestine up to 12 h.
Pharmacokinetic studies results revealed a significant increase in relative bioavailability of encapsulated drugs in comparison with free drugs.

Optimized formulations were found to be stable. Degradation of the isoniazid was faster as compared to rifampicin. So the shelf life can be predicted by the isoniazid shelf life, which was calculated as 1.69 year.

The oral sustained release dosage form was prepared by incorporating an equivalent amount of isoniazid and rifampicin microparticles in a hard gelatine capsule shell as in the marketed FDC product.

This drug delivery system was successful in preventing the drug interference to a great extent due to reduced release of isoniazid in the gastric environment from microparticles. Rifampicin was released to the maximum extent in the gastric environment.

Results obtained from in vitro antimycobacterial study revealed that the developed antitubercular formulation was successful in inhibiting the growth of mycobacterium in simulated gastrointestinal tract environment. In this way we can say that the developed antitubercular formulation can be used for the treatment of abdominal/intestinal tuberculosis.