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Research Article

Theme: Lipid-Based Drug Delivery Strategies for Oral Drug Delivery
Guest Editor: Sanyog Jain

Beta-carotene-Encapsulated Solid Lipid Nanoparticles (BC-SLNs) as Promising Vehicle for Cancer: an Investigative Assessment

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Abstract. Beta-carotene (BC), a red-colored pigment found in plants and animals, is one of the most extensively investigated carotenoids due to its provitamin-A, antioxidant, and anticancer properties. The anticancer activity of BC through oral administration is severely affected due to its low bioavailability and oxidative degradation. The present study aimed to formulate and characterize solid lipid nanoparticles (SLNs) of BC for enhanced bioavailability and therapeutic efficacy. Beta-carotene-loaded solid lipid nanoparticles (BC-SLNs) were prepared employing different combinations of glyceryl monostearate and gelucire. The characterization studies were performed for particle size, morphology, release behavior, and stability. BC-SLNs were also studied for *in vitro* cytotoxicity in human breast cancer cell lines (MCF-7) and pharmacokinetic studies in Wistar rats. The cytotoxicity studies confirmed that encapsulation of BC within the lipid bilayers of nanoparticles did not affect its anticancer efficacy. An improved anticancer activity was observed in BC-SLNs as compared to the free BC. BC-SLNs enhanced the bioavailability of BC on oral administration by sustaining its release from the lipid core and prolongation of circulation time in the body. Similarly, area under the curve (AUC_{total}) enhanced 1.92-times more when BC was incorporated into SLNs as compared to free BC. In conclusion, solid lipid nanoparticles could be an effective and promising strategy to improve the biopharmaceutical properties of carotenoids for anticancer effects.

KEY WORDS: beta carotene; breast cancer; solid lipid nanoparticles; cytotoxicity; particle size.

INTRODUCTION

Breast cancer is one of the fatal malignancies affecting millions of women across the world (1). The therapeutic options available for the treatment of metastatic stage are nowhere to be found and therefore, treatment of metastatic cancer poses a major challenge to the researchers and clinicians. Additionally, cancer growth rate, response time, and failure of therapeutic strategies are the other challenges being faced by clinicians (2,3). Breast cancer is a major global concern and development of effective therapeutic interventions remains a priority. Although a number of treatment therapies are available for breast cancer, the adverse effects associated limits their application (4).

A number of literatures and experimental and clinical studies in the past have explored the potential of retinoids, carotenoids, and other vitamins for chemoprevention and treatment of varied cancer types (3). The cellular and metabolic effects of retinoids and carotenoids depend upon the dose employed, extent of exposure, and cancer cell type. Despite the immense importance of these agents in cancer cell biology and treatment, their anticarcinogenic mechanisms yet remain unknown (5). Beta-carotene (BC), a red-colored pigment found in plants and animals, is one of the most extensively investigated carotenoid due to its provitamin-A, antioxidant, and anticancer properties (6–9). The bioactivity of BC is restricted due to the poor stability against oxidative degradation and lower bioavailability on oral administration (10).

Among the multitude of lipid carriers, Solid lipid nanoparticles (SLNs) are potential delivery carriers due to

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prospects of bioavailability enhancement and stability of drug molecules incorporated within the lipid bilayers. SLNs generally employ biosafe lipids, which are dispersed in an aqueous surfactant (11–14). SLNs offer multitude of advantages vis-à-vis other lipid-derived nanocarriers not limited to substantially higher drug loading improvised drug stability and passive drug targeting (15). SLNs possess the potential to overcome stability and drug-leakage challenges associated with other vesicular delivery systems like liposomes and emulsions (16,17). These nanocarriers can solubilize poorly water-soluble drug molecules and provide controlled drug release in a temporal manner for substantially longer periods (18). The lipid core of SLNs stimulates chylomicron formation and facilitates lymphatic uptake to bypass hepatic first-pass drug metabolism.

Considering these challenges, the present envisioned to design to develop and evaluate the potential of SLNs as an effective delivery system for BC. BC loaded SLNs were developed and characterized for micromeritics, entrapment potential, and release behavior. The developed nanoparticles were further investigated for stability, *in vitro* cellular toxicity and bioavailability enhancement. In brief, the current study confirms the potential of SLNs as an effective delivery vehicle for BC. The carotenoid encapsulated within the lipid layer of SLNs demonstrated improved bioavailability and anticancer efficacy.

MATERIALS AND METHODS

Materials

BC was procured from Himedia Pvt. Ltd., Mumbai, India. Phospholipid S-100 (M/s Phospholipid GmbH, Germany), Pluronic F-68 (PF-68, BASF Pvt. Ltd., Mumbai, India), and Gelucire® 50/13 (Gattefosse India Pvt. Ltd., Mumbai, India) were obtained as generous gift samples from the respective companies. The dialysis membrane (MWCO 12kDa) employed for studying release behavior was procured from Himedia Pvt. Ltd., Mumbai. Glyceryl monostearate (GMS) was purchased from Sigma-Aldrich Pvt. Ltd., Germany.

Fabrication of Beta-carotene-Loaded Solid Lipid Nanoparticles

Beta-carotene-loaded SLNs (BC-SLNs) were prepared by hot homogenization process, as per earlier reports (19).The lipid phase (A) composed of BC (100 mg), glyceryl mono stearate (GMS; 700 mg), and gelucire50/13 (700 mg) were taken together and melted actually melting point of GMS is > 70°C. The lipid phase (B) comprising of Phospholipid S-100 (500 mg) was solubilized in ethanol, while subjecting to continuous stirring. Further, the ethanolic solution of phospholipid was added into molten lipid phase. This lipid phase was added in a streamline manner to 20 mL of previously heated aqueous mixture of Tween-80 (0.5%, *v*/*v*) and Pluronic F68 (0.1%, *w*/*w*). The mixture composed of lipid and aqueous phase was homogenized for 30 min at 10,000 rpm employing high shear mixer (M/s Heidolph Silent Crusher, Mumbai, India).

In the last stage of BC-SLN preparation process, dialysis bag method was performed to remove organic phase and unincorporated BC (20). The SLNs were lyophilized further employing lyophilizer at − 80°C and 200 Torr pressure.

Characterization of BC-SLNs

Physicochemical Attributes

The particle size of developed nanoparticles was determined employing Nano ZS-90 Zeta sizer (Malvern Instruments Corp; UK). The suspension was diluted with double-deionized water for the measurement of polydispersity index (PDI) and particle size. The surface charge of BC-SLNs was measured by diluting the suspension ten times at an electric field of 20.24 V/cm.

The morphological attributes of BC-SLNs were studied using field emission scanning electron microscopy (FE-SEM). The carbon-coated grids were covered with a drop of diluted nanosuspension and further subjected to 300 Å gold coating using a sputter coater. All the samples were visualized at an accelerating voltage of 10 kV and × 2500 magnification.

Percent drug efficiency (%EE) and percent drug loading (%DL) were determined using direct lysis method (21). The suspension was subjected to dialysis to remove the unentrapped carotene and further centrifuged at 15,000 rpm (4°C) for 10 min. The pellet obtained at the bottom of centrifuge tube was dissolved in ethyl acetate, followed by sonication to lyse the pellets. Additionally, the supernatant was analyzed to quantify the content of unentrapped BC. The pellet mixture and the supernatant were further analyzed employing RP-HPLC to estimate the content of BC. In brief, reversed phase C18 column (10 μm, 4.6 mm × 250 mm) was used for chromatographic separation. The mobile phase consisted of an 80:10:10, *v*/*v*/*v* blend of methanol, ethyl acetate, and acetonitrile. The volume of injection was 20 μL and the analysis was performed at 449 nm at 2.0 mL/min flow rate (6,8). The %EE and %DL were calculated using Eqs. 1 and 2.

$$\%\mathrm{EE} = \frac{\text{Total quantity of BC added} - \text{Quantity of BC in the sample}}{\text{Total quantity of BC added}} \times 100 \quad (1)$$

$$\%\mathrm{DL} = \frac{\text{Quantity of BC encapsulated in SLNs}}{\text{Total quantity of SLNs}} \times 100 \quad (2)$$

In Vitro Drug Release Studies

The release of BC encapsulated from the lipidic core of SLNs was determined employing dialysis bag method (6,13). Briefly, the lyophilized BC-SLNs (equivalent to 50 mg of BC) were suspended in 5 mL of release media and placed in a dialysis bag. The dialysis bag was further suspended into 75 mL of release medium comprising of 0.1 N hydrochloric acid and sodium lauryl sulfate (0.5% *w*/*v*), which was further stirred at 35 rpm. Aliquots of 500 μL were removed at

Table I. Micromeritics and % Entrapment Efficiency of BC-SLNs

GMS: Gelucire	Particle size (nm)	PDI	Zeta potential (mV)	%EE	%DL
1:0.5	311.4 ± 22.2	0.376 ± 0.02	− 6.13 ± 0.09	53.4 ± 5.2	9.22 ± 0.93
1:1	203 ± 7.23	0.185 ± 0.009	− 7.21 ± 0.82	68.3 ± 3.4	12.89 ± 1.03
1:2	397.7 ± 18.1	0.504 ± 0.03	− 9.31 ± 0.82	61.9 ± 4.1	11.12 ± 0.83

designated intervals of 0, 0.5, 1, 2, 4, 8, 12, 24, and 48 h, respectively. An equal volume of fresh medium was replenished to maintain the sink condition. The content of BC was estimated by RP-HPLC technique.

Stability Study

The developed nanoparticles were subjected to short-term stability study at three different temperature conditions, *viz.* 5 ± 2°C, 25 ± 2°C, and 40 ± 2°C for a period of 3 months. BC-SLNs were also studied for physical attributes to ascertain the plausible changes in color and residual content.

Antioxidant Activity by DPPH Method

DPPH assay was employed to ascertain the antioxidant activity of lyophilized nanoparticles (22). A stock solution (1 μg/mL) of BC was prepared in methanol. The lyophilized BC-SLNs were suspended in methanol to lyse the particles and separate the encapsulated BC. Further, 100 μL of methanolic solution of each of BC solution (free BC and the removed BC from BC-SLNs) was treated individually with DPPH (0.3 mM) reagent. The mixtures were kept under dark and the resulting absorbance (A) was measured at 517 nm employing UV spectrophotometer (Shimadzu, Japan). Similarly, a control sample was also prepared for comparison purposes to estimate the radical scavenging as mentioned in Eq. 3.

$$\text{Scavenging activity}\ (\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \tag{3}$$

The antioxidant activity of free BC and BC-SLNs was measured at different time intervals up to a period of 3 months.

Cell Culture and Cell Survival Study

MCF-7, human breast adenocarcinoma cells, were obtained from NCCS (National Center for Cell Sciences), India. The cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS), 1% streptomycin and 3 mM glutamine at a cell density of 3×10^4 cells/mL in 96 well plates (Sigma, Germany). The cell culture plates were kept in an incubator at 5% CO_2 atmosphere and 37°C. Further, culture medium of each well in 96-well plate was replaced with 200 μL of complete medium containing free BC and BC-SLNs at a concentration of 1, 10, 25, and 50 μM (equivalent to free BC). The cells were incubated with free and lipid incorporated BC for a period of 48 and 72 h. After the

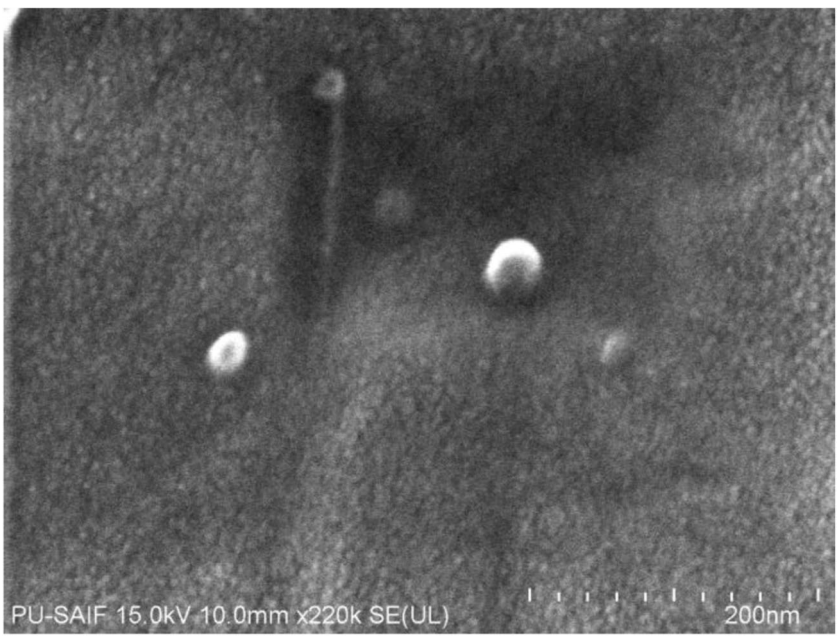

Fig. 1. FE-SEM photomicrograph of BC-SLNs

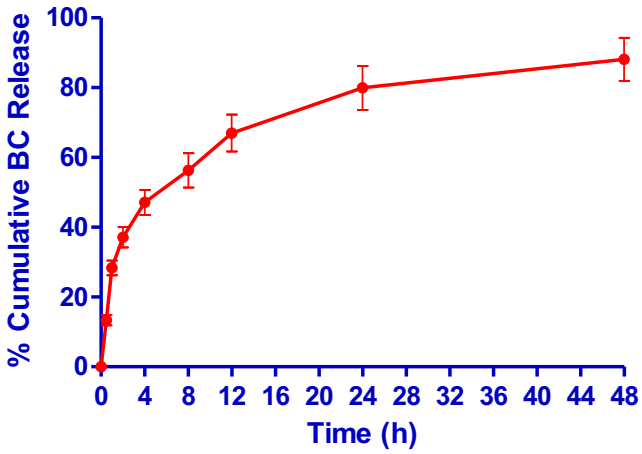

Fig. 2. *In vitro* release profile of BC from BC-SLNs in 0.1 N HCl. Each cross bar indicates average value ± SD ($n = 3$)

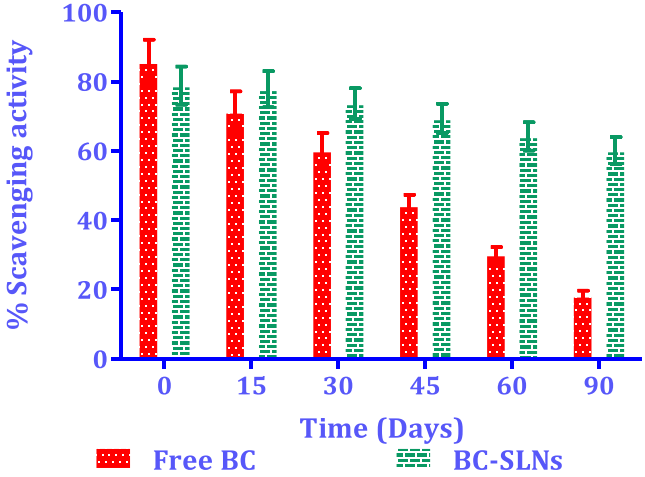

Fig. 3. Antioxidant activity of free BC and BC-SLNs over the period of 3 months

designated time intervals, the cells were rinsed with PBS (pH 7.4) and incubated with 0.15 mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution for 4 h. The incubation resulted in the formation of purple-colored formazan crystals which were further solubilized in 200 μL of DMSO. The absorbance of the resulting solution was determined employing an ELISA plate reader (BioTek, USA) at 550 nm (23).

In Vivo Oral Plasma Quantification Profile

Albino Wistar rats (unisex, 150–200 g) were employed for *in vivo* plasma estimation of BC. All the experimental procedures were carried out in accordance with institutional guidelines as indicated by the Institutional Animals Ethical Committee, Panjab University, Chandigarh, India, with prior approval (PU/IAEC/S/15/05).

The experimental animals were divided into two groups each containing six animals each. Both the groups were administered an oral dose of free BC (15 mg/kg) and lyophilized BC-SLNs (equivalent to 15 mg/kg free BC), respectively. The oral dose was prepared by suspending the formulations in 1 mL of 0.5% sodium carboxymethyl cellulose solution. At pre-defined intervals (0.5, 1, 2, 4, 6, 8 12, 18, and 24 h), 0.2 mL of blood samples were withdrawn from the retro-orbital plexus. The samples were placed in centrifuge tubes containing an anticoagulant and centrifuged at 1000*g* to separate the plasma. The separated plasma was mixed with 0.1 mL of trichloroacetic acid (10%, *w*/*v*) and 3 mL of acetonitrile in order to precipitate the protein and disrupt the nanoshells. The beta-carotene is fairly soluble in acetonitrile, therefore, come out in the solution form. The mixture was vortexed for 12 min and then centrifuged at 1000*g* for 10 min. The supernatant was analyzed for BC content by HPLC (6,24).

Statistical Analysis

The experimental results are represented as mean ± SD with statistical analysis completed by one-way analysis of variance (ANOVA) with Turkey–Kramer multiple comparison post-tests.

RESULTS AND DISCUSSION

Fabrication of BC-Loaded Solid Lipid Nanoparticles

BC-loaded SLNs were fabricated employing hot homogenization technique. The lipid components were chosen on the basis of solubility of the beta-carotene in lipids. The quantity of lipid employed in the preparation process was optimized by varying the ratio of both the lipids (GMS:Gelucire 50/13) from 0.5:1 to 2:1. The formulations were further characterized for micromeritics including particle size, PDI, and zeta potential. The formulation containing GMS:Gelucire 50/13 in the ratio of 1:1was selected as desired ratio for formulation development with particle size of 203 ± 7.23 nm and PDI value of 0.185 (Table I). The formulations containing an equal ratio of GMS and Gelucire demonstrated an escalation in particle size as well as PDI. The incorporation of Tween 80 as a surfactant helped to minimize the interfacial tension between aqueous and organic phases to allow the formation of tiny droplets of molten lipid in the

Table II. Stability Information of the BC-SLNs After 3 Months of Storage

Formulation	Storage condition	Time	Particle size (nm)	PDI	ζ (mV)	% EE
BC-SLNs	Initial	–	203 ± 7.23	0.185 ± 0.009	− 7.21 ± 0.82	68.3 ± 3.4
	2–8°C	After 3 months	199.4 ± 9.3	0.193 ± 0.018	− 7.03 ± 0.9	68.18 ± 5.9
	RT; 25 ± 2°C/60 ± 5% RH		286.7 ± 21.3	0.396 ± 0.02	− 7.29 ± 0.6	66.7 ± 3.2
	HT; 40 ± 2°C/75 ± 5% RH		353.8 ± 19.6	0.468 ± 0.04	− 6.92 ± 0.5	64.8 ± 3.8

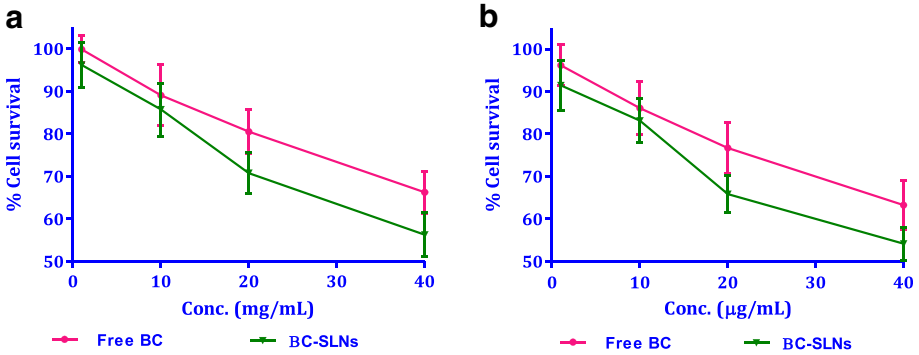

Fig. 4. Cell cytotoxicity of BC and BC-SLNs following **a** 48 h and **b** 72 h. Each data point represented as mean ± SD ($n = 6$; $p < 0.05$)

heated aqueous phase, resulting in decrease of particle size (25).

Physicochemical Attributes

The average particle size of BC-SLNs was found in the range of 200–400 nm with a unimodal distribution (Table I). BC-SLNs prepared with 1:1 ratio of GMS/Gelucire having average particle size of 203 ± 7.23 nm and PDI of 0.185 ± 0.009 was selected as the optimum. The optimized BC-SLNs formulation exhibited zeta potential of −7.21 ± 0.82 mV (Table I). FE-SEM images of BC-SLNs also confirmed the nanometric size range of particles with circular 2-D morphology and uniform surface as depicted in Fig. 1.

A number of different combinations of GMS and Gelucire were employed to prepare BC-SLNs with an aim to achieve higher value of %EE and %DL. The lipid nanoparticles prepared with 1:1 ratio of GMS and Gelucire exhibited high entrapment efficiency (68.3 ± 3.4%) and better drug loading capacity (12.89 ± 1.03%) as compared to other formulations (Table I). The high entrapment efficiency with GMS and Gelucire can be ascribed the more solubility of carotene in the lipidic mixture.

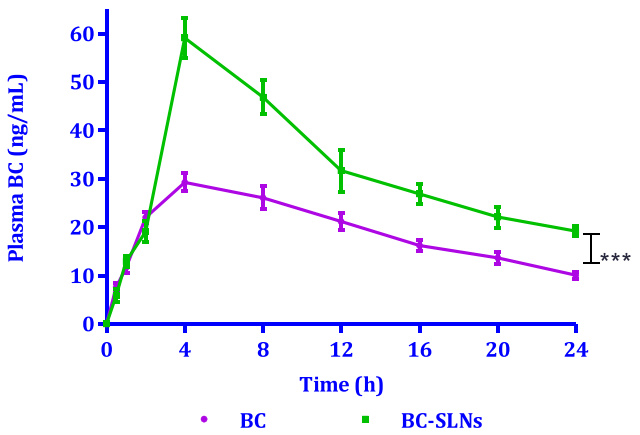

Fig. 5. Plasma profile of BC after oral administration of free BC and BC-SLNs

In Vitro Drug Release Study

The release behavior of encapsulated BC from lipid nanoparticles was studied up to a period of 48 h employing dialysis bag technique. The nanoparticles exhibited an initial burst release (28.34 ± 2.1%) for 1 h followed by relatively slower and controlled release sustained over a period of 48 h (Fig. 2). The initial burst release was due to the BC adsorbed at the surface of particles or its presence just in the first layer of the SLNs. The second phase of sustained release may be ascribed to the diffusion of incorporated BC molecules through the lipid matrix of SLNs. A number of literature studies in the past have demonstrated that the presence of gelucire promoted an increased release of incorporated drug molecules from lipid nanoparticles as gelucire is recognized for faster dissolution (13,26,27).

Stability Study

The developed formulations were subjected to stability studies over a period of 3 months at three different temperatures as depicted in Table II. No significant variations in physical appearance and drug assay were observed, which demonstrates the good physical stability of SLNs (28).

Antioxidant Activity by DPPH Method

Figure 3 represents the free radical scavenging activity of BC and BC-SLNs. Plain BC depicted high antioxidant activity, which was retained even after its localization within the SLNs. Figure 3 depicts a considerable difference in the extent of antioxidant activity of plain BC and BC-SLNs. The results indicate that the antioxidant activity of BC was not

Table III. Various Pharmacokinetic Parameters in Serum of Wistar Albino Rats

Pharmacokinetic parameters	Plain BC	BC-SLNs
C_{max} (µg/mL)	29.3	59.1
T_{max} (h)	4.0	4
AUC_{total} (µg/mL h)	633.81	1215.32
$t_{1/2}$ (h)	11.86	16.30
MRT (h)	18.98	24.47

decreased due to its encapsulation within the biocompatible lipidic layers. The reduced percent scavenging activity of free BC further confirmed that antioxidant activity of BC was exclusively contributed by lipidic system which maintains the biological activity of BC during the course of experiments.

Cytotoxicity Study

The cytotoxicity associated with BC and BC-SLN formulations was determined by MTT assay after 48 and 72 h, respectively (Fig. 4). The results suggested an increased death of MCF-7 cells with an increase in concentration of BC either free or encapsulated in nanoparticulate system. The cytotoxicity studies confirmed that encapsulation of BC within the lipid bilayers of nanoparticles did not affect its anticancer efficacy. An improved anticancer activity was observed in BC-SLNs as compared to the free BC. Both free BC and BC-SLNs failed to show significant inhibitory effect on the cancer cells at a concentration below 10 μM/mL. However, when the concentration of BC was increased, a significant increase in cell death was observed when incubated for a period of 72 h (Fig. 4). The current results sustain the hypothesis that small-sized particles (~ 200 nm) can more efficiently escape phagocytosis and enter cancerous cells at a faster rate as compared to large-sized particles.

In Vivo Oral Plasma Quantification Profile

The plasma profile obtained validates the enhanced bioavailability of BC when incorporated into lipid matrix (Fig. 5). Higher plasma concentration level of BC was observed with BC-SLNs as compared to plain BC. After 4 h of oral dose of free BC, 29.3 ± 1.8 μg/mL of BC appeared in rodent plasma. Further, a rapid decrease (12.2 ± 1.95 μg/mL) was observed, after the time interval of 12 h, which might be attributed to the faster elimination of BC from the central compartment. The administration of BC-SLN formulation resulted in higher BC levels in the serum. A concentration of 59.1 ± 4.2 μg/mL BC (from BC-SLNs) after 4 h of oral administration was reflected in plasma, which was further decreased after 24 h. The results are evidently supportive of the prolonged circulation time of SLNs and validate the importance of nanostructured delivery systems.

The C_{max} values offered by BC-SLNs were substantially higher than plain BC, which was reflected in AUC_{total} too. The group receiving BC-SLNs offered approx. 2-fold enhanced AUC vis-à-vis the group receiving plain BC (Table III). Contrarily, the concentration of BC in rat serum with BC-SLNs was considerably higher than the free BC group except for the time interval up to 2 h (Fig. 5).This might be due to the difference in the absorption pathways, where plain BC is absorbed from relatively faster routine GIT absorption, whereas BC-SLNs followed lacteal pathways, resulting in initial lag (29). In conclusion, the results of pharmacokinetic study in Wistar rats support the potential advantages of SLNs for the sustained delivery of BC. Furthermore, lipid-based nanoparticulate delivery of BC exhibits enhanced bioavailability and prolonged retention in the experimental animals.

CONCLUSIONS

The present study reports successful development of BC-SLNs which possess a potential to enhance the efficacy and safety of BC. The developed system substantially improved the oral bioavailability of BC in animal models and improved the efficacy indicators in *in vitro* models. The initial delayed absorption inferred the absorption from lacteal pathways, after incorporation in SLNs. The present work provides the insight as well as evidences of improved delivery of carotenoid employing lipid-based nanoparticles, which can be further extrapolated and validated.

FUNDING INFORMATION

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COMPLIANCE WITH ETHICAL STANDARDS

All the experimental procedures were carried out in accordance with institutional guidelines as indicated by the Institutional Animals Ethical Committee, Panjab University, Chandigarh, India, with prior approval (PU/IAEC/S/15/05).

Conflict of Interest The authors declare that they have no conflict of interest.

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