RETRACTED ARTICLE: Brain-targeted glycyrrhizic-acid-loaded surface decorated nanoparticles for treatment of cerebral ischaemia and its toxicity assessment

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ABSTRACT

Objective: Enhancement of CS-GA-PCL-NPs (Glycyrrhizic Acid-encapsulated-chitosan-coated-PCL-Nanoparticles) bioavailability in brain.

Methods: Double emulsification solvent evaporation method in order to develop CS-PCL-NPs (Chitosan-coated-PCL-Nanoparticles) followed by characterization of particle size and distribution, zeta potential, encapsulation efficiency and drug release (in vitro). To determine drug-uptake and its pharmacokinetic profile in brain as well as plasma, UHPLC (triple quadrupole Q-trap) MS/MS method was developed and optimized for CS-GA-PCL-NPs as well as to follow-up examined effective role of optimized NPs in reduction of all brain injury parameters after MCAO through the grip strength, locomotor activity, inflammatory cytokines levels, measurement of infarction volume and histopathological changes in neurons with safety/toxicity after i.n. in animals.

Results: The developed NPs showed an average particle size, entrapment efficiency with PDI (polydispersity index) of 201.3 ± 4.6 nm, 77.94 ± 5.01% and 0.253 ± 0.019, respectively. Higher mucoadhesive property for CS-GA-PCL-NPs as compared to conventional and homogenized nanoformulations was observed whereas an elution time of 0.37 min and m/z of 821.49/113.41 for GA along with an elution time of 1.94 min and m/z of 363.45/121.40 was observed for hydrocortisone i.e. Internal standard (IS). Similarly, %CV i.e. inter and intra assay i.e. 0.49–4.41%, linear dynamic range (10–2000 ng/mL) and % accuracy of 90.00–99.09% was also observed, AUCG0–24 with augmented Cmax was noted (**p < .01), in Wistar rat brain as compared to i.v. treated group during pharmacokinetics studies. In MCA-occluded rats, enhanced neurobehavioral activity i.e. locomotor and grip strength along with a decrease in cytokines level (TNF-α and IL-1β) was observed, following i.n. administration.

Conclusions: CS-coated GA-loaded PCL-NPs when administered i.n. enhanced the bioavailability of the drug in rat brain as compared to i.v. administration. The observation from toxicity study concludes; the developed NPS are safe and free of any health associated risk.

Abbreviations: CS-GA-PCL-NPs: Chitosan-coated-Glycyrrhizic Acid-loaded and Encapsulated-PCL-Nanoparticles; UPLC-MS/MS/UHPLC-triple-quadrupole-Qtrap-MS/MS: Ultra high performance liquid chromatography triple-quadrupole-Q-trap-mass spectroscopy and mass spectroscopy; GRA or GA:

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Introduction

The loss of brain functions i.e. stroke [1], may be caused due to physical inactivity, alcohol intoxication, diabetes, smoking, hypercholesteremia and hypertension [2]. Further to this, oxidative stress due to high oxygen use as well as reduced level of endogenous antioxidant agents also make the brain vulnerable to injury [3,4]. During ischaemia, an increase in free radical levels with a marked decrease in antioxidant enzymes is noted; hence administration of antioxidants from any external source may reduce neural damage [5].

Glycyrhiza glabra, has been reported for the presence of various phytochemicals such as saponins, triterpenes and flavonoids i.e. Glycyrrhizic acid (GA) (RxBiosciences, Gaithersburg, MD) and glycyrrhetinic acids [6]. The antioxidant and neuroprotective potential of GA, [7–11], may be used to treat neurological disorders such as cerebral ischaemia [7,8].

Intranasal drug delivery is found to be a noninvasive route for drugs which are active low doses and have no or decreased oral bioavailability. Avoiding the range of therapeutic compounds can be intranasally administered for systemic, local and CNS actions [12,13]. The drug enters directly from the nasal mucosa to the spinal cord and brain using pathways through olfactory and trigeminal nerves with the purpose of treating CNS disorders and decreasing systemic exposure [14]. The systemic drug delivery to the CNS is not found to be effective for about 10% large molecules and 98% small drug molecules due to the presence of barrier in the brain that stops the therapeutics to enter the brain from circulating blood or some time separate the brain from the circulating blood. These barriers are blood-cerebrospinal-fluid barrier and BBB [12–17]. As a result, different invasive strategies like intrathecal delivery (BBB disruption), intraparenchymal, intraventricular and some noninvasive techniques like prodrug approach, chemical modifications, and conjugation of a drug along with antibodies or with ligands have been used to increase the CNS targeting of the drugs [12,13]. Several researchers have attempted nose-to-brain drug delivery to exploit the advantages of this route like avoidance of hepatic first-pass metabolism, circumvention of the BBB, convenience of administration and noninvasive nature [18–21].

The hindrance associated with the use of oral route such as headache, nausea and vomiting as well as dizziness makes the intranasal (i.n.) route as a preferred way of drug administration, in order to reach CNS and brain [12,13]. The i.n. administration of GA help increase bioavailability however, physiological shape and anatomy of nasal cavity may allow a limited contact or retention for drug which may result a decrease in absorption rate [18,19]. In the current scenario, a formulation with high mucoadhesive and viscous nature may help increase the mucosal contact time so that more drugs are absorbed. Like insulin, an in-situ gel formulation consisting of biodegradable and biocompatible nanoparticles may be an effective approach for GA [20]. Polymeric nanoparticles possess the properties of high surface area hence faster drug release, enhanced drug loading capacity and drug encapsulation whereby the drug is shielded from the negative effects of chemical and hepatic first-pass metabolism, biological biodegradable, and P-gp efflux proteins in the nasal mucosa. In addition, the smaller diameter helps NPs to cross the mucosal membrane conveniently and access the brain effectively through endocytic mechanism [21]. To understand the effectiveness of NPs, various studies are available which showed an intensified bioavailability for various drugs such as; PVA-NPs and PLGA-NPs [22–26] and PCL-NPs as applied i.n. for brain targeting [27–30]. Chitosan (CS), a mucoadhesive agent is gaining more interest nowadays [31,32] and this may be attributed to the extra advantages associated with the use of CS i.e. high mucoadhesive nature hence a prolonged release and an increased absorption due to prolong contact time and hence an enhanced bioavailability [21].

This study aims to prepare NPs, applied as i.n., for increasing GA availability to brain. The results of the study are expected to reduce the untoward effects of GA and reduce the dose required. The selection of CS is based on its distinguishing adhesive property and cationic charge which help facilitate surface coating. Whereas the combination of CS and PCL-NPs, further enhances the solubility and permeability phenomenon. This study aims to evaluate comparatively the GA-solution with GA-CS-PCL-NPs for its PKs parameters i.e. bioavailability to the brain, i.n. application of GA-CS-PCL-NPs and i.n. along with i.v. administration of GA-solution. In order to study PK parameters, i.e. GA quantification in blood and brain samples, a rapid and easy as well as a sensitive and reproducible analytical method is required. Though a lot of studies regarding GA quantification have been reported however, most of the studies did not report an individual determination of GA [33,34]. Few of the studies have determined GA quantification individually but still there is lack of information in terms of GA-quantification in plasma samples [35–41]. Lack of reliable and reproducible method for GA-determination in plasma and brain tissues (at nanogram level), is the driving factor for current research.

For the first time in this study, a novel nanoformulation (CS-coated-GA-PCL-NPs) will be prepared and administered via i.n. route and a rapid, easy and sensitive analytical method will be developed and validated in order to determine the GA quantity in plasma as well as brain samples, using UHPLC-MS/MS.

Materials and methods

Materials

GA (from RxBiosciences, 18908 Bonanza way, Gaithersburg, MD20879) and Hydrocortisone (Ranbaxy Research
Laboratories, Gurgaon, Haryana, India), Poly-ε-caprolactone with a molecular weight of 14,000, medium molecular weight Chitosan with degree of acetylation = 85% and PVA i.e. polyvinyl alcohol with a molecular weight of 25,000 LC/MS Grade solvents (ACN and MeOH), MS-Grade solvents AF, AA, DCM and formic acid (purity = 99%) were made available from Sigma Aldrich (St Louis, MO). Whereas, water was purified using Milli-Q system (Millipore, Bedford, MA). For in vitro studies, the analytical Grade reagents were used.

**Preparation of GA-PCL-NPs**

The double emulsion technique (W1/O/W2) was adopted with slight modification in order to prepare GA-PCL-NPs (Glycyrrhizic Acid-PCL-NPs) [42,43]. Table 1 shows the composition of individual formulation. In brief, GA (10 mg) was dissolved in purified water (0.50 ml) and added to PVA-solution (1.0% w/v, pH 3.0 adjusted, aqueous phase) and PLGA (100.00 mg) dissolved in DCM (4.0 ml) with sonication under an ice bath conditions for 2.0 min. GA-S was emulsified in the presence of sonication over an ice bath (1 min). This formed primary w/o emulsion was more emulsified through drop by drop 8.0 ml 2% PVA solution (external aqueous phase) with sonication under an ice bath conditions for 120.0 s. Finally formed formulation was kept mild 400 rpm magnetic stirring at room temperature. In order to separate GA-loaded-PCL-NPs, centrifugation (REMI, Mumbai, India) of the bulk aqueous phase for 30 min at 15,000 rpm, a washing step with cold distilled water and freeze drying (-45°C, 24 h) (Labconco, TriadTM, Kansas City, MO) was carried out, Table 1 shows the composition of developed PCL-NPs.

In order to prepare CS-PCL-NPs, GA1-PCL-NPs as well as GA2-PCL-NPs in a specific amount, was incubated (2 h) with drug of an equal volume (CS (2 mg/mL) in 65% acetic acid) [43,44]. The GA1- and GA2-PCL-NPs resulted in this process followed a centrifugation procedure with a washing step and dispersed again in an equal volume of distilled water. Finally they were stabilized using a freeze dryer (Labconco, TriadTM, Kansas City, MO) for 3 days at –60°C.

**Size, distribution and zeta potential of nanoparticles**

The size, distribution or PDI i.e. polydispersity index as well as zeta potential for NPs was performed with the help of dynamic light scattering technique (DLS) having the facility of computerized inspection system and “DTS nano” software (Malvern Zetasizer, Nano-ZS, Malvern, UK).

**Shape and surface morphological analysis**

The morphological analysis (surface morphology and shape) for optimized NPs was evaluated with the help of TEM and SEM technique as reported [45–47].

**Drug loading capacity and (LC) entrapment efficiency (EE)**

Drug loading capacity and (LC) Entrapment efficiency (EE) of optimized and prepared nanoparticles were evaluated by UHPLC-MS/MS as per the method reported by Ahmad et al. [47] In order to calculate EE and LC, the following equation was applied for the triplicate measurements observed for nanof ormulations [45–47].

\[
EE\% = \frac{GA\text{total} - GA\text{free}}{GA\text{total}} \times 100
\]

\[
%DL = \frac{\text{Entrapped amount of GA NPs weight}}{\text{initial dry weight of starting material}} \times 100
\]

To calculate the yield for CS-coated-GA-loaded-PCL-NPs, the following formula was applied where \( W_1 = \) recovered dried NPs weight and \( W_2 = \) initial dry weight of starting material;

\[
%\text{Yield} = \frac{W_1}{W_2} \times 100
\]

**NPs release model (in vitro)**

The release profile for optimized NPs was evaluated according to the reported method [21,45–49]. In brief; GA-release (in vitro) profile of the GA-S and CS-GA–PCL-NPs was performed using dialysis-sacs (MWCO 12,000.00 gram per mole; company Sigma-Aldrich). Dialysis-Sac (cellulose membrane) was filled through equivalent volume of GA (2 mg) and CS-coated-GA-loaded-PCL-NPs (2 mg equivalent dose) and dissolution apparatus-2 (Veegeo, Mumbai, India) was used to perform the experiment having PBS (500.00 ml, pH 6.5, 37°C ± 0.5°C). All the samples were collected at predetermined sampling time points. All the sampling time points collected 3-times for UHPLC-ESI-Q-TOF-MS/MS (in-house developed method) analysis.

**Ex vivo nasal mucosa permeation studies**

**Ex vivo** nasal mucosa permeation studies of optimized and prepared nanoparticles were evaluated as per the method reported by Ahmad et al. [21,45–49]. In brief; from the local slaughterhouse collected the fresh nasal cavities of goats immediately after fresh nasal tissues taken out. The tissue cells area was fixed (0.785 cm²) for GA-permeation (Logan Instrument Corporation, Piscataway, NJ). We fixed tissue cells area (0.785 cm²) for the permeation of GA (Logan Instrument Corporation, Piscataway, NJ).
Corporation, Piscataway, NJ). PBS (pH = 7.40, 20.0 ml, 37.0 °C) was transferred in to the receptor chamber GA (2 mg) and CS-GA-PCL-NPs (2 mg equivalent dose) were put in the donor chamber (two-millilitre) after a preincubation time (20 min. for each case). Five-hundred microlitres of all test samples were taken from the receptor chamber at specified time points through a successive exchange of the sampled volume with PBS for a period of 24.0 h. All the test samples (in triplicate) were examined using the mentioned method UHPLC-ESI-Q-TOF-MS/MS in this manuscript.

**Development and validation of bioanalytical method**

The US-FDA guidelines as reported [50–52], were followed for developing bioanalytical procedure along with its validation, in order to determine GA in plasma, brain and lung homogenate. For linearity of the method, eight levels of non-zero concentration were used whereas for calibration curve (CC) and calculate peak area ratio, weighed \(1/x^2\) linear least square regression was applied. S/N (signal to noise ratio) was set at 10:1 ratio in order to find the lowest concentration of the calibration curve (LLOQ) whereas LQC, MQC and HQC were used to observe the efficiency of GA-extraction i.e. a comparative evaluation was applied with the help of six pre-spiked replicates of extracted samples (mean area response) vs. post-extraction spiked drug-free plasma and brain homogenate samples. Likewise, the IS recovery was also determined. Furthermore, for brain and plasma samples of GA, a replicate analysis (including six replicas of the samples i.e. MQC, LQC, HQC, LLOQC, along with a CC sample) was performed to determine intra-day accuracy and precision.

**UHPLC**

Waters ACQUITY UPLCTM (Waters Corporation, MA, USA) and MS detector (Synapt; Waters, Manchester, UK), was used in the study. For chromatography, C-18 column (Waters ACQUITY UPLCTM BEH) with particle size and dimension of 1.7 μm and 2.1 mm \(\times\) 100 mm respectively, was utilized. An isocratic LC-MS Grade degassed mobile phase was used for UHPLC as; acetonitrile:5 mM ammonium acetate in a ratio of 50%/50% v/v whereas the flow rate as well as injection volume of 0.30 ml/min and 10 μl/min, respectively were applied throughout the chromatographic run (3.0 min).

**Q-TOF-MS conditions**

For mass determination, Waters XEVO-TQD (MA, USA) (triple quadrupole) mass spectrometer (Micromass MS Technologies, Manchester, UK, and QCA896) was used with operating conditions as; collision gas (Argon) with a pressure of 1.0 bar.
5.3 × 10⁻³ Torr, scan time (1 min), inter-scan delay (0.02 s), whereas for quantification the parameters used were as; collision energy (CE; 58.0 eV), cone voltage (2.60 kV) and ion mode (–ve). The analysis as per mentioned parameters showed a transition at m/z 821.49/113.41 and 363.45/121.40 for GA and hydrocortisone (IS), respectively (Figures 1 and 2). To calculate an accurate mass as well as for the determination of ions (precursor and fragment ions) Mass-Lynx software (SCN918, V4.1) was used.

Quality control (QC) and standard sample preparation
In order to prepare a standard GA solution (1 mg/mL), the required amount of GA was dissolved in methanol and sonicated for 20 min (44 kHz/250 W). For CC, the set of eight different levels of concentration (A–H) were prepared as; spiking of 2% analyte (aqueous) in brain homogenate and plasma (blank) of the rat (20 ml of analyte (aqueous) + 980 ml homogenate of the rat brain (blank)). The method yielded a concentration range of 10.00–2000.00 ng/mL for GA with an individual final concentration for each analyte as; 10.0, 20.0, 100.0, 440.0, 850.0, 1300.0, 1700.0 and 2000.0 ng/mL. Regarding QC samples; four individual samples (ng/mL) were prepared as HQC (1600.00), MQC (850.00), LQC (29.00) and LLOQC (10.10). An internal standard (IS i.e. 50.00 ng/ml) was produced through further dilution of stock solution (methanol) in water: methanol mixture (1:1). Any solution used in the process was prepared freshly and stored at a temperature of 2–8 °C.

Preparation of sample
To prepare a sample of brain homogenate or plasma i.e. CC standards, fresh QC as well as unknown brain homogenate or plasma samples were selected in the study. Each sample (800 μl aliquot) was taken in a glass tube and added with IS i.e. 100 μl (50 ng/mL). Formic acid i.e. 5% solution (300 μl) was added successively and vortexed (300 rpm; 5 min) in order to unlock protein binding. A separately prepared 5 ml extraction mixture (ethyl acetate (700 ml) + acetonitrile (300 ml)) was added to previously prepared sample and shake for 20 min at 100 rpm, in a reciprocating shaker. Following a centrifugation step (400 rpm; 10 min; 4 °C), the supernatant (4 ml), was separated and transferred to a clean glass tube whereby it was evaporated in a water bath (37 °C), till dryness with the help of nitrogen stream. The final residues thus obtained were reconstituted in mobile phase (500 μl), vortexed (300 rpm; 5 min) and transferred to UHPLC vial (10 μL) for further bioanalysis.

In vivo study
Animal ethical approval and experiment
For animal experimental studies ethical approval was obtained from “the ethical committee for animals, Jamia Hamdard, India. Six rats (Male Albino Wistar Rats; weight (240–400 g); age (7–10 weeks)) were kept in a dark-light cycle for 12 h at a temperature and humidity of 25 ± 2 °C and 60 ± 5%, respectively. All the animals were fed with a standard diet and water for seven days however they fasted overnight before any actual experiment.

Pharmacokinetic (PK) study
For PK study, four groups were arranged and administered individually Group-I: GA-S (i.n.), Group-II: GA-S (i.v.), Group-III: CS-coated-GA-PCL-NPs (i.n.), and Group-IV: CS-coated-GA-PCL-NPs (i.v.) with a dose GA = 10 mg/kg body weight. Each sample time point for each four groups (e.g. 0.5 h or 1.0 h or 2.0 h) was taken 3 rats. First collected the blood and sacrificed the rats, immediately removed the brains and lungs for each sample time point of each four groups. After that the brain/ lungs homogenate was prepared, and rest of the extraction procedure is the same as mentioned in the “Preparation of sample” section for bioanalysis/pharmacokinetic analysis. The blood, brain and lungs homogenate collected at pre-dose (0.0 h) were treated (0.5, 1, 2, 4, 8, 12 and 24 h), processed and analyzed further with the help of developed bioanalytical method for determination of Cmax, AUC0–t, and t1/2. In order to study biodistribution; 24 animals in each group (3 animals at each point intervals × 8 total sampling time point including predose = 24) (24 animals in each group × total groups IV or 4 = 96 total animals used for PK study) were dosed as mentioned above. Total 96 animals were used for PK study. Following the collection of blood samples from three animals, brain and lung homogenate were extracted from these animals (sacrificed). The homogenate thus obtained were further analyzed with the developed bioanalytical method [21].

PDs study
The animals were grouped as (5 × 6); G-1 (control i.e. SHAM), G-2, (substantial control i.e. SHAM + Placebo CS-coated-PCL-NPs), G-3 (MCAO induced), G-4 (MCAO + GA solution i.e. 10 mg/kg body weight) and G-5 (MCAO (i.n.) + CS-coated-GA-loaded-PCL-NPs i.e. 10 mg/kg body weight). MCAO was produced with the help of Intraluminal filament model [53] as follows; at the very first step rats were anaesthetized with 400 mg/kg i.p. chloral hydrate, a monofilament i.e. silicon-rubber-coated (4 0-3033REPK10; DOCCOL, MA) placed in the external carotid artery and accessed to MCA, till a slight resistant is observed (indicates occlusion). The filament (inducing ischaemia for 2 h) was withdrawn and the animals in SHAM group were sacrificed except MCAO. Following a reperfusion period of 22 h, the animals were observed for neurobehavioural activity such as grip strength and locomotor activity and sacrificed thereafter in order to remove brain for further studies i.e. histopathological studies, antioxidant activity (data not shown here) and determination of various cytokine levels (TNF-α and IL-1β). A neurological score-scale was utilized to evaluate neurological functions on the basis of points (0–5) as: 0 = absence of neurological dysfunction; 1 = no full extension of left forelimb when lifted by tail; 2 = circling to the contralateral side; 3 = falling to the left; 4 = no spontaneous walk or in a comatose state and 5 = death. These scores were evaluated in a blind fashion as reported [46,54]. To determine cytokines level; a buffer (20 mM Tris-HCl, 100 mM KCl, 5 mM NaCl, 2 mM EDTA, 1 mM
EGTA, 2 mM dithiothreitol and 2mMPSF; pH 7.6) was used to homogenize brain tissues followed by centrifugation of homogenate (12,000 g, 4°C for 15 min), removal of supernatant and assaying of supernatant with TNF-α and IL-1β kits (eBioscience, USA). Antigen (pg)/protein (mL) was used to express tissue cytokine concentration. The data were expressed as mean ± SEM whereas student’s t-test (p < .05) was used to find group difference. ANOVA was used for differences in more than two groups and any difference greater at p < .05 was considered as significant.

**Locomotor activity (closed field activity monitoring).**
Digital photo-actometer was used to describe spontaneous locomotor activity as reported [55]. A square closed arena, (containing infrared light-sensitive photocells) placed in light and sound attenuated room, was selected to observe each animal for ten minutes.

**Grip strength.** Previously reported and modified procedure (Ahmad et al) was used where a grip strength metre is used to record the activity [21]. On the grid of grip-strength-meter, front paw of the animal was placed and moved down until the grasping of grid is released. At the moment, the force achieved by animal paw as displayed on screen is recorded (Kg).

**Histopathological studies.** For histopathological studies, brain was removed quickly from individual group of animals, preserved in fresh buffer i.e. sucrose (30%) as reported, further sliced (12-μm-thick coronal sections) on a cryostat (Leica, Nußloch, Heidelberg, Germany) and finally cortex region (ten sections) was mounted on glass slide and stained with the help of haematoxylin and eosin [56,57].

**Analysis of infarct volume.** Following a two hour occlusion of animals, the brain was removed through scavenging and reperfusion (22 h) and placed in brain matrix. The preserved brain was sliced further into coronal sections (1.5 nm), with the help of sharp blades and stained with 0.1% TTC i.e. triphenyl tetrazolium chloride (37°C for 15 min). The enzyme in red lipid soluble formazan decreases the tetrazolium salt. The stained sections after scanning with a high-resolution scanner revealed two types of tissues as, infarcted tissues (without staining) and visible tissues (intense red stained). The infarct volume was determined as, average infarct area on both sides x section thickness whereas for total brain infarct volume calculation, the infarct volume from each section is added and fixed for oedema [56–59].

**Toxicity study**
For placebo CS-coated-PCL-NPs (Group-B) and CS-coated-GA-loaded-PCL-NPs (Group-C), an in vivo toxicity evaluation was performed in order to assess any mortality observed in nasal and brain histology, resulted with the use of GA dose (10 mg/kg). In detail, every morning (9.00–10.00 am) the rats were dosed, intranasal with 50 μL suspension (10 mg/kg of GA) for 14 days and before any treatment, the animals were observed daily for any morbidity and mortality as well as any abnormal behaviour. In this study, Group-A served as normal control i.e. untreated group. At completion of 14 days period, animals were sacrificed to extract out brain and nasal mucosa. The extracted samples were fixed (10% neutral-buffered formalin solution), transverse sections were prepared and stained with eosin and haematoxylin, in order to examine it microscopically for any histological changes as compared to control group [46].

**Neurotoxicity studies by rotarod method**
In order to evaluate the effect of developed NPs upon motor coordination, previously reported Rotarod test procedure was applied [60] to observe the integrity of motor system. Briefly; Rotarod apparatus (30 cm) long rod with 3 cm diameter) sub-divided to three compartments with the help of disc (24 cm in diameter), rotates at a constant speed (10 rpm) and the trained animals (i.e. administered CS-GA-PCL-NPs) are observed for motor coordination on day 7th and 14th, in 14-day schedule.

**Statistical analysis**
The data obtained were analyzed for mean ± standard error of mean (SEM) and a student’s t-test (unpaired observation) and ANOVA was applied to determine the difference in two means (p < .05).

**Results**

**Chitosan-coated-PCL-nanoparticles preparation and characterization**
GA-PCL-NPs were prepared with the help of double-emulsion-solvent-evaporation technique, the physicochemical characters i.e. mean particle size (100–300 nm) and PDI i.e. dispersion index (0.1–0.50) of which are shown in Table 1. The results revealed an increase in nanoparticle size with each increment in PCL content as observed in the case of GA1–GA3 where an increase in particle size from 163.4 ± 9.4 nm; 100 mg–309.7 ± 21.1 nm was observed, respectively. The phenomenon of increase in particle size as observed is in-line with a previous report [43]. Thus GA1 and GA2-PCL-NPs were selected for further chitosan coating based on the results of mean particle size, PDI, optimum DL

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**Table 2.** Chitosan coating above selected GA1 & GA2-CS-coated-PCL-NPs.

| Formulation Code | Chitosan (%) | Mean Particle Size (nm) ± SD | Polydispersity Index (PDI) ± SD | Zeta Potential (mV) | Entrapment Efficiency (EE%) ± SD | Drug Loading (DL%) ± SD |
|------------------|-------------|-----------------------------|-------------------------------|-------------------|-------------------------------|------------------------|
| GA1(GA-CS-PCL-NPs) | 2 | 201.3 ± 4.6 | 0.253 ± 0.019 | 17.8 ± 2.06 | 77.94 ± 5.01 | 4.17 ± 0.37 |
| GA2(GA-CS-PCL-NPs) | 2 | 283.6 ± 7.3 | 0.409 ± 0.067 | 25.9 ± 3.63 | 74.43 ± 4.87 | 4.84 ± 0.33 |
(4.38%–6.59%), high process yield (79.63%–81.36%) as well as highest EE (73.31%–78.94%) as shown in Table 1.

With the aim to increase brain bioavailability, different CS-coated-GA-loaded-PCL-NPs (with varying amount of CS and PCL as shown in Table 2) were prepared as reported [12,43,61]. The NPs thus prepared were characterized for a nanoparticle size range and low dispersity <0.45. Following application of CS, it was observed that the initial particle size for GA1 (163.4 ± 9.4 nm) and GA2 (234.6 ± 14.1 nm) increased to 201.3 ± 4.6 and 283.6 ± 7.3 nm, respectively. As reported, the change in particle size is attributed to the high weight of CS polymer [12,62] as shown in Figure 3 whereas the increase in particle size for CS-NPs may be due to the presence of CS which enhances the polymer content [47,63] thus the viscosity of dispersed phase [64,65]. As shown in Table 1 and Figure 3, a positive zeta potential for GA1 (17.8 ± 2.06) and GA2 (25.9 ± 3.63 mV) with a narrow particle size distribution (PDI) of <0.45 was observed. The phenomenon of positive zeta potential is suggested to be a property of amine group present in CS [66].

Figure 3. Dynamic light scattering techniques for determining the particle size distribution of GA-loaded-PCL-NPs globule size (a) & zeta potential (c); CS-coated-GA-loaded-PCL-NPs globule size (b) & zeta potential (d) images.

Figure 4. (a) In vitro release profile of GA-solution, GA-loaded-PCL-NPs and CS-coated-GA-loaded-PCL-NPs performed by using dialysis bag method, revealing sustained release pattern of GA-loaded-PCL-NPs and CS-coated-GA-loaded-PCL-NPs (mean ± SD, n = 3). (b) Ex-vivo permeation profiles of developed CS-coated-GA-loaded-PCL-NPs as compared to pure Glycyrrhizic Acid through goat nasal mucosa.
In vitro drug release study for glycyrrhizic acid (GA) revealed a steady release pattern from CS-coated-GA-PCL-NPs after 24 h (∼79%) and based on the release pattern GA1-CS-coated-GA-PCL-NPs are considered as more significant formulation. The release pattern is supposed due to a strong GA and CS-coated-PCL polymer interaction.

Ex vivo nasal mucosa permeation studies
As shown in Figure 4(b), an enhanced permeation of >77.59% (p < 0.001) was observed on the case of optimized CS-coated-GA-PCL-NPs as compared to a permeation of 19.59% from GA (API). Here the property of more permeation is observed because of CS (increases the permeability).

Table 3. Precision and accuracy data of glycyrrhizic acid (GA) in different biomatrixes.

| Biomatrix        | Quality Controls Samples | Theoretical Concentration (ng mL⁻¹ or ng g⁻¹) | Observed Concentration (ng mL⁻¹ or ng g⁻¹) ± SD | Accuracy (%) | Precisionb (%) | Inter-batch precision | Recoveryc (%) |
|------------------|--------------------------|---------------------------------------------|-----------------------------------------------|--------------|----------------|------------------------|---------------|
|                  |                          |                                             |                                               |              |                |                        |               |
| Brain homogenate | LLOQC                    | 10.1                                        | 9.91 ± 0.30                                   | 98.12        | 3.03           | 98.13                  | 2.45          |
|                  | LQC                      | 29.0                                        | 27.01 ± 1.18                                  | 93.14        | 4.37           | 90.00                  | 4.25          |
|                  | MQC                      | 850.00                                     | 826.24 ± 5.93                                 | 97.20        | 0.72           | 96.23                  | 0.83          |
|                  | HQC                      | 1600.00                                    | 1585.49 ± 7.82                                | 99.09        | 0.49           | 1576.84 ± 8.34         | 98.55         |
| Lungs homogenate | LLOQC                    | 10.1                                        | 9.92 ± 0.31                                   | 98.22        | 3.13           | 98.02                  | 2.63          |
|                  | LQC                      | 29.0                                        | 27.81 ± 1.08                                  | 95.90        | 3.88           | 92.38                  | 4.40          |
|                  | MQC                      | 850.00                                     | 828.19 ± 4.30                                 | 97.43        | 0.52           | 97.03                  | 0.60          |
|                  | HQC                      | 1600.00                                    | 1579.67 ± 8.94                                | 98.73        | 0.57           | 1571.27 ± 7.64         | 98.20         |
| Plasma           | LLOQC                    | 10.1                                        | 9.54 ± 0.39                                   | 94.46        | 4.09           | 94.16                  | 3.05          |
|                  | LQC                      | 29.0                                        | 27.67 ± 1.22                                  | 95.41        | 4.41           | 26.19 ± 1.01           | 90.31         |
|                  | MQC                      | 850.00                                     | 833.18 ± 7.61                                 | 98.02        | 0.91           | 830.96 ± 6.84          | 97.76         |
|                  | HQC                      | 1600.00                                    | 1584.19 ± 9.34                                | 99.01        | 0.59           | 1575.01 ± 10.89        | 98.44         |

Values (Mean ± SD) are derived from 6 replicates. *Accuracy (%) = Mean value of [(mean observed concentration)/(theoretical concentration)] × 100; ‡Precision (%): Coefficient of variance (percentage) = standard deviation divided by mean concentration found × 100; ‡Recovery (%) = Mean value of (peak height (mV) obtained from extracted biological sample)/(peak height (mV) obtained from aqueous sample) × 100.

In vitro drug release study for glycyrrhizic acid
GA revealed a steady release pattern from CS-coated-GA-PCL-NPs after 24 h (∼79%) and based on the release pattern GA1-CS-coated-GA-PCL-NPs are considered as more significant formulation. The release pattern is supposed due to a strong GA and CS-coated-PCL polymer interaction.

Ex vivo nasal mucosa permeation studies
As shown in Figure 4(b), an enhanced permeation of >77.59% (p < 0.001) was observed on the case of optimized CS-coated-GA-PCL-NPs as compared to a permeation of 19.59% from GA (API). Here the property of more permeation is observed because of CS (increases the permeability).

UHPLC-ESI-triple quadrupole Qtrap-MS/MS
The method developed and validated is shown in Figure 1 (MS and MS/MS scans of analyte) and Figure 2 (MS and MS/MS scans of IS) whereas Figure 5 represents different chromatograms as: A: Extracted Blank Plasma, B: Blank Brain Homogenate, C: Plasma Extracted Glycyrrhizic Acid (PGA), D: Extracted Brain Homogenate Glycyrrhizic Acid (BHGA), E: Plasma Extracted Hydrocortisone (PH) and F: Extracted Brain Homogenate Hydrocortisone (BH). GA mean recovery from plasma and brain homogenate (n = 6) as found was >78% whereas the method for PL, LH and BH was found selective with r² > 0.997, over a range of 10–2000 ng mL⁻¹ for GA. LOD and LOQ were 1.0 ng mL⁻¹ and 1.64 ng mL⁻¹, respectively. Similarly, a selectivity of the method was proved from
Table 4. Stability data of glycyrrhizic acid (GA) in different biomatrices.

| Exposure condition | Brain homogenate | Lungs homogenate | Plasma |
|--------------------|------------------|------------------|--------|
| MQC (850.00 ng/mL or ng g⁻¹) | MQC (850.00 ng/mL or ng g⁻¹) | MQC (850.00 ng/mL or ng g⁻¹) | MQC (850.00 ng/mL or ng g⁻¹) |
| LQC (29.00 ng/mL or ng g⁻¹) | LQC (29.00 ng/mL or ng g⁻¹) | LQC (29.00 ng/mL or ng g⁻¹) | LQC (29.00 ng/mL or ng g⁻¹) |
| HQC (1600.00 ng/mL or ng g⁻¹) | HQC (1600.00 ng/mL or ng g⁻¹) | HQC (1600.00 ng/mL or ng g⁻¹) | HQC (1600.00 ng/mL or ng g⁻¹) |

**Long-term stability; recovery (ng) after storage (−40 °C)**

| Condition | Brain homogenate | Lungs homogenate | Plasma |
|-----------|------------------|------------------|--------|
| Previous day | 833.41 ± 6.19 | 832.79 ± 5.38 | 830.48 ± 7.01 |
| 30th Day | 811.46 ± 6.37 | 819.46 ± 5.77 | 817.64 ± 6.43 |

**Freeze-thaw stress; recovery (ng) after freeze-thaw cycles (−40 °C to 25 °C)**

| Condition | Brain homogenate | Lungs homogenate | Plasma |
|-----------|------------------|------------------|--------|
| Pre-cycle | 838.79 ± 5.64 | 833.73 ± 6.33 | 831.73 ± 6.01 |
| First cycle | 822.16 ± 6.48 | 821.43 ± 5.16 | 824.43 ± 7.00 |
| Second cycle | 811.49 ± 5.36 | 809.94 ± 4.99 | 818.43 ± 5.46 |
| Third cycle | 803.41 ± 6.17 | 801.94 ± 5.79 | 806.44 ± 6.01 |

**Bench-top stability; recovery (ng) at room temperature (25 °C)**

| Condition | Brain homogenate | Lungs homogenate | Plasma |
|-----------|------------------|------------------|--------|
| 0 h | 838.49 ± 5.01 | 833.37 ± 4.91 | 833.37 ± 4.91 |
| 24 h | 821.79 ± 4.48 | 821.79 ± 4.36 | 821.79 ± 5.91 |
| 4 h | 803.27 ± 5.58 | 803.18 ± 6.01 | 803.01 ± 5.11 |

**Post-processing stability; recovery (ng) after storage in auto sampler (4 °C)**

| Condition | Brain homogenate | Lungs homogenate | Plasma |
|-----------|------------------|------------------|--------|
| 0 h | 831.27 ± 5.58 | 830.18 ± 6.01 | 833.01 ± 5.11 |
| 24 h | 810.29 ± 5.10 | 818.26 ± 5.78 | 818.99 ± 6.11 |

Values (Mean ± SD) are derived from six replicates. Figures in parenthesis represent analyte concentration (%) relative to time zero. Theoretical contents; LQC: 29.00 ng/mL; MQC: 850.00 ng/mL; and HQC: 1600.00 ng/mL.
reduced infarct volume significantly. For MCAO + GA-solution rats and rats, a significant reduction in tissue damage \( \bar{C}_3 \bar{C}_p < .05 \), \( \bar{C}_3 \bar{C}_p < .01 \) was observed comparatively to MCAO group. As compared to MCAO group, pretreatment decreased significantly the infarct volume, however in comparison to MCAO group alone, MCAO + CS-GA-PCL-NPs revealed a significant decrease in tissue damage.

Morphological changes
Following ischaemia-reperfusion injury, histopathological changes in neuron was examined with the help of haematoxylin-eosin staining. No pathological changes were observed for SHAM group whereas brain damage along with loss of neurons and vacuolated spaces were observed for MCAO group. The corresponding area in MCAO + CS-GA-PCL-NPs sections exhibited a partial loss of neurons as well as intact neurons between vacuolated spaces. The neuronal abnormalities in MCAO + CS-GA-PCL-NPs group were more properly treated using CS-coated-GA-loaded-PCL-NPs as compared to only MCAO group animals as shown in Figure 8.

Inflammatory cytokines levels (TNF-\( \alpha \) and IL-1\( \beta \))
Based on behavioural studies and quantification of cytokines level, the results for PDs studies are presented in Figure 9. As compared to SHAM group, a poor neurological function was observed in MCAO group however, neurological deficits were improved significantly compared to pure-GA, following a treatment with CS-GA-PCL-NPs. Enzyme-linked immunosorbent assay (ELISA) showed an increase in TNF-\( \alpha \) and IL-1\( \beta \) levels in the infarcted areas, in MCAO-group compared to SHAM group \( p < .001 \). Following a treatment with CS-GA-PCL-NPs (\( p < .001 \)), decrease in TNF-\( \alpha \) and IL-1\( \beta \) level was observed compared to MCAO-group, however, no alteration was seen following a treatment with pure-GA (Figure 9).

Toxicity study
There were no mortalities of rats observed in any of the groups during the 14-day treatment period with intra-nasal administration of developed nano-formulation (CS-GA-PCL-NPs). Clinical examination of the rats’ brain tissues revealed no signs of irritation or tissue damage for all the rats as compared to the vehicle control groups (CS-PCL-NPs (placebo)). Macroscopic examination of the brain tissues exposed to the CS-GA-PCL-NPs nano-formulation, the vehicle also did not show any change in the morphology of tissue microstructure. As compared to vehicle control, the CS-GA-PCL-NPs nano-formulation treated groups showed no visible sign of inflammation or necrosis demonstrating the safety of CS-GA-PCL-NPs nano-formulation (Figure 10(a,b,c)).

Figure 10(d,e,f) showed dissected nasal mucosa treated with various treatments. No nasociliary damage and the nasal membrane remained intact. In placebo group as well as CS-GA-PCL-NPs, no damage to nasal mucosa in the form of intact ciliated pseudo-stratified nasal epithelium (no cilia erosion) observed, thus proving the used excipients safety in the used nano-formulations in this study.

Neurotoxicity studies by rotarod method
The rotarod test (i.n. CS-GA-PCL-NPs) with endurance time in seconds showed lack of neurotoxicity at time intervals of day

| Parameters | C_{max} (ng/mL) | T_{max} (h) | t_{1/2} | AUC_{0-\infty} (ng h/mL) | AUC_{0-\infty} (ng h/mL) | K_{el} (h^{-1}) |
|------------|----------------|------------|--------|--------------------------|--------------------------|-----------------|
| GA-S (iv) (Control) | 123.81 ± 6.80 | 2.00 | 7.74 | 1002.42 | 1081.84 | 0.09 |
| GA-S (in) | 189.21 ± 7.80* | 2.00 | 23.23 | 2024.17* | 3389.16* | 0.03 |
| GA-coated-CS-PCL-NPs (iv) | 321.67 ± 12.61** | 2.00 | 40.00 | 3052.60** | 6975.29** | 0.02 |
| GA-coated-CS-PCL-NPs (in) | 1065.21 ± 26.43*** | 2.00 | 27.05 | 13623.69*** | 24458.72*** | 0.03 |

*\( p < .05 \); **\( p < .01 \); ***\( p < .001 \).
7th and 14th. The experiment was performed after 1 h of treatment (Figure 11).

**Discussion**

In our findings, GA1 (−21.22 ± 2.34 mV) and GA2 (−26.22 ± 3.06 mV) revealed a negative zeta potential due to the presence of polymeric carboxylic group over NPs surface in GA-PCL-NPs. However, a shielding effect of CS over NPs surface was also observed as suggested due to conversion of zeta potential into positive charge (17.8 ± 2.06 mV (GA1) and 25.9 ± 3.63 mV (GA2-CS-PCL-NPs)) in the presence of amine group in CS [68]. Furthermore, the presence of CS and PVA imparted a property of higher EE% with an optimum DL to CS-GA-PCL-NPs, as compared to other NPs. It is suggested due to electrostatic attraction among CS (+ve charge) and RAS (−ve charge) which makes GA stick to NPs surface [12].

A smooth surface with spherical shape (SEM analysis) as shown in Figure 12(a) and a proper sphericity (TEM analysis) with a particle size <300 nm, as shown in Figure 12(b), was observed for CS-GA-PCL-NPs. In contrast, a crystalline structure [69,70] with slow aqueous permeability of PVA led towards a steady degradation as well as drug release for PCL-NPs [24,43]. However, for GA, the addition of CS (high water permeable nature) resulted an enhanced drug release rate from PCL-NPs [21] i.e. an initial high release after 12 h followed by a sustained release. Previous studies also suggested the high release rate because of CS addition as CS attributes the property of hydrophilicity as well as hydration towards NPs matrix [45,71].

For the permeation studies, three major pathways of brain-drug uptake from nasal mucosa have been reported; (I) systemic pathway where the drug is absorbed into blood circulation and subsequently reaches the brain across BBB (II) lymphatic pathway, and (III) direct pathway where the drug from nasal mucosa epithelium reaches to brain via trigeminal or olfactory nerves while bypassing the BBB [12,13]. Literature reports different studies related to nanoformulations as, a study conducted by Ahmad et al. showing significant enhancement of safranal delivery to CSF and olfactory bulb after preparation and i.n. administration of safranal mucoadhesive nanoemulsion showing better results compared to drug standard solution [44,48] whereas Md et al. reported more efficient uptake of intranasal delivered nanoformulations compared to Bromocriptine-solution administered through i.n. and i.v. route, respectively [72]. Chitosan (with mucoadhesive property) has received increasing attention due to its increased use in protein and adsorption metal
field [21,49,51,67,72]. Chitosan and the surface modifications of NPs with chitosan provide many advantages; prolong release of drug delivery systems along with mucoadhesive nature, thus increasing the absorption of drug and delayed drug release, lowering the burst drug release due to coating of chitosan as well as increase permeation, and retention of NPs (small size of particle i.e. <210 nm) due to the attraction between the –vely charged membrane and +vely charged chitosan [21,49,51,67,72,73]. Hence, CS-GA-PCL-NPs (GA1) with a high %age yield, small particle size, better EE, as well as enhanced permeation potential, was marked as final optimized formulation. Similarly, an enhanced C_max for CS-GA-PCL-NPs (i.n.) is suggested because of mucoadhesive, small particle size and hydrophobic nature of NPs which assists an increase in absorption. Furthermore, the release pattern for GA revealed a fast initial release of 39.44% in 2 h followed by a delayed release within the next 24 h (total release i.e. ~79%). The initial fast release as observed is due to drug-release from outer covering of NPs whereas the delayed release pattern is attributed to swelling and

**Figure 8.** Effect of CS-GA-PCL-NPs (10 mg/kg) administration on haematoxylin and eosin staining in the brain sections of the SHAM, MCAO, and CS-GA-PCL-NPs + MCAO groups. (a) Cortical area of SHAM group animal showed uniform distribution of neurons. Normal neurons with the characteristic conical outlines with no abnormal features are seen. (b) Tissues around infarcted area in the MCAO group show a focal area of vacuolation and neuronal loss. (c) CS-GA-PCL-NPs + MCAO group rats show partial neuronal loss. (d) Quantification of neuronal damage of SHAM, MCAO, and CS-GA-PCL-NPs + MCAO groups. Original magnification × 20 and scale bar = 20 μm.

**Figure 9.** CS-GA-PCL-NPs pre-treatment for 21 days improves performances in the neurological deficits after stroke: (a) neurological scores and (b and c) quantification of TNF-α & IL-1β activity by ELISA in the SHAM, MCAO and MCAO + CS-GA-PCL-NPs groups. (###p < .001 (SHAM vs. MCAO); *p < .01 and ***p < .001 (Treatment vs. MCAO group)).
hydration of polymer which increases the time for drug release [21,74]. Faster dissolution i.e. 27.36% in one hour as shown in Figure 4(a) is also a contributing factor for the burst release of GA. Therefore, a rapid and sensitive UHPLC-ESI-triple quadrupole-Qtrap-MS/MS method was used successfully during biodistribution and PKs studies of CS-coated-GA-PCL-NPs which can detect up to nanogram level of the GA [47].

The interesting finding was an enhanced drug bioavailability from CS-PCL-NPs as achieved in brain due to this burst release which makes GA an efficient agent for treating cerebral ischaemia. In addition, the in vitro drug release (Higuchi model, \(r^2 = 0.981\)) is suggested due to swelling and diffusion process and this is in-line with the previously reported studies [46].
Another site for pharmacodynamic study like, SHAM-group (without GA nanoformulation), showed a marked improvement in locomotor and grip strength activity whereas MCAO was observed with the least activity. Furthermore, the group administered with intranasal CS-coated-GA-loaded-PCL-NPs, exhibited a comparatively more improvement as compared to GA-solution administered group (Figure 7(b)). No brain toxicity was observed for SHAM + Placebo group.

TTC method was carried out whereby morphological features of infarct tissues are determined following an ischaemic injury. This technique help measure ischaemic stroke consequences (severe neuronal damage) leading to neurological impairment [75,76]. MCAO group with infarct as well as behavioural changes, showed a reduced infarct size and improvement in behavioural properties after treatment with GA. Thus it is evident that CS-GA-PCL-NPs as well as GA-Solution effectively reduce infarct volume and improves neurological measures as shown in Figure 7(c,d) [59,77].

MCAO-group animals exhibited a poor neurological activity as compared to SHAM group however, treatment with NPs added a significant improvement. The pure-GA treated group, showed no significant improvement. Thus it may be suggested; i.n. administration of CS-PCL-NPs enhanced GA bioavailability. It is a well-known fact that pro-inflammatory cytokines (TNF-α and IL-1β) are over-expressed in ischaemic conditions as reported by Ahmed et al. [68] and in our study we found a significant decrease in these proteins following a pre-treatment with CS-coated-GA-loaded-PCL-NPs. Moreover, the 14-days toxicity study, after i.n. administration of CS-coated-GA-loaded-PCL-NPs revealed no toxicity or inflammation which proves the safe nature of developed NPs.

Conclusion

The optimized CS-coated-GA-PCL-NPs prepared via emulsification technique showed an optimum particle size, loading capacity and entrapment efficiency with a sustained release (over twenty-four hours). The optimized NPs evaluated for successful administration (i.n.) exhibited a superior in vivo potential in comparison to GA-S (i.v. and i.n.). Furthermore, a rapid and sensitive UHPLC-ESI-triple quadrupole-Q-trap-MS/MS-based bioanalytical method, with an ability to detect up to nanogram level of the GA was developed for PKs and PDs of CS-GA-PCL-NPs. In addition, on the basis of results from neurobehavioural activity, levels of cytokines and histopathological studies, an enhanced PDs features were observed for developed and optimized NPs. Finally, a safe nature for our developed NPs was proved from the results obtained during toxicity studies and hence it may be concluded, CS-coated-GA-loaded-PCL-NPs as developed in this study may be effectively utilized as a novel, safe, non-invasive and effective therapy for targeting brain and treatment of cerebral ischaemia.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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