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Baicalein loaded in tocol nanostructured lipid carriers (tocol NLCs) for enhanced stability and brain targeting

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ABSTRACT

The objective of the present work was to investigate the specific brain targeting of baicalein by intravenous injection after incorporation into nanostructured lipid carriers (NLCs). The NLC system, composed of tripalmitin, Gelucires, vitamin E, phospholipids, and poloxamer 188 (referred to as tocol NLCs), was characterized in terms of its physicochemical properties, differential scanning calorimetry (DSC), stability, in vivo pharmacokinetics, and brain distribution. 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 tocol NLCs had a slightly disordered crystal arrangement. The nanoparticulate dispersions demonstrated good physical stability during storage for 6 days. The incorporation of vitamin E in the formulations greatly reinforced baicalein's stability. The aqueous control and tocol NLCs were intravenously administered to rats. The plasma level of baicalein in NLCs was much higher and the half-life much longer than those in the free control. In the experiment on the brain distribution, NLCs respectively revealed 7.5- and 4.7-fold higher baicalein accumulations compared to the aqueous solution in the cerebral cortex and brain stem. Greater baicalein accumulations with NLCs were also detected in the hippocampus, striatum, thalamus, and olfactory tract. A 2–3-fold increase in baicalein amounts were achieved in these regions. Tocol 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 disorders.

1. Introduction

Strokes remain a leading cause of death and adult disability worldwide. Ischemic strokes account for approximately 80% of all strokes (Feigin et al., 2003). Ischemic insult produces excessive free radicals, which are neurotoxic by inducing the apoptotic cell death of neurons. Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Asian medicine. The flavonoid was shown to protect against ischemic injury by its anti-inflammatory and antioxidant effects (van Leyen et al., 2006; Liu et al., 2010). Baicalein is well known as a 12/15-lipoxygenase and xanthine oxidase inhibitor. In addition, it protects against neuronal cell damage induced by β-amyloid protein, oxidative stress, and glutamate (Park et al., 2010). 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 (Liu et al., 2007; He et al., 2009; Mu et al., 2011). 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 (Tsai et al., 2002). Some solvents used for baicalein administration, such as dimethyl sulfoxide (DMSO), are inadequate and toxic and thus unsuitable for clinical situations (Chen et al., 2008; Xu et al., 2010). In addition, the treatment of

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brain diseases is often constrained by the inability of potent drugs to pass the blood–brain barrier (BBB).

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 nanocompartments (Müller et al., 2002; Hsu et al., 2010). 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 (Date et al., 2011). NLCs are cost-effective and provide easy administration for drugs that cannot be formulated as aqueous solutions. Lipid nanoparticles appear suitable as a delivery system due to prolonged release, targeted efficiency with lower side effects, and less toxicity than polymeric nanoparticles (Sawant and Dodiya, 2008). 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. Vitamin E (α-tocopherol) and other tocols have recently been investigated as materials for drug delivery (Constantinides et al., 2004). Tocols can be excellent solvents for water-insoluble drugs and are compatible with other cosolvents, lipids and surfactants. In general, they are biodegradable, physically stable, and relatively easy to produce on a large scale (Li et al., 2008). Commonly used solid lipids to produce NLCs include tripalmitin, glyceryl behenate (Compritol), glyceryl distearate (Precirol) and cetyl palmitate. Gelucire is one of a family of lipid-based excipients comprising a mixture of polyethylene glycol (PEG)-ylated fatty acid esters and glycerides. Because it produces unique compositions with surfactants, cosurfactants and lipid phases, it has interesting properties such as emulsification, drug solubility enhancement and granule formation (Chambin and Jannin, 2005). 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 the present work was to develop NLCs for baicalein with vitamin E and Gelucire as the alternative inner phases, which differ from conventional NLCs. We assessed the feasibility of tocol 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 tocol NLCs.

2. Materials and methods

2.1. Materials

Baicalein was purchased from Wako Chemical (Tokyo, Japan). Vitamin E and poloxamer 188 were provided by Sigma–Aldrich (St. Louis, MO, USA). Gelucire 48/9 and Gelucire 62/5 were supplied by Gattefossé (St. Priest, France). Tripalmitin was from Tokyo Kasei (Tokyo, Japan). Hydrogenated soybean phosphatidylcholine 80% (SPC) was purchased from American Lecithin Company (Oxford, CT, USA).

2.2. Preparation of lipid nanoparticles

The lipid and aqueous phases were prepared separately. The lipid phase consisted of 1 mg baicalein, 20 mg tripalmitin, 20 mg Gelucire and 50 mg SPC. Vitamin E at 4 or 8 mg was added if necessary. The aqueous phase consisted of 2 ml double-distilled water with 10 mg/ml poloxamer 188. Both phases were heated separately to 80 °C until the dispersions were completely melted. The aqueous phase was added to the lipid phase and mixed for 3 min. The mixture was homogenized using a probe-type sonicator (UP50H, Hielscher Ultrasonics, Teltow, Germany) for 20 min at 50 W. The heating temperature was maintained at 80 °C. The final product was obtained after cooling to room temperature. The compositions and their amounts of lipid nanoparticles are listed in Table 1.

2.3. Particle size and zeta potential

The average particle size and zeta potential of the nanocarriers were determined by photon correlation spectroscopy (Zetasizer 3000HS, Malvern, Worcestershire, UK) using a helium–neon laser with a wavelength of 633 nm at 25 °C. The sizes are given as a number distribution. A 1:150 dilution of the particles was made using double-distilled water before the measurements.

2.4. Total baicalein content in the dispersions

With the aim of quantifying the total compound content after nanoparticle production, 0.1 ml of dispersion was added to 0.3 ml methanol and stirred for 1 min to completely extract the baicalein present. After incubation at room temperature for 5 min, the sample was centrifuged at 1.2×10^5 rpm and 4 °C for 1 h. The supernatant was then withdrawn and analyzed by high-performance liquid chromatography (HPLC). The Hitachi system (Tokyo, Japan) was used for the HPLC analysis. A 15-cm-long stainless steel C18 column with an inner diameter of 4.6 mm (Atlantis® C18, Waters, Milford, MA, USA) was used as the stationary phase. The mobile phase was acetonitrile: 0.01 M 1-pentanesulfonic acid sodium salt in a water system (29:71) at a flow rate of 1 ml/min. Quercetin was used as an internal standard. The ultraviolet wavelength was set to 275 nm.

2.5. Transmission electron microscopic (TEM) examination

The size and morphology of the lipid nanoparticles were observed using a Jeol JEM-2000 electron microscope (Tokyo, Japan). One drop of dispersion was deposited on a carbon film-covered copper grid to form a thin-film specimen, which was stained with 1% phosphotungstic acid. The sample was then examined and photographed under the microscope.

2.6. Differential scanning calorimetric (DSC) characterization

The lipid nanoparticles with or without baicalein were freeze-dried before the DSC measurement. The DSC analysis was performed using a PerkinElmer DSC calorimeter (DSC7, Waltham, MA, USA). Powdered nanoparticles accurately weighed to 5 mg were put in aluminum pans. The thermal analysis profiles were obtained as the temperature was increased from 35 to 120 °C at a rate of 5 °C/min under nitrogen.

2.7. Physical and chemical stabilities

The prepared lipid nanoparticles were stored at room temperature in a desiccator for 6 days. The relative humidity was maintained at 50 ± 5%. An aliquot of a sample was taken at predetermined time intervals to investigate the particle size, zeta potential, and total baicalein content. The average size and surface charge were determined by photon correlation spectroscopy as described in the previous section. The total content was determined by the extraction method described above.

Table 1
The compositions and their amounts in lipid nanoparticles (with 1 mg baicalein).

Code	Tripalmitin (mg)	SPC (mg)[a]	Gelucire 48/9 (mg)	Gelucire 62/5 (mg)	Vitamin E (mg)	H_2O with 1% poloxamer (ml)
G48/E0	20	50	20	–	–	2
G48/E4	20	50	20	–	4	2
G48/E8	20	50	20	–	8	2
G62/E0	20	50	–	20	–	2
G62/E4	20	50	–	20	4	2
G62/E8	20	50	–	20	8	2

[a] SPC, soybean phosphatidylcholine.

2.8. Animals

In vivo experiments were performed with male Wistar albino rats (200–250 g). The animal experiment protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University. Animals were housed and handled according to institutional guidelines. All animals went without food overnight prior to the experiments.

2.9. In vivo pharmacokinetics

Baicalein in the control solution and tocol NLCs was given intravenously (6 mg/kg) to the rats. The medium of the control solution consisted of 10% *N*,*N*-dimethylacetamide, 20% polyethylene glycol 400, 20% tetraglycol, and 50% double-distilled water. Wistar rats were anesthetized with 25% (w/v) urethane intraperitoneally at a dose of 2.5 ml/kg. An aliquot of a 0.3-ml blood sample was withdrawn from the jugular vein into a heparin-rinsed vial according to a programmed schedule at 5, 10, 20, 30, 60, 120, 240 and 360 min after dosing. Each blood sample was centrifuged at 3000 rpm for 10 min. The resulting plasma sample (100 μl) was vortex-mixed with 20 μl of ascorbic acid (10 mg/ml) and 20 μl of the internal standard (quercetin) in methanol (100 μg/ml). Then 20 μl of 0.1 N HCl and 80 μl methanol were added to the sample and shaken (15 s). The resulting solution was centrifuged at 3000 rpm for 10 min, and 100 μl of the supernatant in the test tube was withdrawn for the HPLC analysis.

2.10. Data analysis

Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software, WinNonlin Standard Edition version 1.1 (Pharsight, Mountain View, CA, USA) using a two-compartmental method. The maximum concentration (C_{max}) was determined by observing individual animal concentration-versus-time curves. The area under the plasma concentration curve from the time of administration ($AUC_{0\to\infty}$) was calculated using the trapezoidal rule with extrapolation to infinity. The mean residence time (MRT) was calculated as $AUMC_{0\to\infty}/AUC_{0\to\infty}$. The clearance (Cl) was calculated from the elimination rate constant (k_e) multiplied by the volume of the distribution.

2.11. In vivo brain distribution

Animals to which 6 mg/kg baicalein was intravenously administered were decapitated at 60, 120, 240 and 360 min after the injection. The skull was cut open, and the cerebellum, cortex, hippocampus, striatum, thalamus, olfactory tract and brain stem were carefully isolated and weighed. Subsequently, the regional brain tissue was mixed and homogenized with 200 μl ascorbic acid (10 mg/ml), 180 μl 0.1 N HCl, 800 μl methanol, and 200 μl methanol with the internal standard (100 μg/ml quercetin) for 3 min. The subsequent sample reparation for brain extraction was the same as that described for the plasma extraction method.

2.12. 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).

3. Results

3.1. Physicochemical characterization of lipid nanoparticles

The lipid nanoparticles were prepared by heating and then subjected to a probe sonicator. The preparations were manufactured with the rationale that lipids, polymers, and surfactants used should be biocompatible and biodegradable. Tripalmitin is a triglyceride commonly used as the solid lipid in SLNs and NLCs to facilitate emulsification and form rigid nanoparticles. Because of their biocompatibility and stability, naturally derived purified phospholipids continue to be the main emulsifiers employed in injectable emulsions (Driscoll, 2006). The incorporation of certain drugs in lipid nanoparticles with phospholipids as the sole emulsifier was found to reduce stability and cause phase separation. A combination of phospholipids and poloxamer 188, a non-ionic copolymer, leads to the formation of a mixed film with high surfactant coverage and sufficient viscosity to promote stability (Jumaa and Müller, 2002; Wang et al., 2009). Hence, both SPC and poloxamer 188 were loaded in the nanoparticulate dispersions as the emulsifier systems. Gelucires are multifunctional lipid excipients composed of mono-, di-, and triglycerides and mono- and di-fatty acid esters of PEG (Siepmann et al., 2006). Gelucires have interesting properties and can be used as surfactants, cosurfactants, and lipid matrixes in drug delivery systems (Shimpi et al., 2009). Gelucires are generally recognized as safe (GRAS). We utilized Gelucire 48/9 and Gelucire 62/5 to form SLNs and NLCs because of their multiple roles. Gelucires 48/9 and 62/5 are characterized by melting points of 48 and 62 °C and hydrophile–lipophile balances (HLBs) of 9 and 5, respectively. Both Gelucires were used in the present work for comparison since the HLBs may affect the physical characterization and stability of the particles. The type of the Gelucires may affect the proper association with the other additives such as liquid/solid lipids and emulsifiers. Vitamin E was added to stabilize baicalein when stored. It was also used as a liquid lipid in the inner core to form tocol NLCs. Lipid nanoparticles without vitamin E could be characterized as SLNs.

The mean diameter, zeta potential, and total baicalein amount of the resulting formulations are given in Table 2. All formulations

Table 2
The characterization of the lipid nanoparticles by particle size, zeta potential, and total baicalein amount in the systems.

Code	Size (nm)	Zeta potential (mV)	Total baicalein amount (%)
G48/E0	102.3 ± 0.9	−50.6 ± 5.3	92.0 ± 1.3
G48/E4	96.5 ± 0.6	−53.8 ± 3.8	94.0 ± 3.7
G48/E8	93.3 ± 1.5	−55.6 ± 3.1	96.0 ± 2.1
G62/E0	93.5 ± 0.8	−56.7 ± 1.4	92.1 ± 2.3
G62/E4	94.9 ± 1.1	−53.7 ± 3.6	91.5 ± 4.9
G62/E8	102.5 ± 1.2	−53.3 ± 4.1	94.4 ± 2.2

Each value represents the mean ± SD ($n = 3$).

possessed a mean size of about 100 nm. There was a gradual decrease in the particle size with an increase in the vitamin E content for Gelucire 48/9-containing systems ($p < 0.05$). However, a contrary trend was observed for Gelucire 62/5-containing systems. Differences in size were not large among the different nanoparticles. Results of zeta potential measurements showed that the surface charges of all samples were consistently negative. The absolute zeta potentials of nanoparticles ranged between −51 and −56 mV. No effect of the lipid core (with different Gelucires and with or without vitamin E) on the surface charge was observed ($p > 0.05$). Baicalein contents of freshly prepared dispersions were in the range of 92–96%, indicating a high content uniformity of baicalein in the lipid nanoparticles.

To obtain more information about the particle size and morphology, a TEM analysis was performed on Gelucire 62/5-containing formulations. Sizes of nanoparticles with 0%, 4% and 8% vitamin E (G62/E0, G62/E4 and G62/E8), which were about 100–150 nm in diameter, can be observed on TEM micrographs shown in Fig. 1A–C, respectively. The G48 series were not used for TEM observation since only G62 series were chosen for further in vivo experiments. The particle size measured by TEM was generally well correlated with that found using photon correlation spectroscopy, although a slightly larger particle size was observed in TEM. This may be due to the artifact process before TEM observation. The sample for TEM needed to be dried before measurements were taken. This was quite different from the laser scattering method. TEM showed that most particles had round, uniform shapes. A dense, well-distributed pattern was observed.

3.2. DSC characterization

A DSC analysis was used to investigate the crystalline structure of the lipid nanoparticles. Fig. 2 shows DSC curves of the pure materials and lipid nanoparticles. The thermal behaviors of bulk tripalmitin, Gelucires, and SPC were first studied as depicted in Fig. 2A. For tripalmitin, the melting process took place with a maximum peak at 68.25 °C. Neat SPC revealed a peak at 44.25 °C with a shoulder. A broad peak was also observed at 55 °C. This indicates that there are mixed phospholipids in SPC. The thermograms of both Gelucires showed a large endothermic event from 37 to 63 °C. Gelucires showed the presence of three or four distinct polymorphs melting at different temperatures. As represented in Fig. 2B and C, peaks of tripalmitin and SPC were observed after their incorporation into the nanoparticles. The melting point of tripalmitin in nanoparticulate form was depressed compared to that of the corresponding bulk tripalmitin. A further depression of the melting point was detected after adding vitamin E to the inner phase (tocol NLCs). Endothermic peaks of Gelucire 48/9-containing systems with 0, 4 and 8 mg vitamin E were 66.35, 65.60 and 64.52 °C, respectively (Fig. 2B). The enthalpy (ΔH) of tripalmitin was presented behind the legends of Fig. 2. The enthalpy was decreased following the increase of vitamin E concentration. Peaks were detected at 65.10, 63.93 and 63.68 °C for Gelucire 62/5-containing systems with 0, 4 and 8 mg vitamin E (Fig. 2C). In the same way as Gelucire 48/9, vitamin E could reduce the enthalpy of tripalmitin. The enthalpy of NLCs with Gelucire 62/5 was lower than that with Gelucire 48/9 when compared at the same vitamin E amount. The nanoparticles with baicalein were also detected by DSC to examine the influence of the drug incorporation. The formulations without vitamin E (G48/E0 and G62/E0) were selected for examination because that any crystalline disruption by the additives may be easily observed if there is completely rigid structure in inner phase. As shown in Fig. 2B, the baicalein loading slightly reduces melting point and enthalpy of tripalmitin in G48/E0. On the other hand, the melting point and enthalpy of tripalmitin in baicalein-loaded G62/E0 approximate the values without the active ingredient (Fig. 2C).

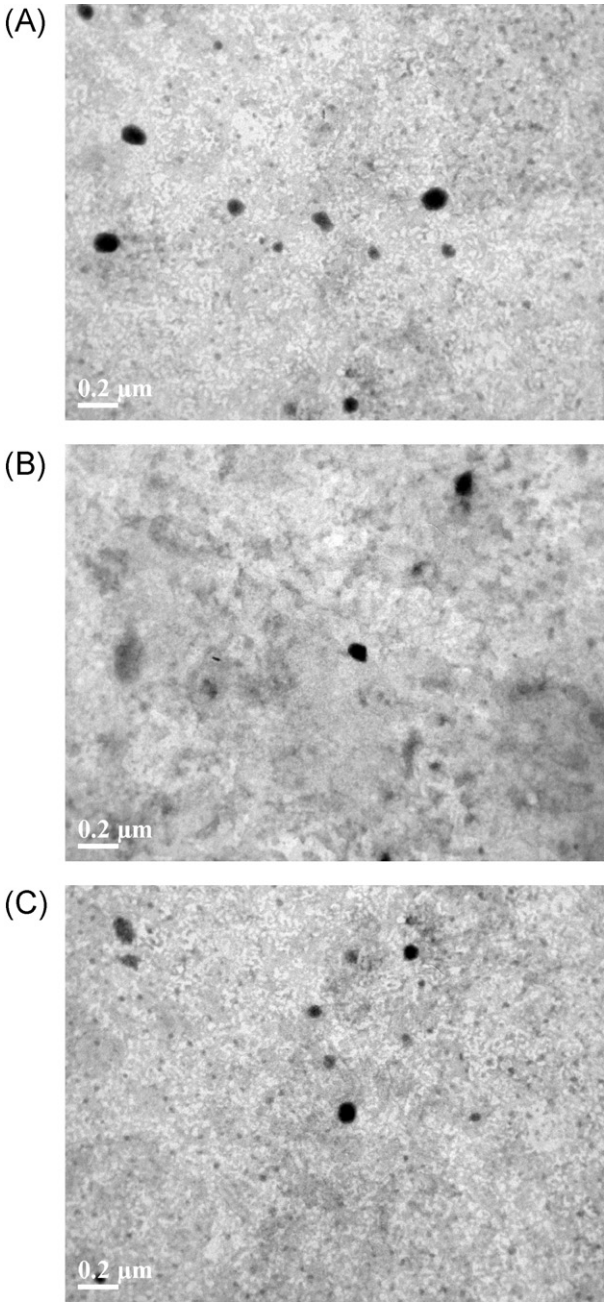

Fig. 1. Transmission electron microscopic micrograph of tocol nanostructured lipid carriers (NLCs) with Gelucire 62/5 and vitamin E at 0 mg (A), 4 mg (B), and 8 mg (C). The scale bar is 0.2 μm.

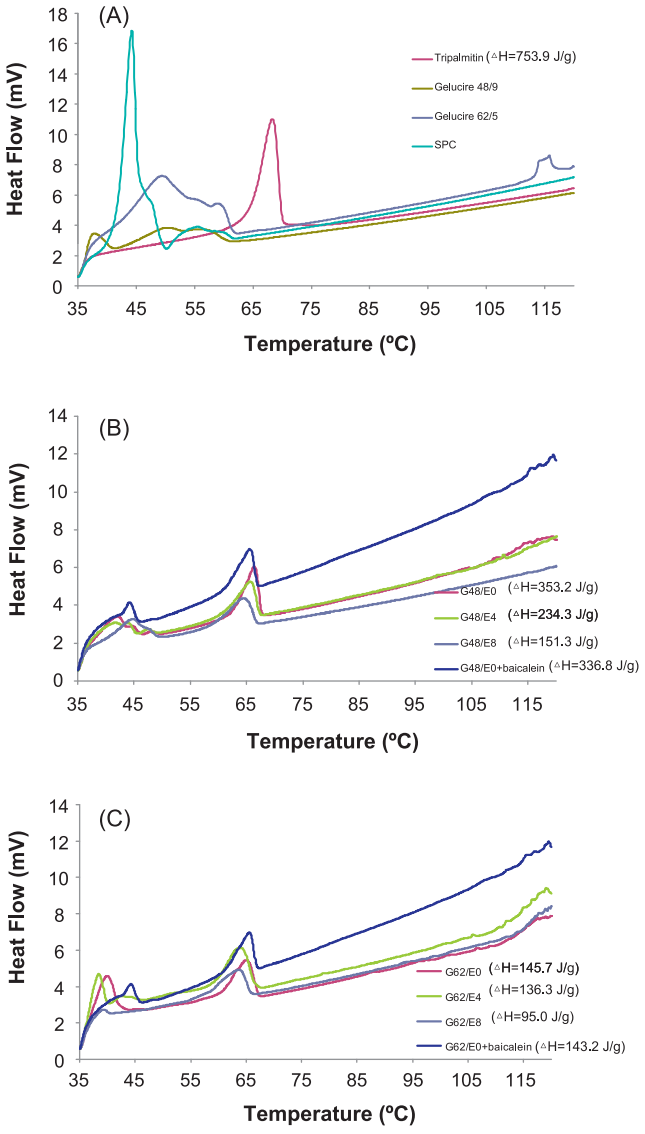

Fig. 2. Heating curves of differential scanning calorimetry (DSC) for pure materials including tripalmitin, Gelucires and soybean phosphatidylcholine (SPC) (A), and tocol nanostructured lipid carriers (NLCs) with Gelucire 48/9 (B) and Gelucire 62/5 (C).

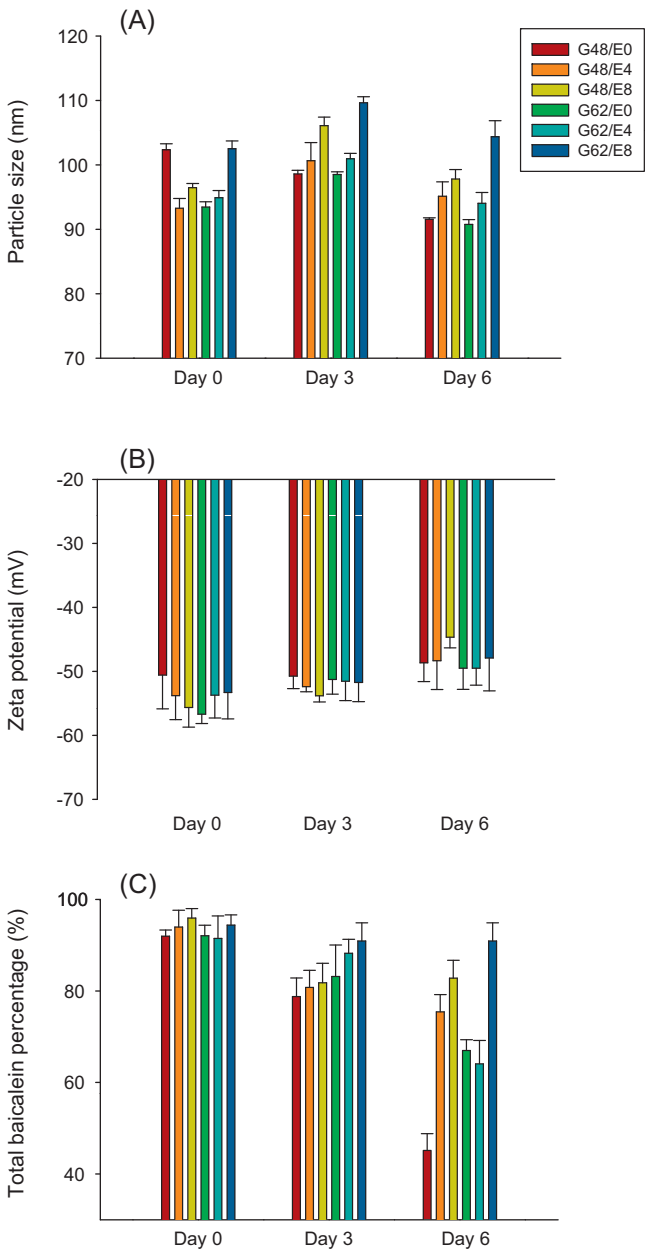

Fig. 3. Changes of particle size (A), zeta potential (B), and total baicalein percentage (C) of lipid nanoparticles during a 6-day storage at room temperature and 50 ± 5% relative humidity. Each value represents the mean and SD ($n = 3$).

3.3. Physical and chemical stability

The short-term stability of all nanoparticles was evaluated for a period of 6 days. The physical stability of nanoparticles was determined by measuring the particle size and zeta potential at 0, 3 and 6 days as shown in Fig. 3A and B, respectively. On visual inspection, all formulations were still homogeneous, and no visible free lipids or breakage of the systems was seen during the short-term stability test. The size of most formulations had slightly increased by day 3; then the size had recovered to the level of freshly prepared dispersions (day 0) by day 6. An exception was SLNs containing Gelucire 48/9 (G48/E0). The size of this system gradually decreased ($p < 0.05$) from days 0 to 6. Except for G48/E8, there was no significant change ($p > 0.05$) in the zeta potential after storage. The G48/E8 system exhibited a decrease in the negative charge from −56 to −45 mV ($p < 0.05$) after storage for 6 days.

The change in the total baicalein content within the nanoparticulate systems for 6 days was examined as shown in Fig. 3C. Baicalein is known to be chemically unstable. The baicalein contents in SLNs with Gelucires 48/9 and 62/5 (G48/E0 and G62/E0) dropped from 92% to 45% and 67% after 6-day incubation, respectively. The addition of vitamin E enhanced the chemical stability of baicalein in the lipid nanoparticles. The results demonstrated that loading 8 mg vitamin E into the nanocarriers (G48/E8 and G62/E8) increased the remaining baicalein content from 45% and 67% to 83% and 91% ($p < 0.05$), respectively.

3.4. In vivo pharmacokinetics

Plasma concentration-versus-time curves of baicalein after a single intravenous injection (6 mg/kg) of the control solution and tocol NLCs (G62/E8) are illustrated in Fig. 4. At all times, the mean plasma concentrations were higher in rats treated with NLCs than in those treated with the free control. Table 3 summarizes the pharmacokinetic parameters obtained by a two-compartmental analysis. Following an intravenous injection of the control group

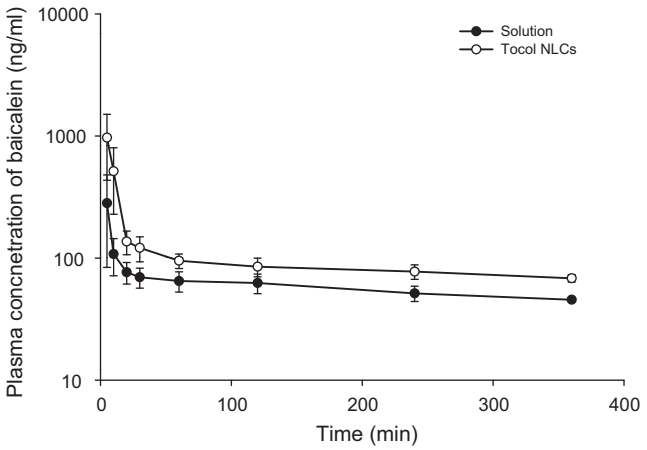

Fig. 4. Mean plasma concentration of baicalein-versus-time curves after an intravenous injection of a baicalein solution (6 mg/kg) and tocol nanostructured lipid carriers (NLCs) with Gelucire 62/5 and 8 mg vitamin E (G62/E8). Each value represents the mean and SD ($n = 5$).

and tocol NLCs, the average peak plasma concentrations (C_{max}) were 0.28 and 0.97 μg/ml ($p < 0.05$), respectively. The $AUC_{0\to\infty}$ values of baicalein for the control and NLCs were 0.93 and 1.73 μg h/ml ($p < 0.05$). It can be seen that the $t_{1/2\alpha}$ of the compound was significantly longer ($p < 0.05$) for NLCs (0.07 h) compared to the control (0.03 h). However, no significant difference ($p > 0.05$) in the MRT was detected between the two groups. Also, a wide inter-subject variation was demonstrated for the free control according to the standard deviation relative to the mean values. Variations in the pharmacokinetic parameters were subsequently reduced after NLC treatment.

3.5. In vivo brain distribution

Baicalein concentrations in different brain regions were determined in order to assess the distribution of the compound when intravenously administered as a free control and as NLCs. Fig. 5 shows the brain accumulations upon administration with the aqueous solution and NLCs from 60 to 360 min. The whole brain was divided into seven sections (Fig. 5A–G). As shown in Fig. 5A, the control solution produced a significant increase ($p < 0.05$) in the amount of baicalein in the cerebellum compared to tocol NLCs. Baicalein concentrations with the tocol NLCs in the main brain were significantly higher ($p < 0.05$) than those with the control solution (Fig. 5B–G). These results indicated that NLCs with Gelucire 68/5 and vitamin E had successfully targeted baicalein to the main brain. Table 4 shows the $AUC_{0\to360}$ values of the brain concentration–time curves of baicalein. The $AUC_{0\to360}$ of baicalein significantly increased with NLC treatment except in the cerebellum. The resulting $AUC_{0\to360}$ in the cortex was 7.5-times higher when applied as tocol NLCs. The brain stem also showed a great enhancement (a 4.7-fold increase) of compound accumulation. A 2–3-fold increase in compound concentration with NLCs was observed in other brain tissues, such as the hippocampus, striatum, thalamus, and olfactory tract.

Table 3
Pharmacokinetic parameters of baicalein solution and tocol nanostructured lipid carriers (G62/E8) by intravenous injection at a dose of 6 mg/kg.

	Baicalein solution	Baicalein NLCs
C_{max} (μg/ml)	0.28 ± 0.20	0.97 ± 0.34
$t_{1/2\alpha}$ (h)	0.03 ± 0.01	0.07 ± 0.03
$t_{1/2\beta}$ (h)	8.95 ± 6.26	10.09 ± 2.03
Cl (l/h kg)[a]	6.24 ± 4.78	3.62 ± 0.64
$AUC_{0\to\infty}$ (μg h/ml)[b]	0.93 ± 0.49	1.70 ± 0.33
MRT (h)[c]	11.97 ± 8.80	13.02 ± 2.03

Each value represents the mean ± SD ($n = 5$).
[a] Cl, clearance.
[b] $AUC_{0\to\infty}$, area under the curve.
[c] MRT, mean residence time.

4. Discussion

Clinical failures of most potentially effective therapeutics for treating central nervous system (CNS) disorders are often not due to an insufficiency of drug potency but rather shortcomings in the method by which the drug is delivered (Hsu et al., 2011). The efficient BBB transport of baicalein is an important step for the practical application of this compound for clinical use. We are attempting to develop an approach that will permit the utilization of baicalein, which is not currently used in clinical practice due to the lack of a suitable drug-carrier system. The use of NLCs should permit effective formulations of this potent agent in therapies for brain disorders. Most lipid nanoparticles use vegetable oils as the inner phase and phospholipids as the emulsifiers. An alternative biocompatible oil, vitamin E, was used in the present study as the liquid lipid for developing NLCs. Our results demonstrated enhanced and sustained brain delivery of baicalein with tocol NLCs. NLC treatment resulted in a significant increase in the elimination of $t_{1/2}$ and $AUC_{0\to\infty}$ of baicalein in the systemic circulation. The experimental results showed that loading baicalein into tocol NLCs offers benefits in terms of increased stability and brain targeting. To the best of our knowledge, this is the first report utilizing vitamin E as the liquid lipid for NLCs.

All nanoparticles developed in this work exhibited a particle size of near 100 nm; thus, they can be defined as nanosized carriers. In addition to the role of the lipid core in these systems, Gelucires can act as a stabilizer for lipid nanocarriers because of their function in emulsification (Date et al., 2011). The surface-active esters in Gelucires facilitate emulsification and form more-rigid surfactant films; they therefore produce small-sized particles. The addition of a liquid lipid tends to promote the formation of a small-particle population as a result of the higher mobility of the matrix (Fang et al., 2008; Puglia et al., 2008). This can explain the smaller size of NLCs with Gelucire 48/9 (G48/E4 and G48/E8) compared to that of SLNs (G48/E0). Nevertheless, this was not the case for nanoparticles with Gelucire 62/5 since a contrary result was observed. Both Gelucires have different HLBs. The emulsifier system with a certain HLB may well have stabilized the lipid nanoparticles that had a moderate polarity (Wang et al., 2009). Gelucire 62/5 with an HLB of 5 may show a lower emulsification ability in particles with vitamin E, resulting in the larger particle size in the presence of vitamin E. Thus, each Gelucire produces a distinguishable lipid-based drug carrier. The particle size of ~100 nm is advantageous for tocol NLCs since the nanoparticles can be sterilized simply by passing them through a sterile syringe-driven filter with no need for thermal treatment. Small particles of <200 nm reveal reduced hepatic uptake and prolonged blood circulation times (Tosi et al., 2008). Based on previous studies, it is also thought that a nanoparticulate size of 100 nm is suitable for brain-cell targeting and controlled release (Lockman et al., 2003).

The emulsifier system can render a determined surface charge to nanoparticles. Since poloxamer 188 is a non-ionic species, SPC could be responsible for the negative zeta potential. The SPC used in this study contained 80% phosphatidylcholine, which is uncharged. Other components (20%) such as phosphatidylethanolamine, phosphatidic acid and phosphatidylinositol are negatively charged (Fang et al., 2006). Some free fatty acids derived from the hydrolysis of glycerides in tripalmitin and Gelucires may have been present and contributed to the additional negative surface charge in the interface. Baicalein has high lipophilicity, and was highly loaded

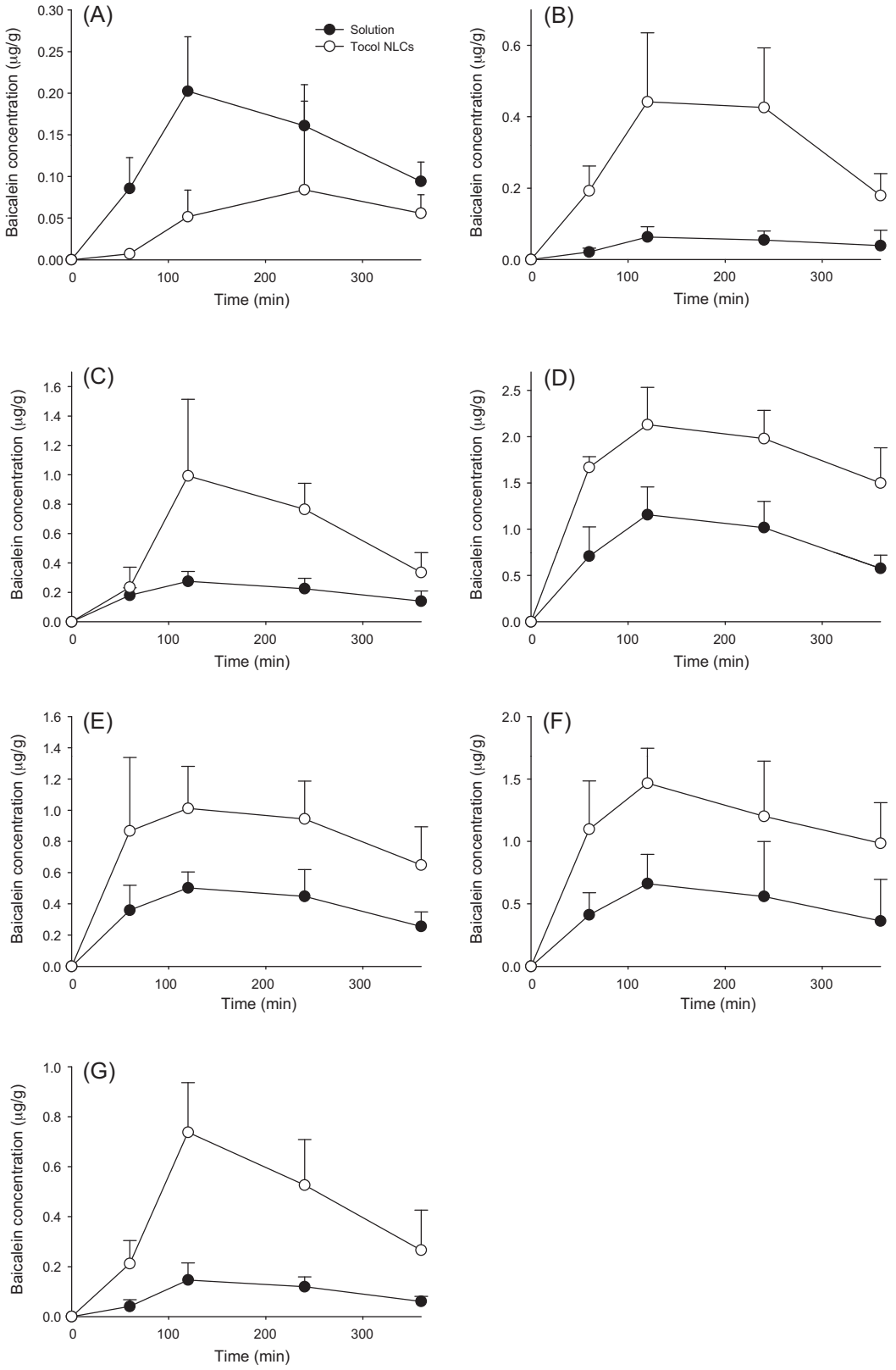

Fig. 5. Baicalein brain concentration-versus-time curves after an intravenous injection of a baicalein solution (6 mg/kg) and tocol nanostructured lipid carriers (NLCs) with Gelucire 62/5 and 8 mg vitamin E (G62/E8) in the cerebellum (A), cortex (B), hippocampus (C), striatum (D), thalamus (E), olfactory tract (F), and brainstem (G). Each value represents the mean and SD ($n = 5$).

Table 4
Comparison of $AUC_{0\to360}$ (μg min/g) of baicalein concentration-time curves in rat brain between baicalein solution and tocol nanostructured lipid carriers (G62/E8) by intravenous injection at a dose of 6 mg/kg.

Area	Baicalein solution	Baicalein NLCs	Ratio of $AUC_{0\to360}$ (LN/solution)
Cerebellum	48.30 ± 10.58	18.86 ± 4.12	0.39
Cortex	15.85 ± 3.29	118.63 ± 15.90	7.49
Hippocampus	71.00 ± 17.71	221.75 ± 33.43	3.12
Striatum	303.23 ± 59.26	636.39 ± 96.66	2.10
Thalamus	135.98 ± 23.32	297.40 ± 58.96	2.19
Olfactory tract	173.22 ± 29.06	410.86 ± 70.54	2.37
Brain stem	33.63 ± 6.36	158.08 ± 34.94	4.70

Each value represents the mean ± SD ($n = 5$).

in the inner phase of the lipid nanoparticles. The lipid core of tocol NLCs was composed of tripalmitin, Gelucires, and vitamin E. More-complex lipids can form less-perfect crystals with many imperfections that offer spaces to accommodate drugs (Kaur et al., 2008). Our DSC results confirmed this phenomenon. Date et al. (2011) also indicated that incorporating Gelucires in the particles resulted in less expulsion of the encapsulated drug from the inner cores.

DSC provides insights into the melting behavior and structure of crystalline material such as lipid nanoparticles. Gelucires showed a wide melting range. An increasing melting range could be correlated with impurities or less-ordered crystals. The melting peaks of Gelucires were not significant after loading into nanoparticles. The structure of lipid nanoparticles can be investigated by observing the melting peaks of tripalmitin because of its purity. A higher melting enthalpy or temperature suggests a more highly ordered lattice arrangement and vice versa (Hou et al., 2003). The thermograms showed that the endothermic peak of tripalmitin was depressed in the nanoparticulate dispersions. The depression increased with greater vitamin E content in the nanoparticles. The melting point of tripalmitin did not change very much after being incorporated into the SLNs (G48/E0 and G62/E0). This suggests a higher-ordered lattice arrangement in the lipid cores. The presence of a glass transition temperature in the DSC thermograms of tocol NLCs points to the crystalline nature of the NLC particles, whereas melting points in the NLCs were more significantly depressed than those in the SLNs. Defects in the crystalline lattice may have contributed to this significant depression. These imperfections in the lattice are beneficial for drug encapsulation, since they create more spaces to accommodate molecules (Hu et al., 2006). Because the melting point of tripalmitin was depressed and the peak width was broadened in the presence of vitamin E, phase separation between the solid and liquid lipids can be excluded (Jenning et al., 2000). The Gelucire 62/5 series had a broader peak width and lower tripalmitin enthalpy compared to Gelucire 48/9 formulations. This indicates more-complete fusion of vitamin E and solid lipids with Gelucire 62/5. Baicalein was also encapsulated in nanoparticles for DSC examination. This incorporation generally did not reduce melting point and enthalpy to a significant level, especially for G62/E0. Baicalein may slightly disrupt the crystalline structure of tripalmitin in G48/E0. This indicates a limited role of the drug on nanoparticulate structure.

The physical stability of the nanocarriers is one of the most important desired product characteristics. Nanoparticles are heterogeneous systems and thermodynamically unstable and, therefore, have a significant tendency to lose physical stability during storage. Particle collisions and partial destruction of the emulsifier interface can increase the possibility of instability of nanoparticles (Freitas and Müller, 1999). A slight increase in size occurred on day 3, which could have been due to aggregation or fusion of the particles. However, this increase was not prolonged since the mean diameter had recovered to the level of day 0 after 6 days of storage. This was likely due to particle destruction, which is commonly observed during storage (Yamaguchi, 1996; Hung et al., 2007). It was noted that Gelucire 48/9-containing SLNs (G48/E0) did not undergo particle aggregation, since the size gradually decreased during 6 days of storage. Although a change in the particle size was observed, the value was still sufficient to maintain the stability of the lipid nanoparticles. A physically stable nanocarrier solely stabilized by electrostatic repulsion will have a minimum zeta potential of ±30 mV (Müller et al., 2001). The negative charge of all systems tested showed a value of <−30 mV, indicating the stability of the prepared products. In the case of poloxamer 188, the steric hindrance is another additional effect that increases the stability of NLCs (Teeranachaideekul et al., 2007).

The key feature of baicalein responsible for its activity is illustrated by major issues of stability. Baicalein is susceptible to oxidation during storage. The $t_{1/2}$ for loss of baicalein in pH 7.5 buffer is 15.2 h (Zhu et al., 2004). NLCs with vitamin E exhibited a protective effect on the residual baicalein content against chemical instability. Vitamin E is a well-known antioxidant that can stabilize the compound from an oxidative environment. SLNs without vitamin E may exhibit expulsion of the compound from the lipid core. This would lead to instability of the active ingredient in the continuous phase during storage. Imperfections in the lipid structure of tocol NLCs proven by DSC may reduce the partitioning of the compound between the dispersed and continuous phases, thus reducing the possibility of oxidation. Tocol NLCs containing Gelucire 62/5 and 8 mg vitamin E (G62/E8) completely maintained the total baicalein content during 6 days of storage with no degradation. This formulation was chosen for further in vivo experiments because of its excellent stability.

Many variables that can affect the use of a drug delivery system cannot be simulated during in vitro experiments (Pardridge, 2010). Therefore, we directly examined the initial in vivo feasibility of tocol NLCs as a CNS drug delivery system using a rat model. Tocol NLC administration in rats simultaneously increased blood levels and brain exposure of baicalein compared to the free control. Baicalein undergoes hepatobiliary excretion and is rapidly excreted into the bile (Tsai et al., 2002). The loading of baicalein by tocol NLCs reduced its metabolism and increased the $t_{1/2}$ and $AUC_{0\to\infty}$ in the circulation. The sustained release of the incorporated drug is a feature quite often correlated with improved pharmacokinetics and efficacy. Baicalein, which was dissolved in lipids, diffused to the interface, and underwent partitioning between the inner and outer phases. Tocol NLCs could thus retard baicalein release and prevent metabolism or excretion of the compound. Gelucires have been particularly widely studied for preparing matrix systems as a means of achieving sustained drug release (Chambin and Jannin, 2005; Qi et al., 2010). This effect is particularly significant for the Gelucires with a low HLB (de Barochez et al., 1989). Gelucire 62/5 fit this criterion. The PEG moiety in Gelucires can cause a steric hindrance effect, decreasing the adsorption of opsonin to NLCs in plasma. The same effect of steric repulsion was expected

for poloxamer 188 (Esposito et al., 2008). Major advantages of a controlled drug delivery system compared to conventional dosage forms include the low intra- and inter-subject variability. This effect can be achieved by incorporating Gelucires in the formulations (Siepmann et al., 2006). Tocol NLCs successfully reduced the variability of baicalein pharmacokinetics among individuals. The low variation may have been due to the uniformity and prolonged-release capability of the nanoparticles.

Baicalein was reported to be slightly distributed in the brain (Tsai et al., 2002). The results of baicalein's brain distribution indicated an improved active ingredient concentration in different regions of the main brain after application by tocol NLCs. This effect was not observed in the cerebellum, suggesting a different delivery route between the main brain and cerebellum. Several mechanisms were proposed for the transport of nanoparticles across the BBB (Wilson et al., 2008). Among them, the prolongation of a drug's $t_{1/2}$ in circulation was proven to be important. The drug can be protected from metabolism and excretion when it is carried in NLCs. A majority of endothelial cells and the surface glycocalyx layer of the BBB are negatively charged, mainly due to sialic acid residues of concentrated acidic glycoproteins (Parikh et al., 2010). Cationic nanoparticles were speculated to be easily attracted by the BBB (Kuo and Wang, 2010). This was not applicable in our case, since a strong negative charge was detected for tocol NLCs. The previous literature (Lockman et al., 2004; Tosi et al., 2008) indicated that both cationic and anionic nanoparticles can cause tight junctions to open, allowing a drug to pass across the BBB. Cationic nanoparticles can disrupt the BBB, whereas anionic nanoparticles exert no effect on BBB integrity. In addition, the ability of cationic nanoparticles to cross the BBB is dramatically decreased by a strong interaction with erythrocytes (Tosi et al., 2008). Anionic particles may be superior to cationic formulations for brain targeting.

Besides the paracellular pathway, nanoparticles can be endocytosed by endothelial cells followed by the release of the drug within these cells (Kreuter, 2001). PEG-coated nanoparticles display an affinity for brain endothelial cells (Brigger et al., 2002). The presence of Gelucires in tocol NLCs may be helpful in inducing this affinity. The poloxamer copolymer system can also enhance drug delivery to the CNS through inhibition of P-glycoprotein efflux (Batrakova et al., 2001). Further studies are required to elucidate the mechanisms of tocol NLCs on brain targeting before arriving at definitive conclusions.

Tocol NLCs revealed the most significant enhancement of baicalein accumulation in the cortex and brain stem compared to the free control. A previous study (Chen et al., 2008) provided evidence that traumatic injury to the cortex can be healed by baicalein. This flavonoid protects rat cortical neurons from β-amyloid-induced toxicity (Lebeau et al., 2001; Park et al., 2010), inferring enhanced memory performance after administering baicalein. The hippocampus is another brain structure involved in memory, which also showed a greater increase in baicalein accumulation by NLCs. The midbrain, pons, and medulla are components of the brain stem. Ischemic brain injury in the midbrain can be ameliorated by baicalein via inhibiting the lipoxygenase pathway (van Leyen et al., 2006; Liu et al., 2010). Loading baicalein in tocol NLCs may strengthen the preventive or therapeutic potential in these regions. Vitamin E loading may have produced antioxidant activity in the brain for additional protection against brain injury or diseases related to oxidative stress. Of course, further investigation is needed to explore this possibility.

5. Conclusions

To develop a brain-specific baicalein nanocarrier, we designed novel NLCs with Gelucires and vitamin E 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 tocol 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. Vitamin E was the key material reinforcing baicalein stability, thus providing considerable stability for practical use. The in vivo results showed that the encapsulation of baicalein in intravenously administered NLCs significantly increased the plasma level and $t_{1/2}$ compared to an equivalent aqueous solution. 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. Gelucires and vitamin E may play important roles in improving the pharmacokinetics and brain transport. Data generated in the current 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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