Original Article

Preparation and characterization of ligand anchored polymeric nanoparticles for the treatment of epilepsy

Ashish Kumar Parashar[1]*, Balak Das Kurmi[2], Preeti Patel[2]

[1]Department of Pharmaceutics, Chameli Devi Institute of Pharmacy, Indore, Madhya Pradesh, India, [2]ISF College of Pharmacy, Moga, Punjab, India

Correspondence:
Ashish Kumar Parashar, Chameli Devi Institute of Pharmacy, Indore, Madhya Pradesh, India.
E-mail: ashish.parashar1@gmail.com

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ABSTRACT

The present work was aimed at developing angiopep-2 anchored nanoparticulate formulation which may improve the efficacy of antiepileptic drug carbamazepine (CBZ) with prolonged circulation and targeting to the seizure sites in the brain. To achieve the above goal, lipoprotein-coated e-caprolactone (e-CL) nanoparticles (NPs) loaded with CBZ were prepared and characterized. The average particle size of the formulation was found to be 96 nm, with an acceptably good polydispersity index (<0.16). The charge values were close to a neutral state with slight negative charges distributed around the NPs (−3.28±0.75 mV). Drug loading was found to be 64%. An initial burst release followed by zero-order release was observed during *in vitro* drug release. The cumulative release for 48 h was 77.9±2.5%. The *in vivo* potential targeting effect of formulation was determined using fluorescence of rhodamine B isothiocyanate-labeled e-CL NPs. The fluorescence signal in the brain of animal with formulation was much stronger at any time post-injection ranged from 2 h to 24 h. It is concluded that this novel delivery system may act as an alternative to the conventional oral and intravenous delivery methods.

Keywords: Angiopep-2, carbamazepine, drug targeting, epilepsy, nanoparticles

INTRODUCTION

Epilepsy is considered as a common and diverse set of chronic neurological disorders and its symptoms can be controlled by antiepileptic drugs (AEDs). About 50 million people all over the world are diagnosed to have epilepsy, and almost 90% of the people with epilepsy are found in developing countries. Epilepsy is a neurological disorder characterized by abnormal electrical activity within the brain, which can result in either generalized or partial seizures. Generalized seizures are widespread, affecting both hemispheres of the brain. In contrast, partial seizures originate at a focus and are isolated to specific areas of the brain. The presence of a focal lesion can sometimes be detected by electroencephalographic readings and functional molecular resonance imaging, allowing for the possibility of targeted treatment to the affected area.[1] In either generalized or partial seizures, the goal is to deliver AEDs to the brain in quantities sufficient to reduce the frequency and severity of seizures without causing side effects.[2,3]

At present, therapy of seizures involves producing high levels of AEDs in the blood, through the use of pills and intravenous (IV) injections. In either case, drug must enter the brain by crossing from the blood into the brain tissue. The presence of p-glycoprotein and multidrug resistance transporters in the blood–brain barrier (BBB) could prevent the entry of AEDs into the brain, causing drug-resistant epilepsy.[3] One theory on the causes of drug resistance in epilepsy is the transporter hypothesis. Experimental and clinical evidence supporting the transporter hypothesis indicated that increased expression of efflux transporters at the BBB in the focal tissue limits the penetration of AEDs to the focus. In addition, molecular efflux pumps, which use cellular energy to pump drugs that might cross the BBB back into the vessel lumen, decrease the ability of many prospective AEDs to accumulate in the brain. Even before drugs can reach the brain, factors such as systemic toxicity and macrophage phagocytosis within the reticuloendothelial system (RES) limit the success of the transvascular route.[4]

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A lot of strategies have been investigated to overcome the BBB to deliver therapeutics to the central nervous system. With the development in nanotechnology and nanoscience, novel approaches have been employed to deliver drug into the brain. Nanoparticles (NPs) are colloidal particles with the particle size between 1 and 1000 nm and can be prepared by encapsulating the drug into a vesicle or by dispersing the drug within a matrix and the product will have significant advantages for drug delivery, such as better bioavailability, systemic stability, higher drug loading, longer blood circulation time, and selective distribution in the organs/tissues with longer half-life. In recent years, nanoparticulate drug delivery systems have been widely studied for brain targeting.[5,6]

The objective of the present study is to deliver AED directly to the seizure focus in the brain to deliver higher, more effective doses to the seizure focus while bypassing the remainder of the body to prevent side effects. To achieve the above goal, we have proposed development of lipoprotein-coated NPs as a carrier to deliver carbamazepine (CBZ) intravenously with the purpose to cross the BBB and deliver drug in the vicinity of seizure, thus to enhance the brain drug concentration and the treatment efficacy.

To localize a higher amount of drug in to the brain AED can be deliver through NPs, due to an optimal size i.e. small enough to travel through the physical restrictions presented by the brain interstitial space (approximately 50 nm) and but large enough to allow for sufficient loading of drug. Drug encapsulated NPs protect the drug from *in vivo* degradation and reduce toxicity. However, once delivered by IV injection, NPs are cleared from the plasma within a few minutes due to opsonization and subsequent phagocytosis by the cells of the RES. And hence, to increase circulation time, polymers such as polyethylene glycol (PEG) can be coated over the surfaces of NPs. The addition of these tethered PEG chains produces "stealth" character: The particles are no longer opsonized or recognized by the RES and therefore circulate for longer periods.[7]

Even though the incorporation of PEG has been shown to increase circulation time, there is no guarantee that PEG-modified NPs through IV injection will cross the BBB. Targeting moieties must also be added to the nanodelivery systems, in addition to PEG, to facilitate penetration of the BBB. One idea for increasing the uptake of NPs into the brain is through targeting by incorporation of ligands, which will facilitate transport through the BBB. Targeting ligands can be added directly or indirectly to the colloidal carriers. Targeting will be most effective when the ligands will conjugate to the ends of the PEG chains; addition of ligands to the surface of the carrier can be hindered by steric effects, precluding target-ligand contact, and recognition.[8,9]

MATERIALS AND METHODS

CBZ was procured as gift samples from Panchsheel Organics Limited, Indore; lipoprotein angiopep-2 was supplied by Duraent Biologicals Ltd., Hyderabad; Methoxy poly(ethylene glycol) (MePEG, MW 2.0 KDa), e-caprolactone, rhodamine B isothiocyanate (RBITC), and stannous octoate were purchased from Sigma. All other chemical solvents and reagents used were of analytical grade and purchased from Kasliwal Brothers, Indore.

Adult male albino rats aged between 6 and 7 weeks and weighing 230–240 g were used for the animal studies. Mice were housed under controlled environment with free access to tap water and standard rodent diet. All the experiments involving animals and their care were approved by the Animal Ethics Committee of Rishiraj College of Pharmacy, Indore (M.P.) (Registration No. 1728/PO/Re/S/13/CPCSEA).

Preparation and characterization of drug-loaded PEGylated NPs

Synthesis of Me-PEG-PCL and maleimide-PEG-PCL

The Me-PEG-PCL and maleimide-PEG-PCL block copolymers were synthesized by ring-opening polymerization of ε-CL in dry toluene under moisture free high purity nitrogen atmosphere, using MePEG or maleimide-PEG as the initiator in the presence of stannous octoate as a catalyst.

Preparation of activated PEG-PCL NPs

Activated PEG-PCL NPs were prepared using a blend of MePEG-PCL and maleimide-PEG-PCL. 22.5 mg MePEG-PCL and 2.5 mg maleimide-PEG-PCL copolymer was dissolved in 1 ml dichloromethane and 20 mg of CBZ was added. Next, 5 ml of 1% (w/v) sodium cholate solution was slowly poured into the solution and then sonicated at 200 w for 50 s. The resulted O/W emulsion was further diluted into 60 ml of a 0.5% aqueous sodium cholate solution and then stirred for 5 min at room temperature by a magnetic stirrer to solidify the NPs. After that, the organic solvent was evaporated by rotary vacuum. The formed NP suspension was centrifuged at 12,000 rpm for 60 min at 4°C and washed twice with deionized water. The pellets were resuspended in 10 ml PBS (pH 7.4) and kept at 4°C for further use.[10] RBITC-labeled NPs were prepared as the same way, except that drug was replaced by RBITC.

Preparation of angiopep-2-conjugated PEG-PCL NPs

For preparation of angiopep-2-conjugated NPs (ANG-NPs), activated NPs were reacted with angiopep in PBS buffer (pH 7.0) for 8 h under nitrogen flow at room temperature. The outer maleimide groups of activated NPs were specifically reacted with the thiol groups of angiopep and the molar ratio of angiopep to maleimide was 1:3. The reaction mixture was then centrifuged at 12,000 rpm for 60 min at 4°C and washed twice by PBS buffer (pH 7.4). The pellets were resuspended in PBS (pH 7.4) and kept at 4°C for further use.[11] The NP concentration was determined by turbidimetry using UV2401 spectrophotometer at 600 nm (Shimadzu, Japan).

High-pressure liquid chromatography (HPLC) analysis

The concentration of CBZ in samples was determined through RP-HPLC (Young Lin, Korea). The mobile phase consisted of water–methanol–acetonitrile (64:30:6, v/v/v) was freshly prepared for each run and degassed before use. The flow rate was set at 1.0 ml/min and the detection wavelength was 235 nm and the total running time was set at 15 min.

Characterization of CBZ-Loaded ANG-NP

Shape and surface morphology

The morphology of the CBZ-ANG-PEG-NP was studied by transmission electron microscopy (Morgagni 268D), Holland at AIIMS, New Delhi, after negative staining with phosphotungstic acid solution.

Particle size and zeta potential

The particle size and zeta potential of the CBZ-ANG-PEG-NP were determined using Zetasizer (Zetasizer 3000; Malvern, UK). The NPs were suspended in PBS pH 7.4. Triplicate measurements were performed for each sample.

Percent drug loading

The drug loading of CBZ in PEGylated NPs was determined by digesting NPs in 5 ml of methanol for 10 min to release the drug content and filtered. The volume was adjusted to 10 ml with methanol. The drug was assayed HPLC at 235 nm and drug encapsulation efficiency was calculated.[12]

In vitro Drug Release

The *in vitro* drug release of the formulations was studied using dialysis membrane (molecular cutoff point 500). NPs (1 ml) free from any free drug were taken into a sack of dialysis membrane and it was suspended in a beaker containing 20 ml of PBS (pH 7.4).[12] The contents of the beaker were shaken using magnetic stirrer at 100 rpm and the temperature was thermostatically maintained at 37±0.5°C. Samples were withdrawn periodically and replaced with the same volume of fresh PBS pH 7.4 and amount of drug was quantified using HPLC at 235 nm.

In vivo Studies

Fluorescence imaging analysis was used to evaluate the BBB crossing and targeting effect of angiopep-modified NPs. ANG-NPs were labeled by RBITC.[6] The rats were anesthetized with 10% chloral hydrate and placed on an animal plate heated to 37°C. The control group received 0.9% w/v saline (1 ml), while the test group received 1 ml of the RBITC-labeled ANG-NP. To compare tissue distributions of ANG-NP, rats were sacrificed at 1 h post-injection. Brain and liver were dissected, washed with saline, and subjected to *in vivo* fluorescence imaging.

Drug Level in Serum

Rats were randomly divided into two experimental groups of five animals each. One of the groups received IV ANG-NP dosage form while the other was controlled.[6] At predetermined time points (15, 30, 60, 90, and 120 min) after CBZ-ANG-NP dosing (0.4 mg/kg), rats were sacrificed by cervical dislocation followed by decapitation and the blood was immediately collected into heparinized tubes while brain and liver tissues were quickly removed and weighed. Blood samples were centrifuged at 4°C and 4000 rpm for 10 min to obtain plasma supernatants that were analyzed for drug content by HPLC.

Brain and Liver Biodistribution Studies

Rat brain and liver tissues were homogenized with phosphate buffer pH 7.4 (4 ml per gram of tissue) using a Teflon pestle tissue homogenizer.[13] Tissue homogenates were centrifuged at 4800 rpm for 15 min (4°C) and the resultant supernatants were analyzed for CBZ.

RESULTS AND DISCUSSION

CBZ-loaded angiopep-conjugated PEG-PCL NPs were prepared through the outer maleimide groups of NPs specifically reacting with the thiol groups of angiopep. The mean diameter of ANG-NP as well as NP was <100 nm, with an acceptably good polydispersity index (<0.16). Such NPs may accumulate more readily in cells due to the enhanced permeability and retention (EPR) effect. The NPs exhibited spherical shape of moderate uniform particle size and the particle size measured from the TEM images [Figure 1].

The charge values were close to a neutral state with slight negative charges distributed around the NPs (−3.28 ± 0.75 mV for CBZ-ANG-NP). Drug loading of CBZ-ANG-NP was found to 64%. The *in vitro* cumulative release profiles of CBZ from NPs are shown in Figure 2. CBZ-ANG-NP presented biphase release behavior. After the initial burst release for about 12 h, the release rate of CBZ slowed down and became an almost zero-order release. The release rate during 48 h was 77.9 ± 2.5% for CBZ-ANG-NP.

The *in vivo* potential targeting effect of ANG-NP was determined non-invasively in nude rats, based on the fluorescence of RBITC-labeled NPs. After given through the tail vein, time-dependent biodistribution was observed using fluorescent imaging in live animals. The fluorescence signal in the brain of animal with ANG-NP was much stronger at any time post-injection ranged from 2 h to 24 h. The fluorescence located on the brain, indicating that PEG-PCL NPs could accumulate in the brain through the EPR effect. However, the fluorescence distributed all over the brain suggesting that PEG-PCL NPs modified with angiopep not only transported across the BBB but also accumulate into the brain through lipoprotein receptor-related protein (LRP) receptor-mediated endocytosis on the BBB and brain cells. Hence, ANG-NP exhibited potential targeting effect *in vivo* in rats.

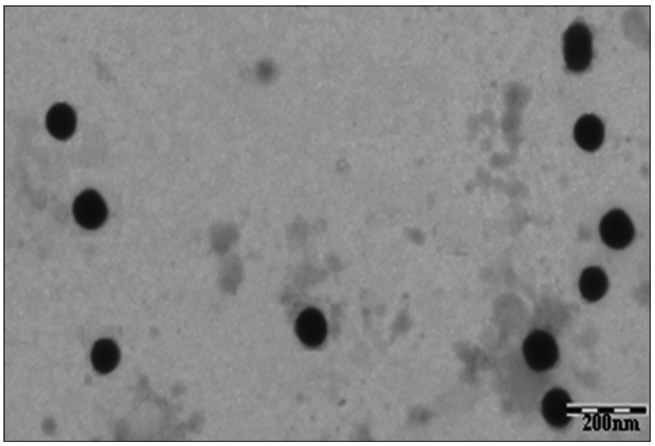

Figure 1: TEM of carbamazepine-angiopep-2-nanoparticles

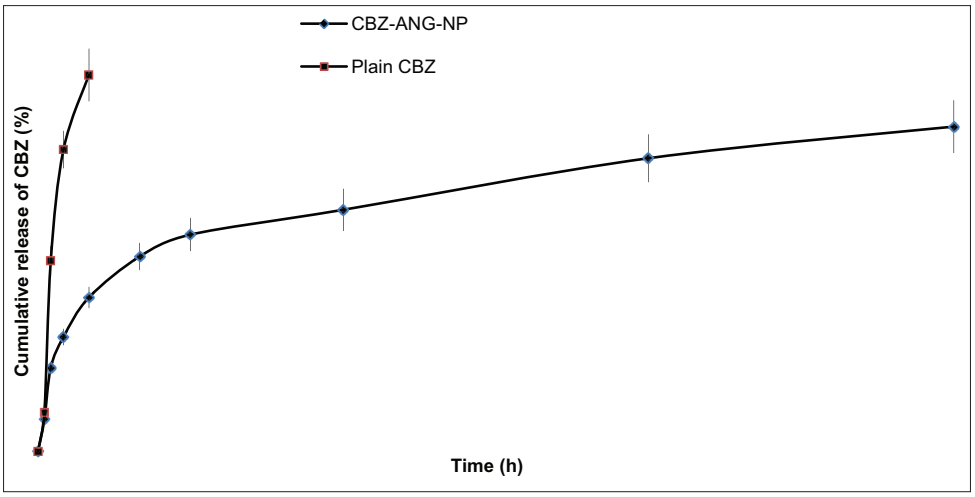

Figure 2: Release profiles of carbamazepine-loaded PEGylated nanoparticles, each point represents average ± SD (n = 3)

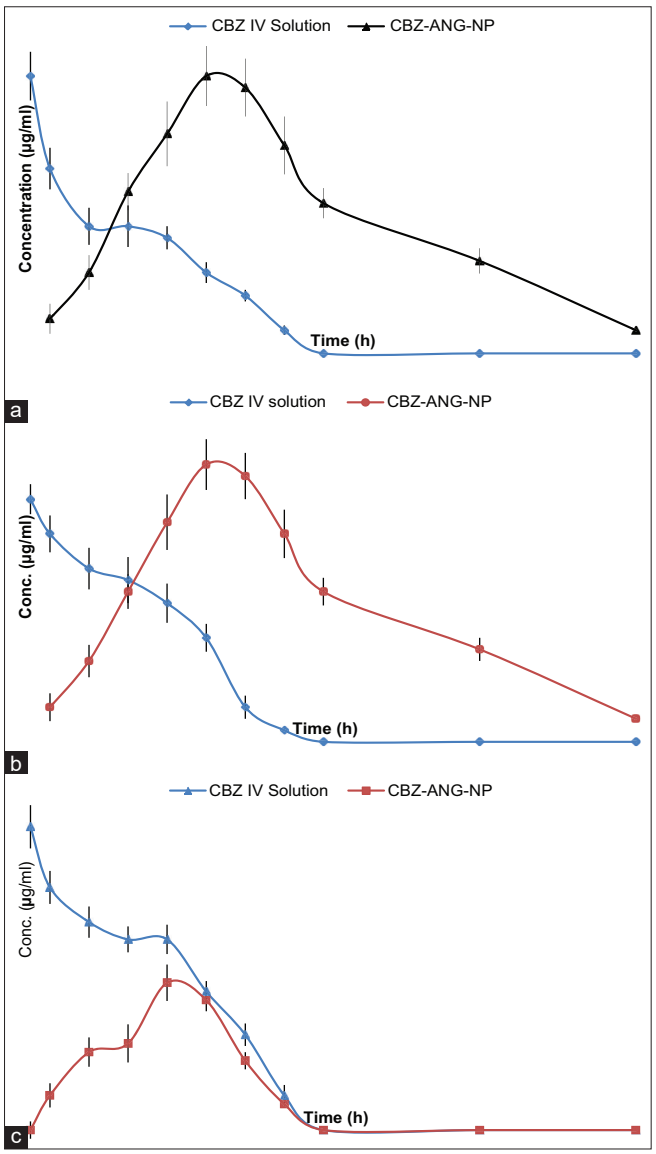

Figure 3: Concentration-time profiles of carbamazepine (CBZ) in (a) plasma, (b) brain, and (c) liver tissues following intravenous administration of CBZ solution and CBZ-ANG-NP formulation ± SD (n = 3)

The mean plasma, brain, and liver concentration-time profiles of CBZ obtained in rat after a single dose of the CBZ containing NPs (0.4 mg/kg) administered as IV formulation is depicted in Figure 3a-c. After reaching the peak, CBZ concentrations in plasma, brain, and liver decreased.

SUMMARY AND CONCLUSION

In spite of decades of research, the quality of life of epilepsy patients is very disappointing since it is too difficult to treat a disease like epilepsy in brain. Conventional drug delivery methods cannot deliver adequate amounts of drugs into brain to treat epilepsy because of the existence of BBB and drug resistance. In recent years, the emphasis for the treatment of epilepsy has been the methods to deliver drug across the BBB. Receptors target could maximize drugs into brain and minimize the systemic toxic effects. Among these receptors, LRP is overexpressed on BBB. It has been demonstrated that LRP could bind numerous ligands including lipoproteins, protease/protease inhibitor complexes, and lipoprotein lipase enriched lipoproteins and mediate transport of ligands across endothelial cells of the BBB.

Among the ligands of LRP, angiopep-2, a novel peptide of 19 amino acids, possesses a higher *in vitro* and *in vivo* LRP-mediated brain penetration capability than other proteins, such as transferrin and aprotinin. In the present study, we developed a novel targeting NPs system by conjugating with angiopep-2, which transported drug across the BBB and then targeted epilepsy. The angiopep-conjugated PEG-PCL NPs exhibited enhanced uptake and accumulation in brain *in vivo*. CBZ-ANG-NP system greatly improved the solubility of CBZ. Second, the surfaces of CBZ-ANG-NP system were modified by hydrophilic PEG chains which extended the circulation time of CBZ in the blood system. Third, in terms of selective delivery, CBZ-ANG-NPs have a potential inherent advantage over free drug molecules as they can diffuse into normal body as well as brain tissues but NPs have a more selective delivery through the EPR effect within brain tissues only.

In the CBZ-ANG-NP system, PCL was chosen as drug carrier as it is a biodegradable polymer approved for human use by the U.S. FDA

and widely used in drug delivery applications. Angiopep was linked to NPs by bifunctional PEG. To maximize the exposure of maleimide groups of NPs to facilitate conjugation with angiopep, the chain of maleimide-PEG (MW, 3500) was longer than that of MePEG (MW, 2000). Prepared NPs were found to be <100 nm, which is optional for improving the pharmacokinetics of NPs and advantageous for endocytosis by brain capillary endothelial cells.

To further verify the targeting effects of CBZ-ANG-NP *in vivo*, albino rats were used to investigate the distribution of RBITC-labeled NPs. The results from *in vivo* imaging photos of brain indicated that angiopep modification enhanced NP accumulation in the brain. It suggested that ANG-NP exhibited targeting effects through transport of the ANG-NP across BBB through LRP targeting. The accumulation of the NPs in the liver could be interpreted as classical passive targeting of the NPs. By presenting PEG on the surfaces of nanoparticles, the rate of RES uptake of the NPs can be greatly reduced, allowing the NPs to have an increased chance to distribute to the target tissue.

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