EVALUATION OF STABILITY, CELLULAR UPTAKE AND ANTI-TUMOUR ACTIVITIES OF AN OPTIMIZED CURCUMIN SOLID LIPID NANOPARTICLES FORMULATION.

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ABSTRACT
Curcumin has a wide spectrum of biological and pharmacological activities such as anti-inflammatory and anticancer activities but its main drawbacks (Low stability and poor bioavailability profiles) have restricted its therapeutic applications. In our previous study an optimized formula of solid lipid nanoparticles containing curcumin (C-SLNs) was prepared to overcome its drawbacks. The aim of the optimized C-SLN formula to increase curcumin’s stability, and enhance its anticancer activity against A2780 ovarian cancer cells. The purpose of the current study was characterization of the optimized C-SLN formula through conducting morphological examination, stability, cytotoxicity and cellular uptake studies. The morphological examination using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) revealed the spherical nature of the optimized C-SLN formula. The stability of the optimized C-SLN formula at different temperatures was evaluated over the storage period of 6 months. The cytotoxicity and cellular uptake studies revealed enhanced antiproliferative activity of the optimized C-SLN formula compared to native curcumin solution in DMSO. The present study indicated the effectiveness of C-SLNs in enhancing the anticancer effect of curcumin in ovarian cancer cells in-vitro.

INTRODUCTION
Curcumin is a naturally occurring phytoconstituent extracted from the root of Curcuma longa linn, commonly known as turmeric that has been used for centuries as a remedy for many ailments (Anand et al., 2007; Nagarajan et al., 2010; Shehzad et al., 2010).

Curcumin has a very broad spectrum of biological activities such as, potent anti-inflammatory, antioxidant, antimicrobial, wound healing, and anticarcinogenic effects (Ghosh et al., 2012; Bansal et al., 2012; Li et al., 2012; Yallapu et al., 2012).

However these great pharmacological potentials of curcumin and its therapeutic applications are restricted because of the molecule’s drawbacks including low aqueous solubility at acidic and physiological pH conditions, rapid hydrolysis in alkaline media and light instability, inherent to its chemical composition (Khalil et al., 2013). Also the hydrophobic character of curcumin results in pharmacokinetic restrictions such as low absorption and bioavailability by oral route, extensive metabolism and rapid elimination (Anand et al., 2007; Sharma et al., 2007).
The main strategies used to overcome the physicochemical limitations of curcumin and to increase its stability and bioavailability are based on loading the compound into nanocarriers, such as liposomes (Kunwar et al., 2006), cyclodextrins (Yadav et al., 2010) and solid lipids (Tiyaboonchai et al., 2007).

Solid Lipid Nanoparticles (SLNs) were developed as the first generation of Lipid Nanocarriers and are being extensively studied as promising approaches for poorly soluble drugs such as curcumin (Almeida and Souto, 2007; Martins et al., 2007; Nayak et al., 2010).

Several production methods can be used to develop SLNs such as Solvent Injection Method (SIM). SIM was found to be of simple implementation, efficient, productive, versatile and offers clear advantages over other existing methods used for SLNs production such as the use of pharmaceutically acceptable organic solvents, no need for high pressure homogenization or technically sophisticated equipments (Schubert and Müller-Goymann, 2003).

In our previous study, SLNs containing curcumin (C-SLNs) were prepared using SIM and 2³ full factorial design was used to obtain an optimized formula. The physicochemical characteristics and the in-vitro release study of the developed CSLNs formulations were evaluated. The optimized C-SLN formula showed a mean particle size of 249 nm with Polydispersity Index (PdI) value of 0.185, Zeta Potential value of -31.81 mV and 74.51 % entrapment efficiency. The optimized C-SLN formula showed a biphasic drug release pattern in the in-vitro release studies (i.e. a burst release at the initial state followed by a sustained release state) following higuchi diffusion model with a total release percent of 85.72 %.

In the current study, morphological examination and the long-term stability profile of the optimized C-SLN formula were evaluated. Furthermore, the therapeutic profile was investigated through conducting cellular uptake and cytotoxicity studies against A2780 ovarian cancer cells.

MATERIALS AND METHODS

Materials

Native Curcumin (NC; 98% pure powder and molecular weight = 368.38 D) was purchased from Acros Organics, NJ, USA; Poloxamer 407 (P407) powder from Spectrum Chemicals MFG CORP, CA, USA; Purified Glycerol MonoStearate (GMS), Hydrochloric Acid (HCl, 38%) and Glacial Acetic Acid were purchased from VWR, West Chester, PA, USA; Phosphate Buffer Saline (PBS; 0.01 M, pH 7.4) powder packets, DiMethyl Sulfoxide (DMSO) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co, St. Louis, MO, USA; Absolute Ethyl alcohol (Ethanol) from Avantor Performance Materials INC., NJ, USA; Methanol and Acetonitrile HPLC Grades from EMD chemicals In., NJ, USA; DeIonized Water (DIW) was obtained from ED Millipore, USA, A2780 Ovarian Cancer Cells were purchased from American Type Culture Collection (ATCC) Manassas, VA, USA; The growth medium (RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin G-streptomycin) from Gibco BRL, Grand Island, NY, USA; The Lysis solution (50% (v/v) N,N-dimethylformamide, 20% (w/v) sodium dodecylsulphate, with an adjusted pH of 4.5) was purchased from Sigma Aldrich, St. Louis, MO, USA. All
other chemicals were standard pharmaceutical grade and used without further purification.

Methods

Preparation of the optimized C-SLN formula

The optimized C-SLN formula was prepared according to the SIM protocols mentioned before (Dawaba et al., 2013). In brief, Curcumin (2% of the lipid phase) and the specified amount of GMS (148.79 mg) were dissolved in the specified volume of Ethanol (1ml) with gentle heating. The resulting solution was rapidly injected into the 10 ml of aqueous phase containing P407 (2% w/v) that was continuously stirred at 400 rpm for 30 min on a magnetic stirrer; 0.1N HCl (4ml) was added to the dispersion and the dispersion then was centrifuged to 10,000 rpm for 30 min at 10°C in Allegra® 64R Benchtop centrifuge (Beckman Coulter Inc, Palo Alto, CA, USA), and aggregates were resuspended to 10 ml distilled water containing 4% poloxamer 407 (by weight) as stabilizer with stirring at 1,000 rpm for 10 min and lyophilized in FreeZone® 2.5 tabletop Freeze dryer (Labconco Corporation, Kansas city, Missouri, USA) for 48 hr. A blank SLN formula (BSLN) was prepared under the same conditions without the addition of curcumin.

HPLC analysis method:

Curcumin analysis was performed according to the methodology mentioned before (Dawaba et al., 2013). Briefly, curcumin was detected at 428 nm with a sample run time of 10 min and a flow rate of 1ml/min using WATERS® HPLC system consisting of Perkin Elmer HPLC column (SPHERI–5 RP – 18, Perkin Elmer LLC, Norwalk city, CT, USA) with the following specifications 5µm, 4.6mm×250mm Preceded by a guard column (SecurityGurad™, Phenomenex Inc., Torrance, CA, USA) filled with C18 cartridges (4 x 2.00 mm ID) at room temperature. The mobile phase was Methanol: H$_2$O (containing 3.6% glacial acetic acid) (73:27, v/v), freshly prepared on the day of use, filtered through a 0.45 µm filter and was degassed by sonication for 15 min (Dandekar and Patravale, 2009).

Morphological Characterization:

For comparison the morphological characterization tests were performed on the optimized C-SLN formula as well as on the BSLN formula.

Transmission Electron Microscopy (TEM):

The TEM analysis was performed using JEOL® Transmission Electron Microscope (JEOL® TEM Model 1010, Tokyo, Japan). The investigated samples were prepared for the microscopic analysis through the Negative Staining Technique where the lyophilized powders were dispersed directly into distilled water then one drop of each diluted dispersion was placed on a 200-mesh carbon coated copper grid. Then the carbon coated copper grid was stained by 2% phosphotungstic acid (PTA) solution for contrast enhancement and dried at room temperature, the sample was ready for the TEM investigation at 70 kV (Zhu et al., 2008, Li et al., 2009; Lv et al., 2009; Mulik et al., 2009; Mulik et al., 2010).

Scanning Electron Microscopy (SEM):

The SEM analysis was performed using Hitachi® Scanning Electron Microscope (Hitachi® Tabletop Scanning Electron Microscope Model TM 3000, Hitachi High-Technology Corporation, Krefeld, Germany). Both the lyophilized formulations (C-
SLN and BSLN) were dispersed separately in water and one drop of each diluted dispersion was evenly distributed onto a conductive tab on a stud and then sputter coated with gold in a cathodic evaporator (Nayak et al., 2010).

**Stability Studies of optimized C-SLN formula:**

**Long – Term Stability Study:**

The prediction of long-term stability for the optimized C-SLN formula was conducted over 6 months period according to the protocol mentioned by Padamwar and Pokharkar, 2006; Tiyaboonchi et al., 2007; Mulik et al., 2009 and Nayak et al., 2010.

Samples of the lyophilized optimized C-SLN formula were stored in air-tight well closed amber glass vials protected from light at room temperature and 40°C and maintained 75% relative humidity in both temperatures. After 3 and 6 months, the curcumin content was determined according to the methodology previously mentioned. The obtained results were compared with fresh formulations and the experiments were performed in triplicate.

**Kinetic treatment of optimized C-SLN formula:**

Degradation reaction of curcumin from the optimized C-SLN formula was studied where the correlation coefficient (r²) was determined according to zero, first, and second-order equations. The decomposition rate constant was then determined according the more suitable kinetic order. Kinetic treatment and parameters were also calculated.

**Cell Culture Study:**

The cell culture study was conducted according to the protocols of Mulik et al., 2009; Mulik et al., 2010; Mulik et al., 2012 and Saxena and Hussain, 2013 where A2780 Ovarian cancer cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/G-streptomycin at 37°C in a humidified, 5% CO2 atmosphere in a CO2 incubator.

**Antiproliferative activity of optimized C-SLN formula (MTT assay):**

The Antiproliferative activity was conducted according to the protocols mentioned by Mulik et al., 2010; Mulik et al., 2012 and Saxena and Hussain, 2013 with slight modifications. The A2780 ovarian cancer cells (1×10⁴/well) were seeded in a 96-well plate and allowed to attach for 24 h. Then the medium was replaced with fresh medium and the cells were treated with different concentrations of NC solution in DMSO and optimized C-SLN formula (0.0001–100μM curcumin/well) and incubated for 24 h at 37°C in CO2 incubator. The growth medium was removed after the treatment, cells were washed three times with PBS and fresh medium was added. 25μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/ml in PBS) was added to the cells and incubated for 3 h at 37°C in CO2 incubator. After the incubation, the cells were lysed and the dark blue crystals were solubilized with 125μl of the Lysis solution. The optical density of each well was measured with NOVOstar Microplate Reader (BMG Labtech Inc. Cary, NC, USA) equipped with a 570nm filter. Percent of cell survival was defined as the relative absorbance of treated cells versus the respective controls. Results were expressed as percentage of cell viability as compared to the control versus concentration. The concentration at which 50% of growth is
inhibited (IC50) was determined using Graphpad prism 5.0 software (Graphpad Software, La Jolla, CA, USA). The obtained results were represented as means ± SD from at least three separate experiments.

**Cell uptake study:**

**Qualitative Cell uptake study:**

The qualitative estimation of curcumin uptake by A2780 ovarian cancer cells treated with NC and the optimized C-SLN formula was carried out using fluorescence microscopy technique mentioned by Mulik et al., 2010; Mulik et al., 2012 and Saxena and Hussain, 2013.

In the qualitative estimation of curcumin uptake, the autofluorescence of curcumin was observed using fluorescence microscopy using green filter. A2780 ovarian cancer cells were seeded (at density of 1x10⁶/well) using 24-well plate and allowed to adhere for 24 h. The medium was replenished with fresh medium, and the cells were treated with NC solution in DMSO and the CSLN formula (10μM curcumin/well) for different time points (6, 12, 24 and 48 h). The medium was replaced with fresh medium by washing the cells thrice with PBS 7.4 after each time point, and the images were captured using Nikon Eclipse Ti fluorescence microscope (Nikon Eclipse Ti Confocal laser Scanning microscope (CLSM), Nikon instrument Inc., Melvill, NY, USA).

**Quantitative Cell uptake study:**

The Quantitative estimation of curcumin uptake by A2780 ovarian cancer cells treated with NC and the Optimized CSLN formula was carried out using Chromatography technique. In the quantitative determination of uptake of curcumin by A2780 ovarian cancer cells, the cells were treated as described above in the Qualitative section. After each time point (3, 6, 12, 24, 48 h), the cells were collected by trypsination and centrifuged for 3 min at 3000 rpm. Afterwards the supernatant was removed; the pellets were resuspended in 1 ml of methanol, and vortexed for 5 min to extract the curcumin in methanol fraction. The lysate was then centrifuged at 5000 rpm for 5 min, and the amount of curcumin was determined using the HPLC analysis method.

**RESULTS AND DISCUSSION**

**HPLC analysis method**

The employed HPLC analysis method gave a sharp peak of curcumin, without tailing, with a retention time of around 6.3 min at 428 nm (Figure 1) indicating the suitability of the analysis method to investigate the presence of curcumin in the pharmaceutical products.
Preparation of the optimized C-SLN formula

The optimized C-SLN formula was successfully prepared by SIM which gave a yellowish sponge product after freeze-drying. The lyophilized formula was easily redispersed in DIW and the resultant suspension was subjected to evaluation tests.

Morphological Characterization:

Transmission Electron Microscopy (TEM):

In the TEM analysis, the investigated samples were visualized in a dry state and the obtained TEM photographs in figure (2) showed that the produced SLNs were spherical in shape with smooth surface in case of BSLN and C-SLN formulations.

The wide size range obtained with TEM analysis may be related to the formation of multiple phospho-lipid layers or the formation of other structures such as liposomes, micelles and drug nanosuspensions (Lv et al., 2009; Das and Chaudhury, 2011; Mehnert and Mäder, 2012).

These results came in agreement with the studies performed by Lv et al., 2009; Mulik et al., 2009; Mohanty and Sahoo, 2010; Mulik et al., 2010; Manju and Sreenivasan, 2011.

Figure (2): TEM Photographs of A) BSLN Formula B) CSLN formula at a magnification power 50000X.
Scanning Electron Microscopy (SEM):

Additional morphological analysis was conducted using Scanning Electron Microscope and the obtained photographs were illustrated in figure (3).

From the performed SEM analysis, it was evident that particles were spherical in shape and homogeneously distributed with size ranging between 200 and 250 nm.

The size observed from SEM micrographs correlated with the size analysis performed by Zeta PALs apparatus. These results came in agreement with the studies performed by Tiyaboonchai et al., 2007; Nayak et al., 2010.

![SEM Photographs of A) BSLN Formula B) CSLN formula at a magnification power 8.00 KX.](image)

Stability Studies:

Long-Term Stability Study:

Curcumin is notorious as light and oxygen sensitive substance (Ansari et al., 2005; Tiyaboonchaia et al., 2007; Nayak et al., 2010) and the major challenges of curcumin delivery in therapeutics grounds involves while defining its stability (Mohanty and Sahoo, 2010). Thus, it is important to maintain the stability during the storage period of 6 months.

Figure (4) showed the results of long-term stability testing performed on the optimized C-SLN maintained 75 % RH and absence of light at room temperature and 40°C respectively whilst table (1) showed the kinetic parameters calculated at the investigated temperatures.
The percentage of curcumin remaining after 6-month storage at room temperature in absence of light was between 99.43% and 93.68%. The percentage of curcumin remaining after 6-month storage at 40°C in absence of light was between 99.35% and 88.70%. These results came in agreement with the studies performed by Padamwar and Pokharkar, 2006; Mulik et al., 2009; Nayak et al., 2010; Shegokar et al., 2011.

These findings are in agreement with the results of other researchers suggesting that production of SLN into a dry powder may prevent the aggregation of nanoparticles and improve the stability of light and oxygen sensitive substances (Mehnert and Mäder, 2001; Hu et al., 2002; Tiyaboonchai et al., 2007; Nayak et al., 2010). These effects of SLNs are in particular evident, if cryoprotective agents are used (Konan et al., 2002; Zhang et al., 2008; Ohshima et al., 2009; Nayak et al., 2010).

Table (1) showed the kinetic treatment of the stability data of the optimized CSLN formula and it was clear that the degradation of curcumin from SLNs followed the Zero-order model best.

Table (1): Kinetic parameters at Room temperature / 75 % RH.

<table>
<thead>
<tr>
<th>Model</th>
<th>Room Temperature</th>
<th>40°C</th>
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<tbody>
<tr>
<td></td>
<td>a*</td>
<td>b*</td>
</tr>
<tr>
<td>Zero Order</td>
<td>1.58</td>
<td>0.054</td>
</tr>
<tr>
<td>First Order</td>
<td>1.99</td>
<td>0.000</td>
</tr>
<tr>
<td>Second order</td>
<td>0.01</td>
<td>5.89E-6</td>
</tr>
</tbody>
</table>

* a = Intercept, b = Slope, r² = Regression Coefficient, k = Rate Constant, t½ in days
Cytotoxicity Studies:

Antiproliferative activity:

The Antiproliferative activity of different curcumin concentrations were observed when incubated with A2780 ovarian cancer cells at 48 and 72 h using MTT assay. In MTT assay, MTT gets reduced to purple formazan by mitochondrial reductase in living cells. This reduction takes place in presence of active reductase enzymes, and hence, this conversion is used as a measure of viable (living) cells (Mulik et al., 2010; Mulik et al., 2012; Saxena and Hussain, 2013). The obtained results were represented in figure (5).

From figure (5), the lower concentrations of treatments (0.0001–0.1 μM) did not show any significant difference in the cell viability between native curcumin and the optimized C-SLN formula.

At 1μM treatment with the optimized C-SLN formula, the cell viability was reduced significantly (76.89 ± 2.139 % after 48 h and 77.69 ± 0.764 % after 72 h) compared to native curcumin (93.16 ± 1.233 % after 48 h and 92.56 ± 0.535 % after 72 h).

At 10μM concentration, the effect was even more pronounced and cell viability was reduced (20.88 ± 0.26 % after 48 h and 20.94 ± 0.551 % after 72 h) with the optimized C-SLN formula, in comparison with native curcumin solution (78.14 ± 0.631% after 48 and 65.47 ± 0.275 % and 72 h).

At the highest concentration of curcumin (100 μM) used in the study (whether after 48 or 72 h incubation), negligible toxicity difference was observed (approximately 2% difference) between the optimized C-SLN formula and the native curcumin solution.

The IC50 of the optimized CSLN formula (3.00 ± 0.187 μM) was found to be 7 fold less than the native curcumin solution in DMSO (22.66 ± 0.267 μM) after 48 h of incubation and the IC50 of the optimized CSLN formula (3.09 ± 0.049 μM) was found to be 5 fold less than the native curcumin solution in DMSO (16.66 ± 0.125 μM) after 72 h of incubation.

In this study, the respective control solutions used were DMSO and the BSLN formula for NC solution in DMSO and the optimized CSLN formula, respectively. Since, these controls showed neglectable effect on cell viability, the antiproliferative effect observed can be attributed to entrapped curcumin rather than the formulation components.
Cell uptake study:

Qualitative Cell uptake study:

Cellular uptake studies are useful to predict the drug delivery potential and biocompatibility of novel formulated nanoparticles (Zanotto-Filho et al., 2013). Taking advantage of the photochemical properties of curcumin, the cellular uptake of the optimized CSLN formula was compared with native curcumin solution using the CLSM (Saxena and Hussain, 2013; Zanotto-Filho et al., 2013).

Figure (6) showed the CLSM images of native curcumin solution in DMSO and the optimized C-SLN formula respectively.

In case of NC solution treated cells, the fluorescence intensity was good after 6 and 12 h but it was reduced with time. After 24 and 48 hr, the fluorescence intensity reduced significantly.

In case of the optimized C-SLN formula treated cells, the fluorescence intensity increased after 6 hr and it remained almost steady even after 48 h, suggesting the sustained intracellular release and retention of encapsulated curcumin from SLNs localized inside the cells.

The obtained images revealed that the cell uptake from the optimized C-SLN formula was more sustained compared to NC solution in DMSO and the qualitative study indicated enhanced uptake of the optimized C-SLN formula in A2780 ovarian cancer cells as compared to NC solution.

This enhanced uptake can be attributed the small particle size of the formula as well as the effect of P407 on the fluidity of the cell membrane. The hydrophobic PPO block of the Poloxamer polymer can incorporate in the lipid membrane and can induce structural changes and fluidization of the membrane (Batrakova et al., 2001; Saxena and Hussain, 2013).

Images of untreated cells were also taken (photos are not represented in this paper) to see possible autofluorescence and No autofluorescence was observed in untreated control cells which confirms that cytotoxicity activity is due to curcumin (Saxena and Hussain, 2013).
NC solution in DMSO  
Optimized C-SLN Formula

A) 
B) 
C) 
D) 

Figure (6): Qualitative cellular uptake of native curcumin solution in DMSO and optimized C-SLN formula after: A) 6, B) 12, C) 24 and D) 48 hr.

Quantitative Cell uptake study:

Figure (7) showed the results of the quantitative intracellular uptake of curcumin from both the native curcumin solution and the optimized C-SLN formula.

The quantitative estimation of curcumin uptake by A2780 ovarian cancer cells from the investigated samples showed prominent difference in curcumin levels and the maximum drug levels were obtained at 12 hr treatment.

Curcumin levels in case of NC solution and the optimized C-SLN formula treated cells after 12 hr were $1.07 \pm 1.74$, $1.55 \pm 2.30 \mu g$ per $10^6$ cells respectively. After
24 hr treatment, curcumin levels were reduced for both the NC solution and the optimized C-SLN formula to 0.66 ± 2.61 and 1.44 ± 1.43 μg per 10^6 cells, respectively. At the end of the experiment (48 hr), the reduction in drug levels in case of NC solution treated cells was more significant (0.49 ± 1.14 μg per 10^6 cells) compared to the optimized C-SLN treated cells (1.32 ± 1.15 μg per 10^6 cells).

In the quantitative estimation of cell uptake, maximum drug levels were obtained at 12 hr treatment. After that, the drug levels were reduced significantly in case of NC solution treated cells compared to the optimized C-SLN formula treated cells. At 12 hr, the optimized C-SLN formula treated cells showed about 1.5 fold increase in drug uptake compared to NC solution treated cells. After 24 hr treatment the increase in drug uptake was about 2 fold compared to NC solution treated cells which further increased to 2.7 fold after 48 hr treatment.

These results showed an increase in the drug levels for the optimized C-SLN formula treated cells at all investigated time points compared to native curcumin solution treated cells. These results were further corroborating the results of previous studies and proposed mechanisms of increased cell uptake, sustained drug release and increased drug retention.

Figure (7): Quantitative intracellular uptake of curcumin from NC solution and the optimized C-SLN formula by A2780 ovarian cancer cells. Data as mean ± SD (n =3).

CONCLUSION

In the present study, the optimized formula of Solid Lipid Nanoparticles containing curcumin (C-SLN) was successfully prepared and evaluated. The morphological examination performed indicated the spherical nature and loading of curcumin into SLN formulation had no effect on particle size and morphology of SLNs. The stability study conducted had proven that the optimized C-SLN formula was stable over the storage period of 6 months at different temperatures. The cytotoxicity studies revealed enhanced antiproliferative activity of the optimized CSLN formula against NC solution indicated by the significant reduction in both cell viability and IC_{50}. The qualitative cellular uptake studies showed enhanced uptake of the optimized C-SLN formula in A2780 ovarian cancer cells as compared to NC solution. The results of
quantitative cellular uptake studies showed an increase in the drug levels for the optimized C-SLN formula treated cells at all investigated time points compared to NC solution treated cells. We conclude from these results that SLNs could serve as a promising delivery system to sustain the release and enhance the stability and bioavailability as well as the antitumor activity of curcumin.

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