Preparation, characterization and *in vitro* cytotoxicity assay of curcumin loaded solid lipid nanoparticle in IMR32 neuroblastoma cell line

Mohamed Habibur Rahman[1], Muthiah Ramanathan[2] and Veintramuthu Sankar[1]
[1]Department of Pharmaceutics, PSG College of Pharmacy, Peelamedu, Coimbatore, India
[2]Department of Pharmacology, PSG College of Pharmacy, Peelamedu, Coimbatore, India

Abstract: Curcumin (diferuloylmethane) possesses low bioavailability due to its poor solubility, permeability and rapid metabolism. Solid Lipid Nanoparticle of curcumin was prepared by high-speed homogenization technique. Stearic acid was used as a lipid, tween 80 as surfactant and various co surfactants were used for the preparation of SLN. The prepared SLN was characterized using zeta sizer, TEM analysis and the average particle size was found to be in the range of 80 nm – 200nm. The entrapment efficiency of the SLN was ~58 to 85%. The characteristic FTIR peaks suggest that the stearic acid is compatible with curcumin. MTT assay was performed on the optimized formulation and the results are indicative that curcumin SLN showed better cytotoxicity in low dose while compared to plain curcumin. The developed Cu-SLN can find its better place in the anticancer therapy.

Keywords: Curcumin, solid lipid nanoparticle, stearic acid, tween 80, co surfactants.

INTRODUCTION

Curcumin [1, 7 – bis (4-hydroxyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], the primary constitute of turmeric, possesses wide range of biological activity with low bioavailability (Ranajit *et al*., 2004; Ireson *et al*., 2002). A role for curcumin in the prevention of cancer and other chronic diseases, through different biological activities, has been reported (Rao *et al*., 1995; Shao *et al*., 2002; Anto *et al*., 2002; Shukla *et al*., 2002; Huang *et al*., 1994).

Its poor bioavailability and brain availability hampered its clinical acceptance in various diseases. Hence enhancement of curcumin bioavailability can improve the therapeutic efficacy of curcumin, as well as reduce their effective dose. There are agreeable data that implicate that Solid Lipid Nanoparticles (SLN) can improve the bioavailability of poor hydrophilic and liphophilic drugs. Quercitin and vinpocetine bioavailability have been improved through SLN delivery (Li *et al*., 2009; Luo *et al*., 2006). Earlier studies suggest that stearic acid has a high degree of potential in increasing the bioavailability of curcumin when it is incorporated in it rather than pure curcumin powder (Begum *et al*., 2008). Many techniques including high pressure homogenization, high shear homogenization and ultra sonication are available, of which the current research work makes use of the high shear homogenization technique (Triplett *et al*., 2009). The high shear homogenizers exert a tangential force to the sample thus causing effective shearing (Burden *et al*., 2008). *Invitro* MTT assay is a better tool to understand the cytotoxic nature of new formulation. The present study utilizes the IMR 32 cell lines in MTT assay to identify the behavior of curcumin SLN. This novel curcumin SLN may provide opportunity to treat cancer in a better manner with lower dose.

**Corresponding author:* e-mail: hablet1@gmail.com

MATERIALS AND METHODS

Curcumin, Pluronic F 168, Polyethylene Glycol 4000, Polyethylene Glycol 6000, Aerosil 200 and Dialysis Membrane 110 were purchased from Himedia Lab, (Mumbai, India); Stearic acid, Tween 80, Polyvinyl Pyrrolidone were supplied by Loba Chemie Pvt. Ltd (Mumbai, India), Propylene Glycol was provided by Qualigens Fine Chemicals (Mumbai, India); Ethanol was procured from SD Fine Chem (India), Sodium Lauryl Sulphate gifted by Sangrose Laboratories Pvt. Ltd (Mavelikara, Kerala, India). Zetasizer (Malvern), High Shear Homogenizer (SONICS Vibracell Polytron PT 3100D), Phase Contrast Microscope (Leica DFC 295), Freeze Dryer (Lyodel), UV Visible Spectrophotometer (UV-1650 PC Shimadzu), FT IR Spectrophotometer (8400 S Shimadzu), IR Hydraulic Pellet Press (Model M15 Technosearch Instruments).

Preparation of solid lipid nanoparticle by high speed homogenization

Stearic acid was mixed with curcumin and warmed up to 75°C for effective melting and mixing. Simultaneously, distilled water to which the surfactant and co surfactant has been incorporated is also heated to 75°C. Thereafter, the aqueous part is added to the lipid part maintaining the temperature at 75°C, with continuous stirring. The two phase system is then homogenized using High Speed Homogenizer at 20,000 rpm for 5 minutes (Trilett *et al*., 2009). The formulations are stored at refrigerated condition until further use.

Lyophilisation

The prepared solid lipid nanoparticle dispersion is lyophilised at 1.03 mbar and −40°C for 24 hrs in the lyodel freeze dryer. The free flowing powder was achieved by the use of aerosil as cryoprotectant (Schwarz *et al*., 1997).

Particle size analysis

The prepared SLN dispersions were diluted with water and the sample was made as a transparent layer on the glass slide and it was focused under 40X in phase contrast microscope for the measurement of the particle size. The size distribution and the charge nature of the prepared solid lipid nano particle loaded with curcumin was analyzed using Malvern zeta seizer. The suitable dilutions of the dispersions were made using water and it was scanned under version 6.30 by using disposable sizing cuvette at the count rate of 317.5 kcps for 60 seconds at the measurement position of 4.6 mm at attentuator 10. High resolution transmission electron microscope analysis was performed for the lipid formulations to examine the internal structure and morphological nature of the lipid nanoparticles.

Drug content determination

1ml of the formulation is diluted with 5ml 0.5% SLS solution and made to undergo sonication in a bath sonicator. Thereafter, the preparation is subjected to centrifugation at 12,000 rpm for 30 minutes at 4°C. The supernatant is collected and absorbance is measured at the corresponding lambda max 430nm for curcumin. The entrapment efficiency for the curcumin SLN was studied by taking 1ml of the formulation and subjected to centrifugation at 2000 rpm for 5 minutes. The supernatant was collected and the settled particles were washed with ethanol. Again, the centrifuges and the supernatant were added together and the absorbance was measured at the corresponding lambda max of 430nm in ultraviolet-visible spectrophotometer. The entrapment efficiency was measured by using the following formula:

% entrapment efficiency = Drug concentration per ml of SLN X Total volume of dispersion/Total drug incorporated X 100

In vitro drug release studies

1ml of the formulation was taken in the semi permeable dialysis membrane clipped at both ends and placed in a beaker containing 50ml of 0.5% sodium lauryl sulphate solution as buffering medium with continuous stirring in thermally controlled magnetic stirrer. The temperature was maintained at 37°C±2°C. 5 ml samples were withdrawn at various predetermined intervals and replaced by buffer to maintain sink conditions. Cumulative drug concentrations in the samples were quantified by measuring the absorbance at 430 nm in ultraviolet visible Spectrophotometer.

Stability studies

Curcumin SLN was stored in vials at 30°C±2°C/65% RH ±5% RH in stability chamber for a period of one year. The samples were tested for particle size, drug content and entrapment efficiency in the interval of 0, 3, 6 and 12 months as per ICH guidelines.

Cytotoxicity studies

IMR 32 cell lines (Human Neuroblastoma cell line) were purchased from National Centre for Cell Sciences (NCCS, Pune, India) and grown in minimum essential medium (MEM) with 1% non essential amino acid, 1mM Glutamine, 1mM sodium pyruvate supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 atmosphere.

MTT assay was performed to determine the cytotoxicity of the prepared curcumin SLN. 500-1000 cells/well were added in 96 well plates from well grown culture and the viability was tested using trypan blue dye with the help of haemocytometer and 95% of the viability was confirmed. After 24 hours, curcumin (1, 10,100µg/ml) was incubated with cultured IMR-32 cell lines separately for 24 hrs. After incubation the curcumin containing medium was removed and 10µl of MTT (5 mg/ml stock solution) was added and the plates were incubated for additional 4 hrs. The medium was discarded and the Formazan blue, which was formed in the cells, was dissolved with 150µl of DMSO. The optical density was measured at 595 nm. The percentage toxicity was calculated by using the following formula:

% Toxicity=1-Drug treated cells/untreated cells x100-----1

The procedure was carried out for lipid and formulation (stearic acid and curcumin SLN) in the same concentration. IC_{50} for curcumin and formulation was calculated by Grapad Prism 5 software and the results were compared (Sargent 2003).

RESULTS

From the preliminary studies, based on the results of drug content, entrapment efficiency, particle aggregation pattern and particle size, the best formulation was chosen and subjected to further studies viz *in vitro* cytotoxicity and stability studies.

The prepared solid lipid nanoparticle was observed under 40X of phase contrast microscope (Leica DFC 295) and the images are represented as supplementary data. The particle sizes were in the range of 80-200 nm. Transmission electron microscopic images of curcumin loaded stearic acid SLN illustrate the particle size of 60-180 which is in good agreement with the phase contrast microscopy particle size. Spherical shape of the curcumin SLN was observed in transmission electron microscopic (fig. 1). The curcumin loaded SLN showed a poly dispersity index of 0.451which indicates the uniformity of nanoparticles.

Table 1: Stability studies of curcumin loaded stearic acid solid lipid nanoparticles using PEG 6000 as co surfactant

Evaluation Parameter	Stability study Intervals			
	0 day	3 months	6 months	12 months
Particle size (nm)	83.33±2.12	83.12±3.21	85.21±3.33	85.32±3.12
Drug content (%)	93.33±2.12	92.32±1.23	90.43±1.21	90.21±1.21
Entrapment efficiency (%)	85.32±3.21	83.12±2.21	82.87±2.32	82.08±2.12

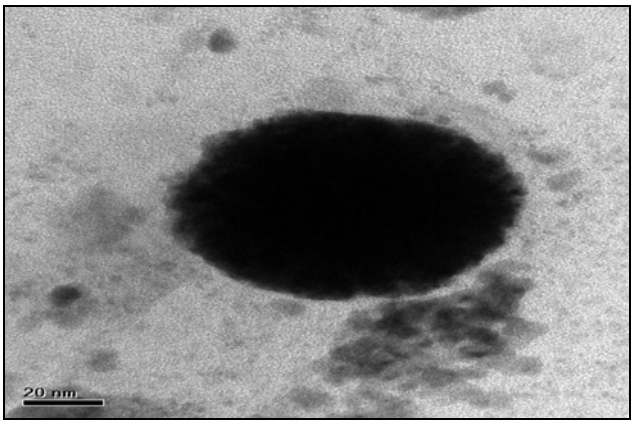

Fig. 1: HR-TEM images under the scale value of less than 200 nm for the curcumin loaded stearic acid lipid nanoparticles

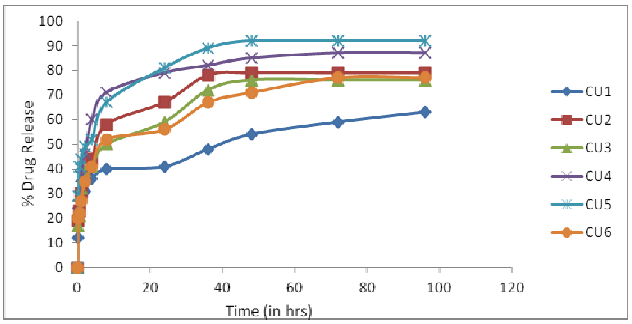

Fig. 2: Dissolution profile of curcumin loaded stearic acid lipid nanoparticles using different co-surfactants in pH 7.4 PBS buffer with 0.5% SLS

Drug content and entrapment efficiency was found to be from 78.12±1.21% to 93.33±2.12 and 58.98±2.12% to 85.32±3.2% respectively. The results indicate that the formulation with propylene glycol as co-surfactant has the capacity to increase the drug entrapment manyfold. It may be noted that in CU6 containing propylene glycol as co-surfactant, the drug loaded is at the maximum. The drug content results suggest that the drug loading capacity of the lipid is significantly influenced by addition of different co-surfactants and the order of co-surfactant influence was found to be PG > PVP > PEG_{6000} > PEG_{4000} > Pluronic for curcumin.

The prepared curcumin SLN was further evaluated for *invitro* release studies. The dissolution studies revealed that the initial fifty percentage release of curcumin from SLN formulations was found to be in the order of CU5> CU4 > CU2 > CU6 > CU3 > CU1. The *in vitro* release studies were continued up to 96 hours for all the formulations (CU1-CU6) and the extent of curcumin release was found to be higher for the formulations prepared with PEG as co-surfactant, i.e., around 92% and 87% for CU5 & CU4 respectively. The influence of different co surfactants in the release of curcumin was found to be PEG6000> PEG4000> Pluronic> PG> PVP. These *in vitro* release studies suggest that the rate and extent of curcumin release was found to be 49% in the first 2 hours and 92% at 48 hrs for the formulation prepared using PEG_{6000} as cosurfactant. Polymeric nanoparticle loaded with curcumin was studied recently, and prolonged release of curcumin was reported (Shaikh *et al*., 2009). The dissolution results of the prepared SLN (fig. 2) reveal that the initial burst release of nearly 50% drug in the first 2 hrs is a promising one to achieve the therapeutic level followed by the sustained rate of curcumin release, which may improve the bioavailability by reducing the metabolism of curcumin. Further, PEG may increase the retention time of lipid nanoparticle in systemic circulation for long time.

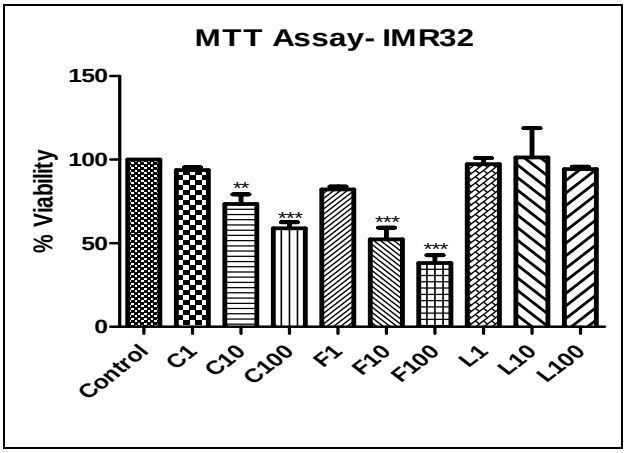

Fig. 3: Cytotoxicity determining MTT assay in IMR 32 neuroblastoma cell lines using different concentration of curcumin loaded stearic acid lipid nanoparticles prepared using PEG 6000 as co surfactant in High Speed Homoginisation technique.

IC_{50} Curcumin = 129.33µg/ml, IC_{50} Formulation = 60.08 µg/ml
C1- Curcumin 1µg/ml, C10-Curcumin 10µg/ml,C100-Curcumin 100µg/ml, F1-Curcumin SLN 1µg/ml, F10-Curcumin SLN 10µg/ml, F100-Curcumin SLN 100µg/ml, L1-Lipid 1µg/ml, L10-Lipid 10µg/ml, L10-Lipid 100µg/ml.

Two way ANOVA was carried out for the dissolution data using Graphpad Prism 5 (Version 5.04) and P value was < 0.0001 for the formulation prepared with PEG_{6000} and its significant, while compared to all other formulations.

Dissolution release kinetics

The order of release rate of curcumin from the prepared SLN was predicted through fitting the dissolution data into the zero and first order equation $Q = k_0t$; $\ln (100–Q) = \ln 100 – k_1t$; respectively, where Q is the amount of drug released at time t and k_0 & k1is the release rate constant. On the other hand, the release mechanism of curcumin from SLN is predicted by fitting the data into Higuchi's & Korsmeyer equation $Q = k_2t^{1/2}$; $\log (Mt/M\infty) = \log k + n\log t$; respectively where K & k_2 is the diffusion rate constant, Mt is the amount of drug released at time t, $M\infty$ is the amount of drug released after infinite time, and n is the diffusional exponent indicative of the mechanism of drug release. (Higuchi T. 1963; Korsmeyer *et al*., 1983)

The stability study results are depicted in table 1. The formulation prepared with PEG_{6000} was stable for 1year, and no significant change was observed on particle size, drug content and entrapment efficiency.

The cytotoxic effect of curcumin and curcumin SLN were explained by MTT assay in IMR32 cell lines and the results are presented in fig. 3. The IC_{50} of curcumin was inflenced by the lipid matrix. Dose dependent cytotoxic effect was observed for both curcumin and curcumin SLN. The fifty percent inhibitory concentration (IC_{50}) was found to be 129.33 µg/ml and 60.08 µg/ml for curcumin and curcumin SLN respectively. The results are indicative that curcumin uptake by neuroblastoma cells was increased sixtyfold through solid lipid nanoparticles. The statistical significance was determined by one way ANOVA with Tukeys multiple comparision test in Graph Pad Prism Version 5.04. The percentage viability of curcumin SLN has not shown any significance in low dose 1µg/ml. There is a notable significance was observed for the higher doses 10, 100 µg/ml. (fig. 3 F10 **, F100 ***, $p<0.05$). Curcumin SLN showed better cytotoxic effect in lower doses while compared to curcumin.

DISCUSSION

The particle size observations were monitored from time to time to understand the aggregation property and stability of the prepared solid lipid nanoparticles. The results suggest that the incorporation of different co surfactant showed a difference in the aggregation pattern of prepared particles. This may be due to the difference in the solubility of lipid in co surfactants. The formulation prepared using PEG_{6000} was found to be more stable while compared to other formulations. Therefore the stable formulation was subjected to lyophilization technique.

The uniformity in the size is a promising nature of the prepared SLN of curcumin which may be useful in target delivery approach for curcumin. Selection of dissolution medium for poor water soluble curcumin is a challenging aspect. The dissolution medium and development of dissolution method was optimized for curcumin (Rahman *et al*., 2009)

Stearic acid is slightly soluble in water which is one of the reasons for the slow and incomplete release of curcumin from stearic acid SLN (Susan *et al*., 2001). There are reports available regarding the polymorphic transition behaviour of lipids which could play a role in the rate of release (Sutananta *et al*., 1995; Hamdani *et al*., 2002). There is a need to understand the influence of co surfactants in the rate of release of curcumin from stearic acid SLN. It is evident from the results that the stearic acid solubility in different co surfactants was found to be indirectly proportional to the formation of lipid matrix which leads to sustained release of curcumin from the lipid matrix.

Previously published reports suggest that the SLN formulations stabilized with 1% non ionic cosurfactant are safe for the cells and acceptable for the incorporation of liphophilic drugs. (Abbasalipourkabir *et al*., 2011). The findings of the present study prove that curcumin loaded SLN is better internalized into the neuroblastoma cells. There are reports claiming that curcumin delivered through SLN was a more effective antiprolifrative while compared to curcumin (Rohit *et al*., 2010). The particle size and the surface charge on the SLN may be the reasons for better cell uptake of curcumin in the form of SLN.

CONCLUSION

To conclude, the SLN technology can be adopted to deliver curcumin (Class IV drug) to achieve better cytotoxicity in lesser dose. PEG can be used as co-surfactant to improve the stability of SLN formulations. Further, the optimized formulation may be subjected to pharmacokinetic studies to claim the *in vivo* fate of the SLN formulation and to establish IVIVC models.

REFERENCES

Abbasalipourkabir R, Salehzadeh A and Abdullah R (2011). Cytotoxicity effect of solid lipid nanoparticles on human breast cancer cell lines. *Biotechnology*, **10**: 528-533.

Anto RJ, Mukhopadhyay A, Denning K and Aggarwal BB (2002). Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: its suppression by ectopic expression of Bcl-2 and Bcl-xl. *Carcinogenesis,* **23**: 143-150.

Begum NA, Jones RM, Lim PG, Morihara T, Kim P, Heath DD, Rock LC, Pruitt AM, Yang F, Hudspeth B,

Hu S, Faull FK, Teter B, Cole MG, Sally A and Frautschy (2008). Curcumin structure-function, bioavailability and efficacy in models of neuroinflammation and Alzheimer's Disease. *J. Pharmacol. Exp. Ther.,* **326**: 196-208.

Burden WD (2008). Guide to the homogenization of biological samples. *Random Primers,* **7**: 1-14.

Hamdani J, Moes AJ and Amighi K (2002). Development and evaluation of prolonged release pellets obtained by the melt pelletization process. *Int. J. Pharm.,* **245**: 167-177.

Higuchi T (1963). Mechanism of sustained action medication, *J. Pharm. Sci.*, **52**: 1145.

Huang MT, Lou YR, Ma W, Newmark HL, Reuhl KR and Conney AH (1994). Inhibitory effects of dietary curcumin on forestomach, duodenal and colon carcinogenesis in mice. *Cancer Res.,* **54**: 5841-5847.

Ireson CR (2002). Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. *Cancer Epidemiol. Biomarkers Pre.,* **11**: 105-111.

Korsmeyer RW, Gurny R, Docler E, Buri P and Peppas NA (1983). Mechanism of solute release from porous hydrophilic polymers. *Int. J. Pharm.*, **15**: 25.

Li HL, Zhao XB, Ma YK and Zhai GX (2009). Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. *J. Control Release,* **133**: 238-244.

Luo YF, Chen DW, Ren LX and Zhao XL (2006). Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. *J. Control Release,* **114**: 53-59.

Rahman SMH, Telny TC, Ravi TK and Kuppusamy S (2009). Role of surfactant and pH in dissolution of curcumin. *Ind. J. Pharm. Sci.,* **71**:139-142.

Ranajit KB, Ishita C, kaushik B and Uday B (2004). Turmeric and curcumin: Biological actions and medicinal applications. *Current Science,* **87**: 44-53.

Rao CV, Rivenson A, Simi B and Reddy BS (1995). Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res.,* **55**: 259-266.

Rohit S, Mönkkönen J, Risto O, Kakasaheb R and Anant RP (2010). Transferrin mediated solid lipid nanoparticles containing curcumin: Enhanced *in vitro* anticancer activity by induction of apoptosis. *Int. J. Pharm.,* **398**: 190-200

Sargent JM (2003). The use of the MTT assay to study the drug resistance in fresh tumor samples. *Recent Results Cancer Res.,* **161**: 13-25.

Schwarz C and Mehnert W (1997). Freeze-drying of drug-free and drug loaded solid lipid nanoparticles. *Int. J. Pharm.,* **157**: 171-179.

Shaikh J, Ankola D, Beniwal DV and Singha D (2009). Nanoparticle encapsulation improves oral bioavailability of curcumin by at least 9-fold when compared to curcumin administered with piperine as absorption enhancer. *Eur. J. Pharm. Sci.,* **37**: 223-230.

Shao ZM, Shen ZZ, Liu CH, Sartippour MR, Go VL, Heber D and Nguyen M (2002). Curcumin exerts multiple suppressive effects on human breast carcinoma cells. *Int. J. Cancer,* **98**: 234-240.

Shukla Y, Arora A and Taneja P (2002). Anti-mutagenic potential of curcumin on chromosomal aberrations in Wistar rats. *Mut. Res.,* **515**: 197-202.

Susan B and Maryadele J (2001). The Merck index: An encyclopedia of chemicals, drugs and biologicals. Merck, Whitehouse Station, NJ. Pp. 6909.

Sutananta W, Craig DQ and Newton JM (1995). An investigation into the effects of preparation conditions and storage on the rate of drug release from pharmaceutical glyceride bases. *J. Pharm. Pharmacol.,* **7**: 355-359.

Triplett DM and Rathman FJ (2009). Optimization of β-carotene loaded solid lipid nanoparticles preparation using a high shear homogenization technique. *J. Nanoparticle Research,* **11**: 601-614.