ORIGINAL ARTICLE

Baicalein Loaded Polysorbate 80 Nanostructured Lipid Carriers Offered Enhanced Stability and *In Vitro* Drug Release

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Abstract

Aim: The aim of this work was to characterize baicalein loaded nanostructured lipid carriers (NLCs) intended for brain targeting by intravenous route of administration. **Materials and Methods:** The NLC system, composed of stearic acid and polysorbate 80, was characterized in terms of its physicochemical properties, differential scanning calorimetry (DSC), and *in vitro* drug release. **Results and Discussion:** The lipid nanoparticles were spherical with an average size of ~100 nm. The zeta potential of the nanoparticles was about −50 mV. DSC studies suggested that the majority of the inner cores of polysorbate 80 NLCs had a slightly disordered crystal arrangement. The nanoparticulate dispersions demonstrated good physical stability during storage for 6 days. **Conclusion:** Polysorbate 80 NLCs improved baicalein's stability and the ability of baicalein to penetrate the brain; thus, this is a promising drug-targeting system for the treatment of central nervous system.

Key words: Baicalein, improved drug release, polysorbate 80, stability

INTRODUCTION

Strokes remain a major cause of death among worldwide. Ischemic strokes result in approximately 80% of all strokes.[1] Ischemic injury produces many free radicals, which are neurotoxic by inducing the apoptotic cell death of neurons. Baicalein (5, 6, 7-trihydroxyflavone) is a flavonoid obtained from the root of *Scutellaria baicalensis* Georgi, a medicinal plant which is used in Asian medicine. Baicalein was shown to protect against ischemic brain injury by its anti-inflammatory and antioxidant effects.[2,3] Baicalein is well known as a 12/15-lipoxygenase and xanthine oxidase inhibitor. In addition, it protects against neuronal cell damage induced by-amyloid protein, oxidative stress, and glutamate.[4] It was speculated that baicalein may be a promising agent for prevention or therapy of ischemic brain damage, traumatic brain injury, Alzheimer's disease, Parkinson's disease, and dementia.[5-7] Baicalein possesses some shortcomings, leading to an irrelevant *in vivo* or clinical effect compared to its powerful *in vitro* efficacy. Its rapid elimination half-life ($t_{1/2}$) in plasma (~10 min) and poor water solubility limit its applicability.[8] Some solvents used for baicalein administration, such as dimethyl sulfoxide, are inadequate and toxic and thus unsuitable for clinical situations.[9,10] In addition, the treatment of brain diseases is often constrained by the inability of potent drugs to pass the blood-brain barrier. One of the possibilities for delivering drugs to the brain is the use of nanoparticles. Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are new generations of lipid nanoparticles produced from solid lipids. SLNs consist of pure solid lipids, while NLCs are made of a solid matrix that entraps liquid lipid nano compartments.[11,12] NLCs are very important in drug delivery due to their ability to accommodate a greater quantity of drugs and their higher stability compared to SLNs.[13] NLCs are cost-effective and provide easy administration for drugs that cannot be formulated as aqueous solutions.

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Lipid nanoparticles appear suitable as a delivery system due to prolonged release, targeted efficiency with lower side effects, and less toxicity than polymeric nanoparticles.[14] Hence, lipid nanoparticles may be feasible as carriers for baicalein delivery. Liquid oils typically used for NLCs consist of digestible oils from natural sources. There is a need for novel, biocompatible formulations that are cost-effective, non-irritating, and capable of being sterilized before application. Polysorbate 80 is a hydrophilic non-ionic surfactant and is frequently used as an emulsifier and dispersing agent for drug delivery.[15] Polysorbate 80 can be excellent solvents for water-insoluble drugs and are compatible with other cosolvents, lipids, and surfactants. Commonly used solid lipids to produce NLCs include tripalmitin, glyceryl behenate (compritol), glyceryl distearate (precirol), and cetyl palmitate. Stearic acid is one of a family of lipid-based excipients comprising a mixture of stearic and palmitic acids. Its incorporation in lipid nanocarriers may be helpful in increasing drug loading of lipophilic compounds. Due to the lipophilic nature of their matrices, NLCs are considered particularly useful for administering lipophilic compounds such as baicalein. The aim of this work was to develop NLCs for baicalein with polysorbate 80 and stearic acid as the alternative inner phases, which differ from conventional NLCs. We assessed the feasibility of polysorbate 80 NLCs to stabilize baicalein and achieve specific brain targeting via the intravenous route. The size, zeta potential, morphology, and thermal analysis of NLCs were characterized. The physical and chemical stabilities of the NLCs loaded with baicalein were investigated. The *in vivo* pharmacokinetics of and targeting efficiency toward the rat brain were examined to elucidate the applicability of polysorbate 80 NLCs.

MATERIALS AND METHODS

Materials

Baicalein was purchased from Wako Chemical (Tokyo, Japan). Stearic acid and polysorbate 80 were provided by Sigma-Aldrich. Ethanol and acetone were purchased from S. D. Fine Chemicals.

Preparation of lipid nanoparticles

The lipid and aqueous phases were prepared separately. The lipid phase consisted of 10 mg baicalein, 120 mg stearic acid, 6 ml ethanol, and 6 ml acetone. The aqueous phase consisted of 120 ml double-distilled water and 2-3 drops of polysorbate 80. Both phases were heated separately to 80°C until the dispersions were completely melted. The aqueous phase was added to the lipid phase by continues stirring on magnetic stirrer. Stirring was done until the solution becomes turbid. Now, lyophilization was done, and SLN were obtained.

Particle size and zeta potential

The particle size of tailored nanoformulations was measured by photon correlation spectroscopy (Beckman Coulter [Delsa Nano]) at a fixed angle of 90°. Briefly, 1 mg sample of each nanoformulation was suspended separately in 1 ml of distilled water and sonicated for 30 s. The zeta potential of each nanoformulation was measured at similar concentration using zeta meter (Malvern Instruments, UK). An electrophoretic velocity of 150 mV was applied to observe the zeta potential. All experiments were carried out in triplicate ($n = 3$).

Powder X-ray diffraction (PXRD) pattern

PXRD patterns were recorded on a powder X-ray diffractometer (X'Pert PRO, Panalytical Company, Netherlands) using Ni-filter, CuKα-radiation, voltage of 60 kV and a current of 50 mA. The scanning rate employed was 1°/min over the 10°-60° diffraction angle (2θ) range. The PXRD pattern of baicalein, blank nanoconstructs, physical mixture of baicalein and blank nanoconstructs, and baicalein nanoconstructs were recorded.

Transmission electron microscopic (TEM) examination

The surface morphology of each nanoformulation was examined using TEM (FTI Tecnai F20). An aqueous suspension of nanoformulation was drop cast onto a carbon coated grid which was then air dried at room temperature before loading into microscope, maintained at a voltage of 80 kV.

Differential scanning calorimetric (DSC) characterization

DSC scans were recorded for baicalein, blank nanoconstructs, physical mixture of baicalein and blank nanoconstructs, and baicalein nanoconstructs using DSC (Mettler Toledo, 822e, Greifensee, Switzerland). The samples were weighed and hermetically sealed in 40 µl aluminum pans, which were further heated over a temperature range of 30-300°C at a heating and cooling rate of 10°C/min.

Fourier transform infrared spectroscopy (FTIR)

The spectrum was recorded for baicalein, physical mixture of baicalein and stearic acid, and baicalein nanoconstructs using infrared spectrophotometer (Bruker, India). Samples were prepared in KBr disk with a hydrostatic press at a force of 40 psi for 4 min. The scanning range employed was 400-4400/cm at a resolution of 4/cm.

Nanoencapsulation efficiency and drug loading capacity

The nanoencapsulation efficiency and drug loading capacity were determined by suspending 10 mg sample of baicalein nanoconstructs in 1 ml of distilled water, shaken and kept aside for 10 days. After this treatment, the sample was centrifuged at 40,000 rpm (Hicom ultracentrifuge) for 1 h. The supernatant liquid was then diluted appropriately and filtered through 0.22 µm membrane filter. The absorbance of filtrate was measured at 277 nm using a UV/visible spectrophotometer (Shimadzu) (Chaudhari *et al*., 2010).

In vitro drug release

In vitro drug release studies were performed using dialysis bag method. Briefly, 10.3 mg sample of baicalein nanoconstructs (~10 mg of baicalein, a single intravenous dose in mice) was dispersed in 1 ml of simulated phosphate buffered saline (without enzyme, pH ~ 7.4 ± 0.1), and then this dispersion was filled into a dialysis bag. The bag was suspended in a beaker containing 10 ml of simulated phosphate buffered saline (without enzyme, pH ~ 7.4 ± 0.1) which was further placed on a magnetic stirrer. The temperature and speed of the stirrer were maintained at 37°C and 50 rpm, respectively, as recommended for dissolution testing of intravenous products (Gupta *et al*, 1987). A sample of 5 ml was withdrawn at different time intervals and replaced with fresh simulated intestinal fluid (without enzyme, pH ~ 7.4 ± 0.1) to mimic finite sink conditions. The absorbance of samples was measured at 277 nm using the UV/visible spectrophotometer (Shimadzu).

Statistical analysis

Statistical analysis of differences between the various treatments was performed using unpaired Student's t-test. The post hoc Newman-Keuls test was used to check individual differences between groups. A 0.05 level of probability ($P < 0.05$) was taken as the level of significance. Data entry and analysis were completed using Winks SDA 6.0 software (Texasoft, Cedar Hill, TX, USA).

RESULTS AND DISCUSSION

Preparation of baicalein loaded nanostructured lipid particles (baicalein nanoconstructs)

The baicalein was successfully loaded in nanostructured lipid particles (NLPs) using solvent diffusion technique. The GRAS (generally recognized as safe) component, stearic acid was used as a lipid material to synthesize the nanostructured particulate system. The stearic acid was dissolved in the equimolar volume of acetone and ethanol (1:1). These solvents promoted the diffusion of the drug in lipid nanocore.

Characterization of nanostructured lipid particles

Particle size and zeta potential analysis

Nanoparticles <100 nm can easily penetrate the blood-brain barrier via tight endothelial junction and thereby improve drug delivery. Hence, particle size plays a key role in therapeutic efficacy of nano-drug delivery systems. The particle size of nanoconstructs and baicalein nanoconstructs was observed to be 14.2 ± 9.3 nm and 16.4 ± 5.4 nm, respectively [Table 1 and Figure 1a and b]. Similarly, the zeta potential of baicalein nanoconstructs (−3.40 ± 1.32) was also reduced significantly as compared to NLPs (−40.7 ± 2.45).

Scale bar ~100 nm. The particle size of NLPs and baicalein nanoconstructs was observed to be 14.2 ± 9.3 nm and 16.4 ± 5.4 nm, respectively. The experiment was performed in triplicate (mean ± Standard deviation, $n = 3$).

PXRD

PXRD was used to determine the crystalline geometry of drug in the lipid matrix. The XRD pattern of free baicalein showed sharp and intense peaks, thus indicating its crystalline structure [Figure 2]. Blank nanoconstructs showed diffused peaks with low intensities. Similarly, the physical mixture of baicalein and blank nanoconstructs showed few sharp peaks with diffused peaks. The sample, baicalein nanoconstructs showed peaks of diminished intensity, indicating that crystal structure of drug was deformed to an amorphous state.

PXRD pattern of baicalein, blank NLPs, physical mixture of baicalein and blank NLPs, and baicalein nanoconstructs. Baicalein showed crystalline structure while blank NLPs showed peaks of diminished intensity, indicating the amorphous structure. Physical mixture of baicalein and blank NLPs showed peaks of individual components. Baicalein nanoconstructs showed that crystalline phase of the drug was deformed into amorphous geometry during encapsulation.

TEM

The particle shape is also a key parameter in therapeutic efficacy analysis of a nanoformulation. TEM was used to visualize the surface topography of lipid nanoformulations [Figure 3a and b]. We observed the spherical and uniform shape of NLPs and baicalein nanoconstructs without any deformation of surface texture. The freeze drying step did not influence the surface texture of nanoformulation.

TEM confirmed the spherical and uniform shape of NLPs and baicalein nanoconstructs, without any deformation of surface texture

DSC

DSC was employed to determine the physical state of the drug in lipid particles. A sharp endothermic peak at 265°C (Melting point ~ 256-271°C) was observed in pure baicalein sample. The thermogram of blank nanoconstructs showed a slightly broad peak at 115°C. As expected, physical mixture only exhibited the peaks of individual components. On the other hand, baicalein nanoconstructs showed a single broad peak at 117°C, thus indicating the molecular dispersion of the drug in baicalein nanoconstructs (Figure 4).

A sharp endothermic peak at 265°C was observed in pure baicalein while blank NLPs showed a slightly broad peak at 115°C. As expected, physical mixture only exhibited the peaks of individual components. The baicalein nanoconstructs showed a single broad peak at 117°C, thus indicating the molecular dispersion of the drug in baicalein nanoconstructs.

Table 1: Particle size, nanoencapsulation efficiency, and drug loading capacity

Sample	Particle size	NE (%)	Drug loading capacity/10 mg
NLPs	14.2±9.3 nm	-	-
Baicalein	-	-	-
Baicalein nanoconstructs	16.4±5.4 nm	44.3±3.4	5.66 mg

NLPs: Nanostructured lipid particles, NE: Nanoencapsulation efficiency

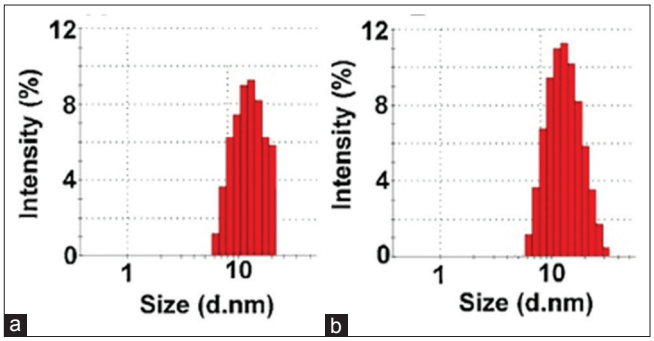

Figure 1: (a and b) Particle size distribution of (a) nanostructured lipid particles and (b) Baicalein nanoconstructs

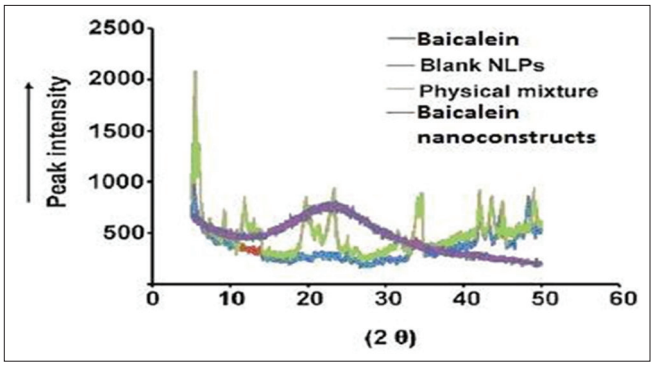

Figure 2: Powder X-ray diffraction

FTIR

FTIR spectrum of baicalein, physical mixture of baicalein and stearic acid, and baicalein nanoconstructs was captured to examine the chemical linkage formed during encapsulation of drug into nanoformulation (Figures 5-8). The FTIR spectrum showed the characteristic peaks of pure baicalein at 3406 cm^{-1} which signifies presence of O-H functional group and peak at 1652 cm^{-1} shows a presence of C=O stretch. The stearic acid showed the characteristic peak of the O-H group at 2954 cm^{-1} and C=O stretching peak at 1695 cm^{-1}. These peaks were remained unchanged in the physical mixture of both components. However, a slight shift was observed at 1703 cm^{-1} and 3404 cm^{-1} from the original peaks of pure baicalein and stearic acid. The baicalein nanoconstructs showed the characteristic peaks of O-H group at 3408 cm^{-1}, OCH_3 group at 2914 cm^{-1}, and C=O streaching at 1699 cm^{-1}.

Baicalein loading in the nanostructured lipid particles

The nanoencapsulation efficiency of baicalein nanoconstructs was calculated to be 44.3 ± 3.4% with drug loading capacity of 5.66 mg/10 mg of nanoformulation [Table 1].

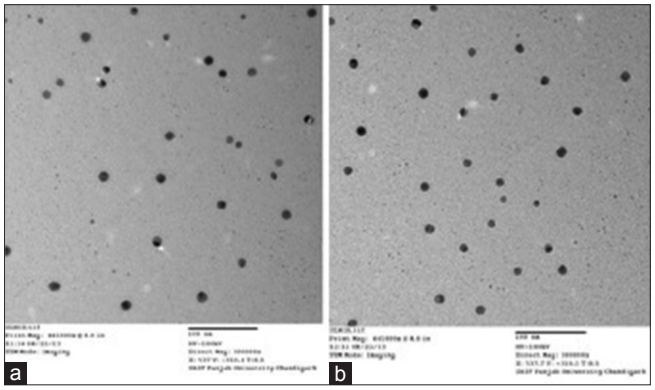

Figure 3: Transmission electron microscopy of (a) nanostructured lipid particles and (b) Baicalein nanoconstructs

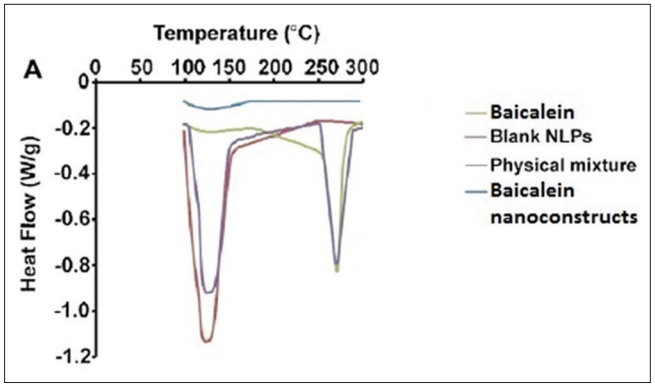

Figure 4: Differential scanning calorimetry of baicalein, blank nanostructured lipid particles (NLPs), physical mixture of baicalein and blank NLPs, and baicalein nanoconstructs

In vitro drug release

We used dialysis method to determine the drug release from lipid nanoformulation. Percent release of drug from two formulations (baicalein solution and baicalein nanoconstructs) was calculated and presented in [Figure 9]. We fitted the first order (log cumulative amount of drug released vs. time) equation to determine the release kinetic of the drug from lipid nanoformulation. The nanoformulation, baicalein nanoconstructs exhibited an initial burst by releasing 30.2% of drug within 2 h, followed by a slow release of 95.4% in 24 h. Release of drug from the dialysis membrane depends on the permeability constant that was calculated by adding a known quantity of drug (10 mg/ml) inside the dialysis bag and then monitoring the drug concentration in the dissolution medium as a function of time.

Release kinetic and percent release of drug from baicalein nanoconstructs in simulated phosphate buffered saline (without enzyme, pH ~ 7.4 ± 0.1). Release kinetic was found to follow first order kinetic, with an initial burst of 30.2% within 2 h, followed by a slow release of 95.4% in 24 h.

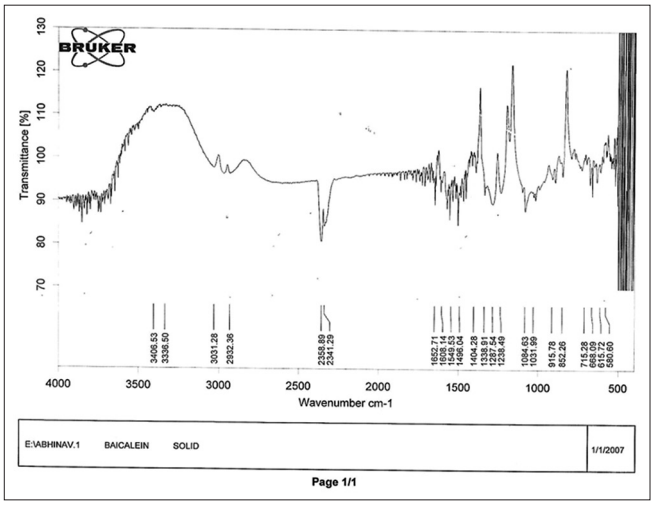

Figure 5: Fourier transform infrared spectroscopy of baicalein

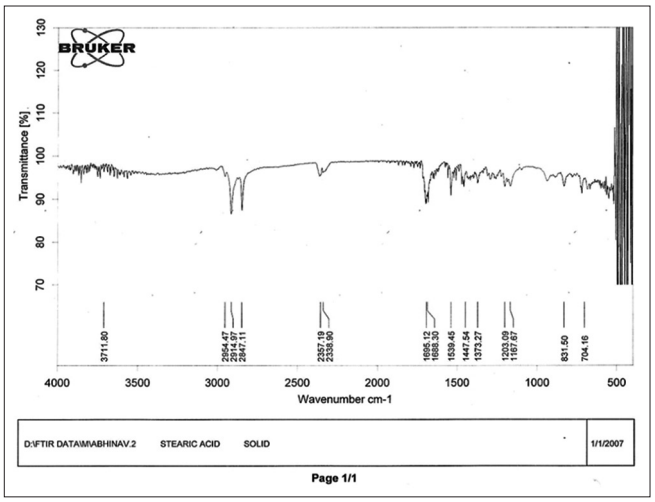

Figure 6: Fourier transform infrared spectroscopy of stearic acid

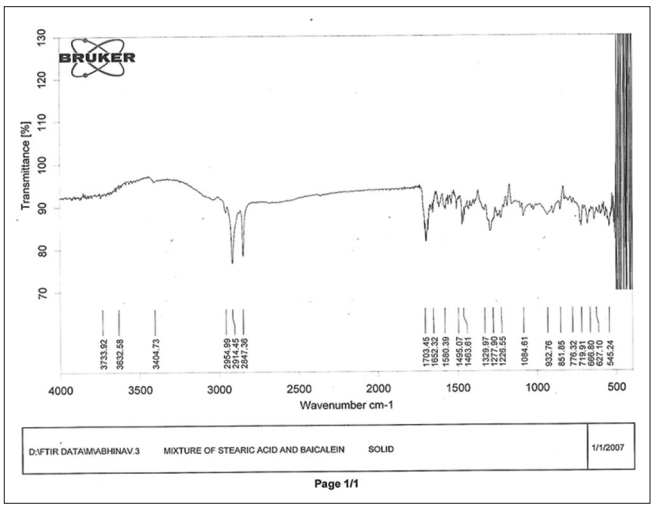

Figure 7: Fourier transform infrared spectroscopy of physical mixture of baicalein and stearic acid

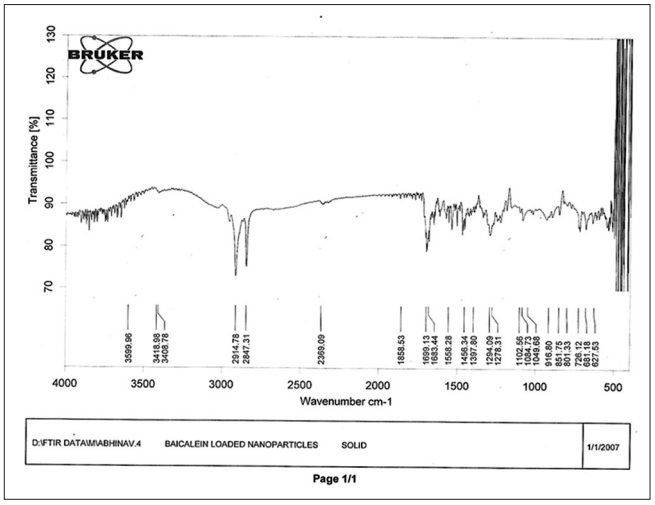

Figure 8: Fourier transform infrared spectroscopy of baicalein loaded nanoconstructs

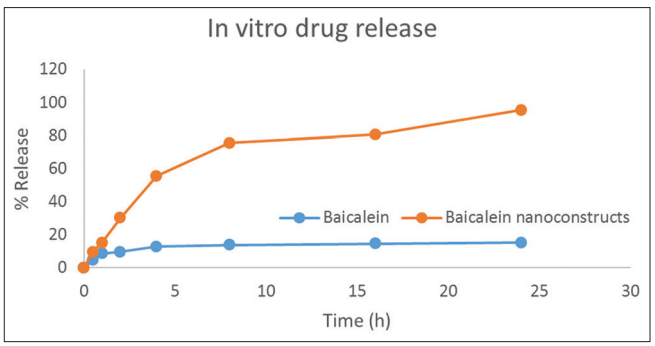

Figure 9: *In vitro* drug release of baicalein and baicalein nanoconstructs

CONCLUSIONS

The objective of this research work was to formulate and characterize nanoconstructs of baicalein for augmenting brain delivery in hypoxia. It may be concluded that to develop a brain-specific baicalein nanocarrier; we designed novel NLCs with polysorbate 80 and stearic acid by solvent diffusion method for brain delivery. The nanoparticulate preparations allowed the formation of almost spherical ultrafine particles of a size near 100 nm with a zeta potential of around − 50 mV. The crystal order of the solid lipids was disturbed in the inner cores of NLCs according to the DSC profiles. The polysorbate 80 NLCs were shown to be promising carriers for baicalein delivery due to the ability to load the compound, thus protecting the compound from degradation, and in delivering the compound to the circulation and brain. The brain-targeting efficiency of baicalein was greatly improved by NLCs based on a brain distribution experiment. NLCs successfully targeted the main brain, especially the cortex and brain stem. Stearic acid may play important roles in improving the pharmacokinetics and brain transport. Data generated in this study represent a novel and effective strategy for baicalein delivery, which may be beneficial for future applications of baicalein to brain injuries and disorders.

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