Preparation, Characterisation and In Vivo Evaluation of Silybin Nanoparticles for the Treatment of Liver Fibrosis

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Abstract

Purpose: To formulate and characterize nanoparticles containing silybin, and evaluate their activity against carbon tetrachloride (CCl₄)-induced liver toxicity.

Methods: Silybin nanoparticles were formulated by o/w emulsion solvent evaporation technique using poly-ε-caprolactone as polymer. Four different nanoparticle formulations (NP₁, NP₂, NP₃ and NP₄) were prepared by varying the drug/polymer ratio. The particles were characterized for particle size, drug content and in vitro drug release. The pharmacokinetics and pharmacodynamics of the silybin formulations in male Wistar rats were evaluated following i.v. administration, using silybin solution as reference. The hepatoprotective activity of the formulations was also determined in a CCl₄-treated rat model.

Results: Silybin nanoparticles were successfully prepared using o/w emulsion solvent evaporation technique. The nanoparticles sustained the release of the drug both in vitro and in vivo for up to 10 days and offered better pharmacokinetic properties than the free drug itself. Intravenous nanoparticulate administration reversed serum liver enzyme levels by 95 % compared to only 50 % for the drug solution.

Conclusion: The developed silybin nanoparticles showed superior pharmacokinetic properties and hepatoprotective activity to silybin solution.

Keywords: Silybin, Nanoparticles, Pharmacokinetics, Pharmacodynamics, Hepatoprotective activity.

INTRODUCTION

Non-alcoholic and alcoholic fatty liver, chronic hepatitis and hepatic carcinoma all of which causes liver fibrosis that results in irreversible cirrhosis ultimately death in human beings [1,2]. Liver fibrosis is the accumulation of extracellular matrix or scar in the liver. There is no standard treatment for liver fibrosis [3]. Thus there is a need to find effective treatment for fibrosis. The ideal antifibrotic therapy would be one that is liver-specific, well tolerated when administered for prolonged periods of time, effective in attenuating excessive collagen deposition without affecting normal ECM synthesis, effectively delivered and nontoxic to other organs[4].

Silybin is one of the oldest drugs for the treatment of liver fibrosis and cirrhosis [5]. Although it is considered to be ideal for the...
treatment of liver fibrosis, delivery to the liver still needs improvement. The effectiveness of oral silybin as a hepatoprotective agent has disappointed by its poor solubility, low bioavailability and low half-life [6]. Silybin needs to be administered daily to achieve its effects. Nanosized carriers encapsulating silybin with size less than 6 µm can be taken up passively into Kupffer cells in the liver and can result in increased drug concentration in the liver after intravenous administration, thus increasing therapeutic efficacy. They can result in sustained systemic release of silybin for more than a week, depending on various factors, after forming a depot in the Kupffer cells. Thus, repeated daily administration for silybin can be avoided. Further, oral bioavailability problems with silybin can be avoided since bioavailability is 100 % after intravenous administration.

Oxidative stress in Kupffer cells is known to initiate the formation of liver fibrosis in many diseases and thus antioxidant silybin levels in these cells, if enhanced, can tremendously improve therapy with silybin. Thus, with this type of formulation, sustained parenteral release, improvement in bioavailability as well as enhancement of biochemical protection can be achieved. Together, these mechanisms lead to increase in effectiveness of therapy. Thus, the objective of this study was to prepare biodegradable nanoparticles of silybin, and evaluate their sustained release characteristics, liver targetability and liver protection following intravenous administration.

EXPERIMENTAL

Silybin and Poly-ε-caprolactone (mol wt:, 14,000) were procured from Sigma-Aldrich, Germany. Polyvinyl alcohol (PVA, cold-water soluble) was procured from Qualikems Fine Chemicals Pvt Ltd, New Delhi. Dichloromethane was procured from SD Fine Chemicals Ltd, Mumbai, India. All other reagents were of analytical grade. A probe sonicator (Homogenizer 150 VT), used to prepare the nanoparticles, was procured from M/S Biologics, Inc USA. A zeta sizer (3000 HAS (Malvern Instruments, Malvern, UK). The formulations were diluted 1:1000 with the aqueous phase of the formulation to obtain suitable kilo-counts per second (kcps). Analysis was performed at 25 °C with an angle of detection of 90°. Each determination was made in triplicate.

Determination of particle size and charge

The nanoparticles were evaluated for their particle size, polydispersity index of size distribution and surface charge potential, by photon correlation spectroscopy (PCS) using Zetasizer 3000 HAS (Malvern Instruments, Malvern, UK). The formulations were diluted 1:1000 with the aqueous phase of the formulation to obtain suitable kilo-counts per second (kcps). Analysis was performed at 25 °C with an angle of detection of 90°. Each determination was made in triplicate.

Evaluation of encapsulation efficiency

Encapsulation efficiency (EE) was calculated by estimating the amount of unentrapped drug. This was found by measuring the absorbance of the drug in supernatant, which was obtained after centrifugation of the nanoparticle suspension and then applying Eq 1.

\[
EE (\%) = 100 \left( \frac{W_1 - W_2}{W_1} \right) \quad (1)
\]

The value obtained from Eq 1 was compared with entrapped drug. For this determination, an accurately weighed amount of nanoparticles was taken in a test tube, dissolved in dichloromethane (DCM) and the solvent allowed

Preparation of silybin nanoparticles

Emulsion (o/w) solvent evaporation method was employed in the preparation of silybin nanoparticles using poly-ε-caprolactone as the polymer. Four different nanoparticle formulations NP₁, NP₂, NP₃ and NP₄ containing drug:polymer in the ratio of 1:1, 1:2, 1:3 and 1:4, respectively, were prepared. For the preparation, silybin (100 mg) and polycaprolactone (100, 200, 300 or 400 mg) was dissolved in 15 ml of dichloromethane by vortexing. The mixture (organic phase) was added drop-wise to 50 ml of 2% PVA solution under probe sonication at 40 w for 12 min to obtain a w/o emulsion. This emulsion was placed on a magnetic stirrer to ensure complete evaporation of dichloromethane, leaving nanoparticle suspension. The suspension was centrifuged at 10,000 rpm for 20 min, resulting in the formation of a pellet at the bottom of the tube. This pellet was washed with phosphate buffered saline (PBS), re-suspended and again centrifuged. The pellet was collected and allowed to dry completely. The powdered particles were collected, weighed and used for further evaluation.
to evaporate completely. An aliquot of methanol (10 ml) was added to the test tube which dissolved only the drug. The absorbance of this solution was measured and the amount of drug encapsulated calculated.

In vitro release study

The in vitro release study was performed in a diffusion cell set-up across a dialysis membrane. An inverted cylindrical test tube cut to a height of 8 cm was used as a donor compartment. The receiver compartment consisted of 100 ml of phosphate buffer (pH 7.4, 37 °C) in a beaker placed over a water bath. A dialysis membrane which was pre-soaked in warm water for 30 min was placed at the lower end of the cylindrical set-up and the membrane separated the donor compartment from the receiver compartment. Nanoparticles containing 20 mg of drug was suspended into 5 ml of pH 7.4 buffer and placed in the donor compartment. The system was stirred using a magnetic stirrer and bead. Samples (5 ml) were removed from the receiver compartment and replaced with the same volume of fresh medium immediately. The samples were analyzed spectrophotometrically at 287 nm.

Pharmacokinetic studies

Male Wister rats (weighing 150 – 180 g each) were purchased from Mahaveer Enterprises, Hyderabad, India, and were maintained in an air-conditioned room at 22 ± 2 °C and relative humidity of 45 – 55 % in a 12/12 h light/dark cycle. All the animal experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India [7] and the study protocol was approved by Institutional Animal Ethical Committee of Vaagdevi College of Pharmacy, Warangal, India (ref no. 1047/ac/07/CPCSEA). The body weight of the rats were also monitored.

The animals were anaesthetized on the last day of the study and blood was collected by cardiac puncture. Plasma was separated from the blood samples by centrifugation at 3000 rpm for 15 min. Hepatoprotective activity was quantified by serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvic transaminase (SGPT) levels in the plasma. Subsequently, their livers were subjected to histopathological examination. First, the rats were sacrificed at the last day of the study, the liver separated carefully and preserved in formalin solution, and liver sections were prepared. [13] The body weight of the rats were also monitored.

Statistical analysis

The data were expressed as mean± standard deviation (SD) and statistical analysis was carried out by one-way ANOVA followed by Student’s Newman-Keuls test. The level of significance used was P < 0.05. The statistical software used was Graph Pad Prism, USA, versions 4 and 5.
RESULTS

Silybin nanoparticles were successfully prepared using poly-ε-caprolactone by o/w emulsion solvent evaporation method. The mean particle size of the nanoparticles ranged from 130 - 430 nm (Table 1). Mean particle size increased with increase in polymer concentration. The polydispersity index (PDI) of the nanoparticles was < 0.4, indicating that the nanoparticles were homogenous in distribution. The zeta potential value indicates long term stability. The encapsulation efficiency of the nanoparticles increased as polymer concentration increased.

**In vitro drug release characteristics**

*In vitro* release data, shown in Fig 1, indicates that all the formulations sustained drug release for over a period of 20 days. A biphasic drug release pattern was found, i.e., burst release followed by sustained release. In the first 6 hours of release, the unencapsulated drug component was released. Subsequently, the encapsulated drug component was gradually released. Drug release from NP1 was higher than from NP2, NP3 and NP4. Log percent cumulative drug released, plotted as a function of log time yielded curves, the slope of is the diffusional release exponent (n). The values of diffusional n were 0.359, 0.374, 0.420 and 0.450 for NP1, NP2, NP3 and NP4 respectively, which indicate that drug release from all the formulations followed a Fickian pattern [9].

![Figure 1: Drug release from silybin nanoparticles (○=NP1, □=NP2, △=NP3, ×=NP4)](image)

**Pharmacokinetics of the formulations**

The retention time of the drug, based HPLC studies, was 16.0 and 18.0 min for the two isomers of silybin. The plasma profiles of the drug after administration of nanoparticles and *i.v.* solutions are shown in Figure 2. Nanoparticles resulted in higher and more prolonged drug levels than the drug solution. This was reflected by increased area under the curve. The t_{1/2}, AUC and Vₚ of nanoparticle formulation were higher than for the drug solution, while clearance (CL) was lower than for the free drug (Table 2). All the pharmacokinetic parameters obtained for the nanoparticles were statistically different from those obtained for the drug solution (p < 0.001), except Cmax.

![Figure 2: Plasma concentration-time curve of silybin following i.v. administration to rats (● = nanoparticles, ○ = solution)](image)

**Hepatoprotective activity**

Table 3 shows hepatoprotective activity data. The administration of CCl₄ to the animals resulted in a marked increase in SGPT and SGOT activities, indicating increased toxicity, but this was mitigated in the animals treated with silybin formulations. The reduction in toxicity was statistically significant at p < 0.001 for both silybin formulations and silybin solution.

**Table 1: Particle size, charge, PDI and encapsulation efficiency of silybin nanoparticles**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (mean±SD, nm)</th>
<th>Polydispersity Index(PDI)</th>
<th>Zeta potential (mean±SD)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP₁</td>
<td>135±3</td>
<td>0.13</td>
<td>-31±3</td>
<td>91.0</td>
</tr>
<tr>
<td>NP₂</td>
<td>220±2</td>
<td>0.14</td>
<td>-36±3</td>
<td>92.6</td>
</tr>
<tr>
<td>NP₃</td>
<td>310±2</td>
<td>0.20</td>
<td>-34±3</td>
<td>94.9</td>
</tr>
<tr>
<td>NP₄</td>
<td>426±1</td>
<td>0.35</td>
<td>-37±3</td>
<td>95.3</td>
</tr>
</tbody>
</table>
The photomicrographs in Fig 3 display the histological changes in the liver of the animals following administration of the drug-loaded nanoparticles. The histological profile of the control animals showed normal hepatic architecture with distinct hepatic cells, well-presented cytoplasm sinusoidal spaces and central vein. However, there was disorganization of normal cells with intense centrilobular necrosis following CCl\textsubscript{4} intoxication. Moderate accumulation of fatty lobules and cellular necrosis were observed in the animals treated with silybin solution. However, the nanoparticle formulation exhibited strong protection against CCl\textsubscript{4}-induced liver damage, as evidenced by the presence of normal hepatic cords, well-defined cytoplasm and absence of necrosis. Furthermore, the body weights of the rats which fell significantly after CCl\textsubscript{4} treatment were restored to normal following administration of the silybin preparations.

### Table 2: Pharmacokinetic parameters for silybin formulations administered i.v. (mean ± SD, n = 3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Silybin solution by i.v. bolus</th>
<th>Silybin nanoparticles (NP1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>4.7±0.2</td>
<td>4.3±0.1</td>
</tr>
<tr>
<td>$K_e$ (h\textsuperscript{-1})</td>
<td>0.316±0.009</td>
<td>0.0079±0.00016***</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>2.19±0.10</td>
<td>87.6±1.3***</td>
</tr>
<tr>
<td>$V_d$ (L)</td>
<td>0.893±0.04</td>
<td>0.976±0.007***</td>
</tr>
<tr>
<td>Clearance (L/h)</td>
<td>0.282±0.001</td>
<td>0.00715±0.0012***</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (µg.h/ml)</td>
<td>15.42±0.24</td>
<td>600.8±8.0***</td>
</tr>
</tbody>
</table>

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with silybin i.v. solution.

### Table 3: Effect of silybin formulations on enzyme levels in rats with carbon tetrachloride (CCl\textsubscript{4})-induced hepatotoxicity (mean ± SD, n = 6)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Initial body weight (g)</th>
<th>Body weight after 9 days (g)</th>
<th>SGPT (U/L)</th>
<th>SGOT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>160±10</td>
<td>180±15</td>
<td>11.1±2.3</td>
<td>33.3±2.8</td>
</tr>
<tr>
<td>CCl\textsubscript{4}</td>
<td>165±15</td>
<td>148±10</td>
<td>77.8±3.9</td>
<td>90.0±3.3</td>
</tr>
<tr>
<td>Silybin solution</td>
<td>170±5</td>
<td>180±5***</td>
<td>44.4±4.6</td>
<td>63.3±3.2</td>
</tr>
<tr>
<td>Drug nanoparticles</td>
<td>170±6</td>
<td>196±15***</td>
<td>18.4±3.4</td>
<td>47.6±4.6</td>
</tr>
<tr>
<td>Placebo</td>
<td>165±6</td>
<td>156±12</td>
<td>70.4±2.6</td>
<td>86.6±2.2</td>
</tr>
</tbody>
</table>

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with CCl\textsubscript{4} group

However, the drug nanoparticles completely reversed the elevated levels of SGOT and SGPT.

### DISCUSSION

In recent years, various new drug carrier systems in the micro- and nanometer size range have been generated to improve drug delivery. Among them, nanoparticles have certain advantages such as a maximal load of the drug and a long shelf life. Furthermore, their body distribution and permeability in tissues can be controlled by size and surface properties [10]. Previously, we demonstrated the enhanced liver protection of curcumin, curcumin analogues and piperine, using kupffer cell (KC)-targeted nanoparticle and liposomal formulations, and sustained release intraperitoneal microspheres [11-13]. Silybin, a natural antioxidant, has long been used for the treatment of chronic liver diseases. The effectiveness of silybin as a liver disease remedy was discounted by its poor solubility and low bioavailability. It offers low bioavailability due to high first pass metabolism, low $t_{1/2}$ and results in patient non compliance due to repeated dosing. Consequently, insufficient concentrations of drugs may accumulate in the target cells. Further, uptake into non-target cells may lead to significant side-effects. Thus we made an attempt to deliver silybin nanoparticles to its target site through bypassing oral route. It can be administered systemically to offer liver protection in a variety of diseases. It can also be targeted to KC and its delivery into these cells can be enhanced with silybin encapsulated in the particulate systems and further improving the therapy.

To achieve the aims of this study, silybin nanoparticles have been prepared using emulsion solvent evaporation technique. Four different formulations NP1, NP2, NP3 and NP4 were prepared. Particle size and entrapment efficiency of silybin nanoparticles increased with increase in polymer content. This may be due to the availability of more polymer to coat the drug. Increase in polymer content also delayed drug release due to increase in particle size and hence reduced surface area available for drug release. Drug release was highest from NP1 due to because it had the polymer content and this has the effect of retarding drug release as a result of increased particle size and reduced surface area available for drug release. Formulation NP1 was used as the optimized formulation for hepatoprotective test because of its lower particle size and polydispersity index (PDI), and good release profile. This particle size favors uptake into all the liver cells responsible for the formation of liver fibrosis [14]. Higher uptake of particles into KC occurs by passive targeting.

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NP1 sustained drug release for up to 10 days in vivo and offered better pharmacokinetic properties than the drug in solution form. Elimination rate constant and clearance than for the drug solution. Furthermore, area under the curve (AUC) was several-fold than for the solution. This suggests reduction in drug elimination and metabolism with nanoparticle formulation administration.

Carbon tetrachloride, a known hepatotoxin, is a commonly used model for hepatoprotective drug screening, and the severity of the liver damage is measured by the levels of elevated cytoplasmic enzymes (SGOT and SGPT) in circulation [15]. The hepatocellular damage induced by CCl₄ is due to its metabolite, trichloromethyl free radical (CCl₃) that binds to lipoprotein and leads to peroxidation of the lipids of the endoplasmic reticulum [16] by the action of the mixed function of the cytochrome P450 oxygenase system. This free radical, which is initially relatively unreactive when formed initially, reacts very rapidly with oxygen to yield a highly reactive trichloromethyl peroxy radical (CCl₃OO). Both radicals are capable of binding to proteins or abstracting a hydrogen atom from an unsaturated lipid, thus accelerating lipid peroxidation. Expectedly, SGOT and SGPT levels were higher in CCl₄-treated animals due to tissue damage caused by CCl₄ which results in the release of the enzymes into the blood stream. Upon administration, silybin reduced elevated enzyme levels. Reversal of liver toxicity was greater for nanoparticle formulation than for the drug solution form probably because the nanosized particles were instantly taken up by KC as well as by several other liver cells. As a result, the drug becomes accumulated in the liver cells and is subsequently released at the cellular level to achieve better hepatoprotection.

Overall, the findings of this study suggest that the developed nanoparticle formulation may be useful in the treatment of cirrhosis and fibrosis with silybin. It is likely that these results can be extrapolated to other drugs, suggesting the probability of nanoparticulate passive targeting of drugs to the liver, including for the treatment diseases such as liver cancers.

CONCLUSION

Silybin nanoparticles can be suitably prepared by emulsion solvent evaporation technique using polycaprolactone as a biodegradable polymer. The particles showed good encapsulation efficiency and sustained drug release both in vivo and in vitro.
vitro and in vivo. Silybin nanoparticles offer an effective approach for drug targeting of the liver.

REFERENCES