8. Comparative evaluation of enteric formulations of Curcumin

The purpose of this section was to perform a comparative evaluation of different optimized enteric formulations of CUR, namely, nanoemulsifying preconcentrate (SNP (SP2); discussed in chapter 5), polymeric Self-emulsifying nanocapsules (PSN (P5); discussed in chapter 6), and polymeric enteric nanospheres (NS (P16); discussed in chapter 7). Evaluation was carried out on the basis of parameters such as stability, *in vitro* drug release as well as the pharmacokinetic profile.

8.1. Stability study

Physicochemical stability of the optimized formulations was carried out, according to the ICH and WHO guidelines. Optimized formulations (P5, P16 and SP2) were kept in screw capped vials and kept in the stability chamber maintained at 40±2 °C and 75±5 %RH for 3 months. Samples were analyzed for drug content and particle size at predetermined time intervals (30, 60 and 90 days).

8.3. *In vitro* dissolution study

*In vitro* release of CUR from the optimized formulations (P5, P16 and SP2) was compared with the CUR reference formulation. Details of the method are described previously in section 6.4.8.

8.4. Pharmacokinetic studies

Pharmacokinetic studies were performed to determine the bioavailability of CUR, following oral administration of optimized CUR formulations (P5, P16 and SP2), and compared with CUR reference formulation. Details of the method are described previously in section 6.4.9.

8.5. *In vivo* roentgenographic analysis

Oral suspension of plain curcumin and optimized formulation; P5 (100 mg/kg), was administered as a suspension in 0.5% carboxy methyl cellulose equally diluted with radiopaque diatrizoate meglumine and diatrizoate sodium solution (76%). Animals (guinea pigs) were fasted overnight with free access to water. Roentgenographic images of the animal's abdomen were captured at predefined time intervals, to localise the movement of the formulation throughout the GIT. Images were captured at 45 kV, 5mAs/200 using Siemens X-ray machine, USA with 300 mA with fluoroscopy.

8.6. Degradation analysis

0.1 g of CUR-SNP formulation (P5) and pure CUR were dissolved in 100 ml of 0.01 mol/L alkali solution (phosphate buffer; pH 7.2), separately. Both the solutions were stored in dark room for 24 h and thereafter analyzed for drug content using HPLC.
8.7. Cell viability assay

Effect of CUR loaded PSN formulation (P5) on cell growth was determined on human colon carcinoma, HT29 cell line. The cell growth inhibitory activity of samples was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Gou et al., 2011). Cell lines were maintained in folate deficient RPMI 1640 medium under suitable conditions supplemented with 2 mM glutamine, 1% PenStrep (Sigma Aldrich, St. Louis, USA) and 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂/95% relative humidity (CO₂ Incubator; Binder, Germany). When the cells were confluent, they were trypsinized and seeded into 96-well culture plates at a cell density of 2 X 10^3 cells per well and the plates were maintained under the conditions previously mentioned. Twenty-four hours later, the old medium was carefully aspirated and the cells were incubated in a logarithmic growth phase with various concentrations ranging from 0 to 30 µg/ml of free CUR, equivalent CUR loaded PSN formulation (P5), plain PSN formulation and DMSO (control). After 24 h of incubation, the old medium was aspirated and replaced with fresh medium. MTT dye (0.5 mg/ml, 20 µL) was added to each well and the plate was incubated for further 4 h at 37 °C allowing viable cells to reduce MTT into purple formazan crystal (Sgouras et al., 1990). After incubation, the medium was removed and 200 µL of dimethyl sulphoxide (DMSO) was added and the optical density was measured at 450 nm using a microplate reader (Sunrise Tecan, Mannedorf, Switzerland). Cell viability was expressed as a percentage compared to a control (cell lines not treated with either formulation or free CUR), using the following equation:

\[
\text{% Cell Viability} = \frac{N_I}{N_C} \times 100
\]

Where, N_I and N_C are the number of surviving cells in the group treated with CUR loaded formulation and in the untreated cell group, respectively.

8.8. Results

8.8.1. Physicochemical stability

Physicochemical stability study of CUR formulations at 40°C ± 2°C and 75± 5% RH for 3 months, indicated no significant change in drug content and mean particle size (Figure 49).

8.8.2. In vitro dissolution study

Comparative cumulative percentage drug release profile of the optimized formulations (P5, P16, SP2 and reference) is presented in Table 37. Figure 51 showed insignificant drug release (<20 %) from all formulations in initial 5 h, representing stability of CUR formulation in gastric pH (1.2).
Figure 49: Stability studies of optimized formulations at 40±2°C and 75±5 %RH up to 3 months (n=3) (Mean±S.D.)
Comparative evaluation of enteric formulations of curcumin

Table 37: In vitro dissolution criteria for selection of optimized formulation(s)

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>% Cumulative drug release</th>
<th>Inference</th>
<th>Selection criteria</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-5 h</td>
<td>6-12 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>15.42 ± 0.16</td>
<td>53.99 ± 2.13</td>
<td>Controlled release</td>
<td>Controlled release</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96.12 ± 2.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12-24 h)</td>
<td></td>
<td>Selected for further study</td>
</tr>
<tr>
<td>P16</td>
<td>10.53 ± 0.16</td>
<td>97.12 ± 1.92</td>
<td>Modified release</td>
<td>-do-</td>
</tr>
<tr>
<td>SP2</td>
<td>12.61 ± 0.72</td>
<td>98.99 ± 1.54 (in</td>
<td>Immediate release</td>
<td>-do-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;30 min.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Formulation</td>
<td>20.34 ± 0.38</td>
<td>82.53 ± 0.76</td>
<td>Modified release</td>
<td>-do-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>For reference purpose</td>
</tr>
</tbody>
</table>

8.8.3. Pharmacokinetic study

Plasma drug concentration time profile of optimized formulations (P5, P16, SP2) and plain CUR is presented in Figure 51, in comparison to similar profile for marketed control formulation. Concentration of CUR was detectable up to 24 h, although insignificant plasma drug concentration was observed in group treated with optimized formulations (P5) respectively. Results are supported with roentgenographic images (Figure 52) at different time points. Images indicated the presence of contrast which represents the distribution of formulation in...
GIT at various time points such as from 0 to 2 h in upper GIT, from 2 to 4 h in the small intestine, and from 4 to 24 h in the lower intestinal region (up to anus), whereas plain CUR showed marked availability in lower GIT after 6 h representing elimination of CUR.

![Cumulative plasma drug concentration profile](image)

**Figure 51**: Cumulative plasma drug concentration profile of optimized formulations, Plain CUR and Reference Formulation (n=5)

**Table 38**: Criteria for selection of appropriate formulation(s) on the basis of plasma drug time profile

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Cumulative Plasma CUR (ng/ml)</th>
<th>Inference</th>
<th>Selection criteria</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5</td>
<td>200.12</td>
<td>Limited systemic absorption</td>
<td>Localized delivery (Insignificant plasma drug concentration)</td>
<td>Selected for further study</td>
</tr>
<tr>
<td>P16</td>
<td>950.97</td>
<td>Significant systemic absorption</td>
<td>-do-</td>
<td>Rejected</td>
</tr>
<tr>
<td>SP2</td>
<td>212.26</td>
<td>Limited but rapid systemic absorption</td>
<td>-do-</td>
<td>Rejected</td>
</tr>
<tr>
<td>Reference</td>
<td>607.53</td>
<td>Significant systemic absorption</td>
<td>-do-</td>
<td>For reference purpose</td>
</tr>
</tbody>
</table>
Figure 52: Roentgenography study conducted in guinea pigs A). Plain CUR, B). CUR loaded Optimized formulation (PSN) (P5) (where, I- blank, II- after 30 min administration of formulation, III- after 1 h administration of formulation, IV- after 2 h administration of formulation, V- after 4 h administration of formulation, VI- after 6 h administration of formulation, VII- after 12 h administration of formulation, VIII- after 24 h administration of formulation)
8.8.4. Degradation analysis
Continuous decrease in drug content was observed in the solution containing pure CUR whereas no sign of degradation was observed in solution of formulation; P5 (Figure 53).

Figure 53: Degradation profile of curcumin IN 0.01 mol/l alkali solution (Phosphate Buffer; pH 7.2) (n=3), (Mean ±S.D.)

9.8.5. Cell viability assay
Viability of cells was measured using the MTT test to evaluate the cytotoxicity of CUR on HT29 cell lines. Results of cell viability assay are shown in Figure 54. The IC_{50} value of the optimized PSN formulation (P5) was found to be 28.32 µM, while that of free CUR had 20.56 µM. Compared to DMSO treated cells, no cytotoxicity was observed in the cells exposed to blank PSN formulation. The test results displays no significant even after 72 h duration.

Figure 54: Cell viability study of CUR loaded PSN formulation (P5) on HT29 cell lines conducted using MTT assay (n=3) (P value <0.05)
8.9. Discussion

The purpose of comparative evaluation was to study the effects of three optimized formulations, namely, polymeric Self-emulsifying nanocapsules, nanoemulsifying preconcentrate and polymeric enteric nanospheres containing CUR, in comparison to the unformulated CUR and reference formulation. Initially, selected optimized formulations were subject to stability analysis. No significant change on stability was observed, in relation to the drug content and/or mean particle size (p<0.05). All the optimized formulations were observed to exhibit 5 h lag time and thereafter showed delayed release pattern up to 12h. Therefore, considering the GI transit time from stomach to colon of 4 to 6 h, present formulations could serve as potential carriers for delivery of CUR to colonic region, without any significant release in stomach. Results suggest that the solubility of poorly aqueous soluble CUR was enhanced with present formulations. As curcumin is a drug with shorter half-life (2 h) and poorly absorbed from GIT, therefore, there is a need to develop controlled release formulation of curcumin, in order to reduce the frequency of administration. In this study, we observed that selected formulations exhibited modified release pattern (Table 37) of which, formulation P5 was found to display controlled release pattern. Therefore, formulation (P5) was selected for in vivo study to determine the therapeutic efficacy in the prevention of local pathology of colon i.e. melanosis coli (Mrsny et al., 1992).

The novel finding in the present study is that CUR-NS formulation (P16) significantly increased curcumin appearance in plasma, in comparison to the marketed formulation. Observed data show significantly higher concentration of CUR in plasma after oral administration of CUR-NS formulation (P16) in comparison to reference formulation. Higher plasma concentration of CUR may be explained by enhanced bioavailability as a function of increased aqueous dispersibility, smaller particle size and controlled release of drug from the nanosphere formulation (P16). However, insignificant amount of drug in plasma suggested limited systemic uptake of the formulation (P5). Results signified either degradation of drug or localized delivery to colonic site.

Therefore, roentgenographic study was performed on animal model (guinea pig) as this model is analogous to human physiology, also having similar GI transit time (4-6 h) (Padilla-Carlin et al., 2008). Images indicate that PSN formulation safely reached colon, which is also presented by formulations, in vitro, with sufficient lag time. Results obtained at different stages (time points) suggest that PSN formulation is available in the GIT starting from initial time point (i.e. 30 min) till 24 h. It can be observed that the formulation reaches intestinal region after 4 h (Figure 52(B) (V)), whereas, in case of CUR, there is no such indication. Further, Figure 52(B) (VIII) represents PSN formulation in lower intestine up to 24 h.

Degradation study indicated that the PSN formulation (P5) remains stable in alkaline conditions (pH 7.2), even after kept for 24h. Cytotoxicity assay on the drug loaded nanocapsules was conducted on HT29 cell lines to estimate cell viability with free CUR, blank nanocapsules, and control (DMSO). Results demonstrated that CUR loaded PSN formulation significantly inhibited
the growth of cell lines; however, cell viability was less than DMSO. Blank nanocapsules were treated with cell lines (HT-29) to verify whether a decrease in % cell viability was due to polymeric nanocapsule. It was observed that the blank polymeric nanocapsules did not impact the cellular viability of HT-29 cells as compared to PSN formulations. This suggests that PSN formulations release the drug in a controlled fashion in the vicinity of proliferating cell lines, compared to blank nanocapsules. Therefore, it may confer that limited systemic absorption (plasma drug profile) and releasing the intact drug in the large intestine (in vitro release) and effective delivery of drug to target site (cell line study) signifies localised delivery of CUR to colonic sites successfully.

8.10. Conclusion

On the basis of observed results, it was concluded that the developed polymeric Self-emulsifying nanocapsule can be used for localized delivery of curcumin to the inflammed colonic region.
IN-VIVO STUDY

Experimental Design

0 Day 15\textsuperscript{th} Day 30\textsuperscript{th} Day

Control Diet for 30 days

Sennosides (25mg/kg) + Suspension of PSN Formulation

Sennosides (25mg/kg) Suspension of PSN Formulation

Sennosides (25mg/kg) + Suspension of Standard Formulation

Sennosides (25mg/kg) Suspension of Standard Formulation

SACRIFICE