Chapter 7.0

SUMMARY AND CONCLUSION

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7.1 Summary of the Study

Turmeric, the source of the polyphenolic active compound curcumin (diferuloylmethane), has been used extensively in traditional medicine since ancient times as a household remedy against various diseases, including hepatic disorders, cough, sinusitis, rheumatism, and biliary disorders. Recently a large number of studies have shown that curcumin has a surprising array of antioxidant, antitumor, anti-inflammatory, anticancer, and other desirable medicinal properties. However, the main drawback associated with curcumin is its poor aqueous solubility (water solubility is only 11 ng/mL) (Kaminaga et al., 2003), permeability and stability in gastrointestinal fluids, which leads to poor bioavailability (~1% in rat) (Pan et al., 1999; Yang et al., 2007).

In solution, curcumin exists primarily in its enolic form characterized by strong intramolecular hydrogen bonds. The physicochemical properties of curcumin are due to the formation/disruption of both intra- and intermolecular H-bonds, along with charge delocalizations that are responsible for its therapeutic potential. Most of the CU (>90%) is rapidly degraded within 30 min of placement in phosphate-buffered saline (PBS) at pH 7.2. Curcumin functions as an anti-cancer agent by activating apoptosis signalling and inhibiting cell proliferation. However, the main shortcoming associated with curcumin is its insignificant aqueous solubility, poor permeability, stability in gastrointestinal fluids and first pass metabolism which leads to poor bioavailability.

The purpose of the present study was to enhance the solubility of the poorly soluble drug curcumin, by lipid based nanoemulsion as the formulation technique with food-acceptable ingredients, to augment the in vitro release by improving solubility and to enhance the bioavailability of curcumin by delivering it at the molecular level in the form of nano globule via lymphatic portal system. The study can be summarized as follows:

1. Physicochemical characterization and identification of procured curcumin by organoleptic properties, solubility, loss on drying, partition coefficient, UV spectroscopy, FTIR spectroscopy and differential scanning calorimetry was done which indicated that curcumin was authentic and pure.

2. An analytical method using UV-Vis spectrophotometry and HPLC was developed and validated. The result obtained indicated that the developed methods were
reproducible, selective and accurate for the determination of CU in various studies. The validated methods were successfully applied to preformulation studies including solid state stability, excipient compatibility and nanoformulation studies. Bioanalytical method was developed and validated for the determination of CU on plasma sample analysis.

3. During preformulation studies it was found that CU was unstable in phosphate buffer (pH 7.4) and physiological medium Krebs Ringer Solution (pH 7.4). Therefore concentration of ascorbic acid, an antioxidant was optimized. The degradation of CU was almost completely inhibited with 0.1% w/v of ascorbic acid. The excipients used for formulation development have no interaction with CU. The solubility of CU was found to be pH dependent and increased with increased in pH.

4. For NE development excipients were selected on the basis of solubility of CU in oil and surfactants and miscibility among the oil, surfactant and co-surfactant. Solubility studies were performed using shake flask method. Good solubility of CU was found in Labrafil lipophile 1349 (18.87±0.82 mg/mL), Captex 500 (16.3±0.52 mg/mL) and Labrafil PG (19.38±0.95 mg/mL) as oil, Solutol HS 15 (93.64±2.92 mg/mL), Unitop FFT 40 (73.16±3.92 mg/mL) and labrasol (81.65±3.01 mg/mL) as surfactant. Labrafil Lipophile WL 1349 and Captex 500 were selected as the oil phase to promote lymphatic delivery. Miscibility studies were performed by measuring transmittance of the sample. On the basis of solubility and miscibility studies Solutol HS 15 and Unitop FFT 40 were selected as surfactants and PEG 400 and Transcutol HP were selected as co-surfactants for NE development.

5. Process parameters (stirring time and speed, temperature and process of addition of surfactant or S_{mix}) were optimized for the preparation of pseudoternary phase diagram by aqueous titration method using Labrafil Lipophile WL 1349, Solutol HS 15 and Transcutol HP (LST combination), Labrafil Lipophile WL 1349, Unitop FFT 40 and PEG 400 (LUP combination), Captex 500, Solutol HS 15 and Transcutol HP (CST combination). In all three combinations, the area of NE isotropic region increased slightly as the ratio of surfactant in S_{mix} was increased up to 2:1 concentration but on further increase in surfactant concentration, area decreased. It might be due to reduction of interfacial tension up to lower level where NE formation was more. Lower interfacial tension may provide a flexible film that can readily deform around
droplets and might provide the correct curvature at the interfacial region for the desired NE type.

6. The NE formulations for oral delivery were developed successfully to satisfactory levels in terms of globule size, globule shape, globule size distribution, poly dispersity index, zeta potential, drug loading, robustness to dilution, *in vitro* studies (drug release) and *ex vivo* permeation studies.

7. The stability of CU loaded NE formulation in phosphate buffer (pH 6.8) were performed and it was observed that no significant (p > 0.05) degradation occurred up to 24 h when CU was loaded in the NE.

8. The maximum quantity of CU that could be successfully incorporated into the NE at ambient storage temperature was found to be 64.29±1.85, 30.56±0.69 and 41.29±0.69 mg/mL for NE-SB1, NE-LA1 and NE-CC3 respectively. The *in vitro* release of curcumin from CU loaded NEs (10 mg/mL) were determined using dialysis membrane in 0.1N HCl and compared with CU suspension. A very significant (P<0.001) increase in percentage drug release was achieved in the case of NEs as compared to CU suspension. In CU suspension the drug was not released even up to a detectable level till 180 min (3 h) and drug precipitation was observed in the dialysis bag. After 3 h, CU released up to a detectable level and 21.7% CU release was observed till 12 h. The highest release 84.9±1.8, 86.0±2.51 and 84.6±3.43% at 180 min (3 h), 100.5±1.4, 98.9±4.13 and 100.4±4.21% at 720 min (12 h) of CU was obtained in case of NE-SB1, NE-LA1 and NE-CC3 respectively.

9. *In vitro* performance of lipid based NE formulation was assessed using *in vitro* lipolysis model under conditions that mimic lipid digestion in the small intestine. Free fatty acid released during *in vitro* lipolysis studies was more than 90% at 10 min from the developed nanoemulsions. The NE contains medium chain triglycerides which was easily digested with in 15 min and released almost all free fatty acids.

10. The bioaccessibility of curcumin in the aqueous dispersed was in the range of 85 - 98% for the absorption from the developed NE of all the three combination.

11. On the basis of globule size, zeta potential, release study, lipolysis and bioaccessibility study, nanoemulsion formulation NE-SB1 from LST combination, NE-LA1 from LUP combination and NE-CC3 from CST combination were selected.
12. The *ex vivo* release of curcumin from CU loaded NEs (10 mg/mL) were determined using duodenum in phosphate buffer (pH 6.8) and compared with CU suspension. It was found that formulation NE-SB1, NE-LA1, NE-CC3 showed 85.8±2.95, 90.82±4.2, 83.8±2.11% CU release at 3 h and 100.3±4.89, 98.1±4.9, 99.5±2.21% CU release at 12 h respectively as compared to 28.2±1.73% release at 12 h from CU suspension. The pattern of drug release in simulated gastric fluid and intestinal fluid was found very similar to each other in all NE formulations. It was further observed that as the droplet size decreased the permeation of drug increased. Thus, NE provides a larger surface area available for partitioning of the drug across the intestinal membrane.

13. The *ex vivo* everted gut sac studies were determined to assess the permeability of CU from suspension and developed NE formulations. A curcumin-loaded NE-SB1, NE-LA1 and NE-CC3 produced a $P_{app}$ up to $4.36 \times 10^{-5}$ cm/sec with flux of 0.436 μg/cm²/h, $3.94 \times 10^{-5}$ cm/sec with flux of 0.394 μg/cm²/h and $3.95 \times 10^{-5}$ cm/sec with flux of 0.395 μg/cm²/h at 2 h, where as drug suspension had $P_{app}$ of $0.488 \times 10^{-5}$ cm/sec with flux of 0.0488 μg/cm²/h. It was observed that the permeability of curcumin from NE was found to be significantly (p<0.05) higher (~8-9 times) compared to drug suspension.

14. All the three optimized formulations were subjected to pharmacokinetic studies which showed $AUC_{0-in}$ and $C_{max}$ of 1027.86±30.76 ng/mL.h and 803±24.06 ng/mL for NE-SB1, 791.26±19.45 ng/mL.h and 679±13.52 ng/mL for NE-LA1, 994.41±23.58 ng/mL.h and 746±16.49 ng/mL for NE CC3 which were significantly (p < 0.05) higher than that from free CU suspension, 173.05±18.5 ng/mL.h and 126±13.56 ng/mL respectively. The bioavailability of curcumin was increased up to 11-12 folds depending on the formulation composition.

15. The contribution of lymphatic transport in bioavailability enhancement was assessed and $AUC_{0-in}$ and $C_{max}$ was reduced to approximately 20 % and 30% for NE-SB1, 14 and 16% for NE-LA1, 17 and 21% for NE-CC3 respectively which revealed contribution of lymphatic transport of curcumin.

16. During *in vitro* cytotoxicity studies against Glioblastoma cell line (U-87), IC50 of curcumin solution was found approximately between 25 μM and 50 μM, while for the NE- SB1 and NE-LA1 it was approximately in between 10 μM and 25 μM, for NE-
CC3 it was approximately 25 µM. These result indicated that NE formulation was easily taken up by cancerous cell in comparison to pure CU which was due to smaller particle size of the formed globule.

17. Accelerated stability studies revealed that after time intervals of 0, 1 (30 days), 2 (60 days), 3 (90 days) and 6 (180 days) months, there was an increased in globule size from 73±8.17 to 105±9.15 nm for NE-SB1, 82±7.79 to 132±21.76 nm for NE-CC3 and 64±6.19 to 164±17.83 nm for NE-LA1 which did not affect the physical appearance of NE. No significant change (p>0.05) in assay of CU occurred. There was no change in physical appearance, phase separation, drug precipitation. The shelf life of the developed NE formulation was found to be 618 days for NE-SB1, 604 days for NE-LA1 and 762 days for NE-CC3.

7.2 Conclusion of the Study

In the current investigation, NE formulation of CU was proposed for increasing its bioavailability by lymphatic transport. Different nanoemulsions (NE) formulation containing curcumin were successfully formulated by aqueous titration method. The developed NE formulations were evaluated for globule size, surface morphology, in vitro and ex vivo release, in vitro lipolysis studies followed by lymphatic absorption and cytotoxicity against glioma cells. TEM studies demonstrated the spherical shape of the globule in the NE system. The drug release studies revealed higher and uniform release of CU from NEs. The results of ex vivo studies showed enhanced absorption of the drug from the intestine with NE as compared to plain drug suspension. The in vitro lipolysis study showed the effect of globule size on the release of free fatty acids and CU. The developed NE formulation also favored the lymphatic transport of drug which enhanced the relative bioavailability of CU by 11 to 12 fold. The CU loaded NE showed good cytotoxic result against glioblastoma cells compared to pure curcumin which indicated the effectiveness of CU in nanoemulsion. The CU loaded NE was found to be physically and chemically stable for 6 months under accelerated condition. Thus, it can be inferred that robust nanoemulsion formulations of curcumin were developed with increased bioavailability and therapeutic effectiveness against brain tumor especially malignant glioblastoma.