Carbohydrate Polymers 89 (2012) 72–79

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Venlafaxine loaded chitosan NPs for brain targeting: Pharmacokinetic and pharmacodynamic evaluation

Shadabul Haque[a,b], Shadab Md[a], Mohammad Fazil[a], Manish Kumar[c], Jasjeet Kaur Sahni[a], Javed Ali[a], Sanjula Baboota[a,*]

[a] Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi 110 062, India
[b] Ranbaxy Research Laboratories, Gurgaon, India
[c] Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India

ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form 18 February 2012
Accepted 20 February 2012
Available online 28 February 2012

Keywords:
Biodistribution
Brain targeting
Chitosan
Confocal microscopy
Intranasal route
Venlafaxine

ABSTRACT

The purpose of the present investigation was to prepare venlafaxine (VLF) loaded chitosan nanoparticles (NPs) to enhance the uptake of VLF to brain via intranasal (i.n.) delivery. VLF loaded chitosan NPs were prepared and characterized for particle size, size distribution, zeta potential, encapsulation efficiency and *in vitro* drug release. In order to investigate the localization of chitosan NPs in brain and other organs qualitatively confocal laser scanning microscopy technique was carried out using rhodamine-123 (ROD-123) as marker. The levels of VLF in plasma and brain tissues were also determined, the brain/blood ratios of VLF for VLF (i.v.), VLF (i.n.), VLF chitosan NPs (i.n.) were 0.0293, 0.0700 and 0.1612, respectively, at 0.5 h, indicative of better brain uptake of VLF chitosan NPs. The higher drug transport efficiency (508.59) and direct transport percentage (80.34) of VLF chitosan NPs as compared to other formulations suggest its better efficacy in treatment of depression.

1. Introduction

Conventional drug delivery methods fail to deliver a number of therapeutic agents to the brain efficiently. Blood brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier restrict the transport of drugs from systemic circulation into the central nervous system (CNS). Though the BBB serves to protect the brain and spinal cord from a variety of pathogens and toxic substances, nevertheless it also presents a significant barrier in treatment of CNS disorders. Consequentially a number of invasive strategies like intraparenchymal, intraventricular, intrathecal delivery (BBB disruption) and non-invasive techniques like chemical modifications, prodrug approach and conjugation of a drug with antibodies or ligands have been used to increase the CNS targeting of drugs (Davis, 1997).

Nose-to-brain delivery of drug moieties have been attempted by several researchers to exploit the advantages of this route, such as circumvention of the BBB, avoidance of hepatic first-pass metabolism, practicality and convenience of administration and non-invasive nature (Illum, 2000). Nose to brain delivery is possible by the olfactory region located at the roof of nasal cavity as its neuroepithelium is the only part of the CNS that is directly exposed to the external environment. From this neuroepithelium drugs are carried to CNS by trigeminal nerve systems by a number of mechanisms (Ali, Ali, & Baboota, 2010; Pires, Fortuna, & Alves, 2009). Although the olfactory route has not been investigated widely in humans because of difficulties in absolute measurements of drug in the CSF or brain tissues, several studies in animals have been published for drugs such as Olanzapine (Kumar, Misra, Mishra, Mishra, & Pathak, 2008a), Risperidone (Kumar et al., 2008b), Buspirone (Khan, Patil, Yeole, & Gaikwad, 2009), Ropinorole (Khan, Patil, Bobade, Yeole, & Gaikwad, 2010) and Didanosine (Al-Ghananeem, Saeed, Florence, Yokel, & Malkawi, 2010).

Venlafaxine (VLF) is a dual action antidepressant, and belongs to class of antidepressants known as serotonin and norepinephrine reuptake inhibitors (SNRI). VLF is amongst the first line drugs used in the treatment of depression. It inhibits central serotonin and norepinephrine neuronal reuptake increasing the diminished levels of neurotransmitters like serotonin and norepinephrine in the synaptic cleft between neurons in the brain (Wilson, Howell, & Waring, 2007). VLF is commercially available as immediate and controlled release tablets and capsules. VLF is one of the most widely used antidepressants; nevertheless oral therapy has a number of drawbacks, such as slow onset of action, side effects like tachycardia, increased blood pressure, fatigue, headache, dizziness, sexual dysfunction, dry mouth and low bioavailability (40–45%) (Stahl, Grady, Moret, & Briley, 2005). Additionally, VLF has an elimination half-life of 4–5 h and therefore needs frequent

* Corresponding author. Tel.: +91 9818529286; fax: +91 11 26059633.
E-mail addresses: sbaboota@rediffmail.com, Shadab87@gmail.com (S. Baboota).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2012.02.051

administration to maintain a blood level for effective therapeutic concentration (Troy, Parekar, Fruncillo, & Chiang, 1995). Moreover efficacy of VLF relies upon its continued presence at the site of action over a prolonged period of time (Kilts, 2003). However the saw-tooth pattern of plasma drug concentrations following oral drug administration often causes adverse events at maxima and loss of therapeutic effect at minima, which leads to intolerability (Kilts, 2003). This fact provides a strong rationale for designing more effective intranasal dosage forms capable of directly transporting the drug to the brain to overcome the aforesaid drawbacks. Reported researches have disclosed some necessary prerequisites for intranasal delivery systems for nose to brain transportation of drugs. The delivery systems should be designed to provide a longer residence time in nasal cavity to overcome the nasal mucociliary clearance and facilitate rapid transport of drug across nasal mucosa. The mucoadhesive chitosan nanoparticles (NPs) have both the attributes as it not only reduces the mucociliary clearance but also opens the tight junctions between cells transiently, which facilitate drug transport across the nasal membrane to brain by the paracellular route (Vllasaliu et al., 2010). Moreover chitosan NPs possess added advantages of small particle size, enhanced permeability across nasal mucosa and ability to encapsulate various ingredients (Genta et al., 2003).

The objective of the present research work was to investigate the potential use of chitosan NPs for enhancing the brain delivery of VLF and the utility of the intranasal (i.n.) nose to brain route for preferential improved delivery of CNS drugs to the brain in comparison with intravenous (i.v.) route. VLF chitosan NPs were developed and characterized for various physico-chemical characteristics to determine their suitability for nose to brain delivery. In order to investigate the localization of chitosan NPs in brain and other organs qualitatively confocal laser scanning microscopy technique was attempted using rhodamine-123 (ROD-123) as marker. Measurements of VLF in blood and brain tissues were determined after administration of VLF chitosan NPs i.n. and VLF solution i.n. and i.v. to confirm their brain-targeting efficiency. Overall the current investigation was aimed at providing a completely safe and effective formulation, taking into consideration the chronic nature of treatment, with brain-targeting potential and reduction in dose and dosing frequency.

2. Materials and methods

2.1. Drugs and reagents

The drug venlafaxine hydrochloride was received as gift sample from Ranbaxy research Lab. (Gurgaon, India). Low molecular weight chitosan glutamate (20–300 cps, ≥85% deacetylated), Sodium Tripolyphosphate (TPP) and Rhodamine 123 were purchased from Sigma Aldrich (Bangalore, India). Glacial acetic acid was purchased from Merck Chemical Ltd. (Mumbai, India). Methanol HPLC grade and Acetonitrile HPLC grade were procured from S.D. Fine Chemicals, Ltd. (Mumbai, India). Potassium dihydrogen phosphate, methanol, sodium hydroxide (NaOH) and 1-Octanol were all purchased from S.D. Fine Chemicals, Ltd. (Mumbai, India). Cellophane tube (mol. wt. cut-off: 12 000 Da, flat with 25 mm, diameter of 16 mm, capacity 60 ml ft) was purchased from Sigma Aldrich Chemicals, Pvt. Ltd (Missouri, USA). All chemicals were of analytical grade.

2.2. Animals

Adult Wistar strain rats of either sex (aged 4–5 months) weighing between 200 and 250 g were selected for pharmacodynamic and pharmacokinetic studies.

2.3. Preparation and characterization of VLF loaded CS NPs.

The chitosan NPs were prepared by ionic gelation of chitosan with sodium tripolyphosphate (TPP) as reported earlier (Al-Ghananeem et al., 2010; Calvo et al., 1997). The preparation of chitosan NPs involves the mixture of two aqueous phases at room temperature. One phase contains a solution of polycation chitosan and the other contains a solution of polyanion TPP. For this purpose chitosan was dissolved in acetic acid (2%, v/v, pH 3.5) at a concentration of 1.75 mg/ml with constant stirring overnight. TPP was dissolved in distilled water at the concentration of 2.0 mg/ml. NPs were obtained by the addition of 4 ml of TPP solution to 10 ml of chitosan solution with constant stirring (800–900 rpm) at room temperature for 30 min. For the association of VLF to CS NPs, it was dissolved into chitosan solution at the drug polymer ratio of 1:1 before the addition of TPP. The resultant NPs were separated from the aqueous medium by ultracentrifugation at 15 000 rpm at 10 °C for 40 min. The supernatant was discarded. The pellets were washed with distilled water and then freeze dried.

Chitosan NPs were prepared and characterized for particle size and size distribution, drug content, pH, and zeta potential. The chitosan NPs size and size distribution were determined using photon correlation spectroscopy with in-built Zetasizer (model: Nano ZS, Malvern Instruments, UK). The amount of encapsulated VLF in NPs was determined in triplicate by HPLC analysis. The encapsulation efficiency (EE) of VLF in NPs was determined as the mass ratio of the encapsulated VLF in NPs to the theoretical amount of VLF used in the preparation and the loading content (LC) of VLF in the NPs was calculated as the mass ratio of the encapsulated VLF in NPs to the practical weight of NPs. The *in vitro* release studies of VLF loaded chitosan NPs were performed by dialysis sac method in phosphate buffer (pH 7.4).

2.4. Ex vivo permeation studies on nasal mucosa

In order to investigate the permeation efficacy of chitosan NPs for rapid transportation of VLF across nasal mucosa *ex vivo* permeation studies were conducted using Franz diffusion cell on porcine nasal mucosa (Sintov, Levy, & Botner, 2010). On experimental day nasal tissue (treated with isopropyl alcohol to remove adhering mucous and fat) was cut to appropriate size and mounted between the donor and receptor compartments of the Franz diffusion cell, with the mucosal side facing the donor compartment, and the receptor compartment was filled with phosphate buffer (pH 6.4) and magnetically stirred at 200 rpm for proper mixing. The diffusion cell was thermostated at 37 ± 0.5 °C. VLF chitosan NPs (equivalent to 10 mg of VLF) and VLF (10 mg) were dissolved in 1 ml of phosphate buffer (pH 6.4) and placed in the donor compartment, respectively. Aliquot (1 ml) was collected from the receiver cell at designated time intervals (0.08, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h) and replaced immediately with the same volume of fresh media maintained at 37 ± 0.5 °C. After appropriate dilutions, the samples were filtered using 0.45 μm membrane filter and the amount of drug in the receptor media was analyzed by HPLC.

2.5. Pharmacodynamic studies

2.5.1. Modified forced swim test

Antidepressant efficacy of VLF chitosan NPs was determined by forced swim test on chronic depression induced rats (Cryan, Markou, & Lucki, 2002; Porsolt, Bertin, & Jalfre, 1977). This study was approved by Institutional Animal Ethics Committee, Jamia Hamdard, New Delhi. Animals were divided into five groups with three animals in each group. Group 1 was considered as naive group which neither received any drug nor depression was induced in it. Group 2 rats were given marketed tablets of VLF as oral standard

at the dose of 1.35 mg/day. Marketed 25 mg tablet was suspended in 10 ml of normal saline containing 0.2% of the carboxymethyl cellulose (suspending agent) to give concentration of 2.5 mg/ml. For 1.35 mg/day dose, 0.54 ml of this suspension was administered orally for 15 days with the help of oral feeding needle. Group 3, 4 and 5 rats were given nasal formulation 50 μl in each nostril with the help of a microlitre syringe attached to polyethylene tubing having 0.1 mm internal diameter. The polyethylene tubing was inserted 7 mm inside the nostril. Group 3 rats were kept as control and were administered saline solution (100 μl/day) 50 μl in each nostril for 15 days. Group 4 rats were administered pure VLF solution at the dose of 0.6075 mg/day dissolved in 100 μl of normal saline solution for 15 days. Group 5 rats were administered VLF chitosan NPs (1.88 mg/day equivalent to 0.6075 mg/day VLF) dissolved in 100 μl normal saline solution 1 h prior to intranasal administration for 15 days. Rats were placed in a cylindrical glass tank (46 cm tall and 20 cm in diameter) filled to a depth of 30 cm water (25 ± 2 °C) individually for 6 min training and were then removed and dried. The water depth of 30 cm allowed the rats to swim or float without their hindlimbs or tails touching the bottom of the tank. The 6 min training was done 30 min after administration of dose in each group for 15 days. After 5 h (on 15th day) animals again were reexposed to the swimming in a similar environment for 6 min and behavioral climbing time, swimming time and immobility time were recorded for 300 s and compared with group 3 control (untreated but trained, i.e. depressed) and group 1 naive (neither untreated nor trained, i.e. non depressed animals).

2.5.2. Measurement of locomotor activity

The locomotor activity was recorded for 5 min using photoactometer on 15th day for all the five groups as mentioned above. Each animal was observed in a closed square (30 cm × 30 cm) arena equipped with infrared light sensitive photocells using digital photoactometer (Hicon instrument, Mumbai, India). Locomotor activity was expressed in terms of total photobeam counts for 5 min per animal. The apparatus was placed in a darkened, light and sound attenuated, and ventilated testing room.

2.6. Qualitative localization and biodistribution studies by confocal laser scanning microscopy (CLSM)

Rhodamine-123 (ROD-123) fluorescent dye was used to determine the localization of CS NPs in brain and other organs qualitatively (Tosi et al., 2007; Vergoni et al., 2009). ROD-123 is widely used in cerebral studies and it is unable to cross the BBB (Lange, Bock, Shinkel, Boer, & Breimer, 1998). For the preparation of ROD-123 loaded chitosan NPs, 0.9 ml of Rhodamine 123 (20 mg/ml in ethanol) was dissolved in 10 ml of chitosan solution (0.175%, w/v) followed by its cross linking with 4 ml of 0.2% (w/v) TPP solution at room temperature with constant stirring at 800–900 rpm for 30 min. The resultant NPs were concentrated by centrifugation at 15 000 rpm at 10 °C for 40 min.

For biodistribution studies animals were divided into two groups, each composed of 3 animals. Group 1 received ROD-123 loaded NPs (i.v.) in the dose of 0.836 mg/day dissolved in 1 ml of sterile isotonic solution by tail vein, group 2 received ROD-123 loaded chitosan NPs (i.n.) at the dose of 0.836 mg/day dissolved in 100 μl of normal saline solution. Then the animals were sacrificed at fixed time point of 20 min, 60 min and 120 min to localize the NPs in different organs (i.e. brain, liver, lung and spleen) and the isolated organs were washed with Ringer's solution. Cerebellum was separated from brain after dissection of right, left and frontal encephalon. Each tissue was cut in its thickness by a microtome into 5 μm slides. The slides were then fixed in 4% (w/v) formaldehyde solution and stored at 4 °C before confocal laser scanning microscopy (CLSM) studies. In order to demonstrate the location of NPs, tissue slides were stained for 10 min with 50 μl (250 ng/ml) of DAPI (4′-6-diamidino-2-phenylindole; Sigma Aldrich, Mumbai, India) solution, which is known to form fluorescent complexes with natural double-stranded DNA, and observed using a fluorescence microscope (Olympus FluoView™ FV 1000, CA, USA) with double band, for DAPI and ROD-123. The red fluorescent spots due to the fluorescent dye ROD-123 were considered as the visible markers of the drug embedded into NP.

2.7. Brain uptake and in vivo pharmacokinetic studies

The *in vivo* pharmacokinetic and brain uptake were studied using an *in vivo* experimental technique described previously (Al-Ghananeem et al., 2010; Khan et al., 2009). Three rats for each formulation per time point were used in the studies. Group 1 received VLF solution (i.v.) in the dose of 0.6075 mg/day dissolved in 1 ml of sterile isotonic solution, group 2 received VLF solution (i.n.) at the dose of 0.6075 mg/day dissolved in 100 μl of normal saline solution and group 3 received i.n. VLF chitosan NPs (i.n.) (1.88 mg/day equivalent to 0.6075 mg/day VLF) dissolved in 100 μl normal saline solution 1 h prior to intranasal administration, respectively. The rats were euthanized at scheduled times by cervical dislocation after sedating them with chloroform to collect blood and brain tissue. The blood samples were collected from retino-orbital vein present in the eye in precoated EDTA tubes. The skull was cut open and brain was carefully excised. It was quickly rinsed with saline and blotted with filter paper to get rid of blood-taint and macroscopic blood vessels as much as possible. The blood and brain samples were obtained after 15, 30, 60, 120, and 480 min, respectively, after administration of dose by different routes. The concentrations of VLF in blood, and brain tissues were analyzed by validated HPLC method.

2.7.1. Bioanalytical HPLC procedure and sample preparation

Chromatographic separation was achieved with a Lichrocart® C_{18} reversed-phase column (250 mm × 4.6 mm, particle size 5 μm). The mobile phase consisted of acetonitrile and phosphate buffer (20 mM, pH adjusted to 3.0 by orthophosphoric acid) (75:25, v/v). The mobile phase was set at a flow rate of 1 ml/min. The run time of the sample was kept 15 min. The ultra-violet detection of venlafaxine was performed at 210 nm.

The extraction of VLF from plasma and brain samples was carried out using the liquid–liquid extraction (LLE) technique. Plasma sample and homogenized brain tissue (0.5 ml) was mixed with 0.1 ml Rivastigmine Hydrogen tartarate (4 μg/ml) as internal standard solution (Subramanium, Arumugam, Reddy, & Udupa, 2008). The mixture was shaken with 2 ml acetonitrile for 3 min on a vortexer and after centrifugation at 5000 rpm for 10 min, the organic layer was transferred to a test tube. The residual water layer and precipitants were subjected to re-extraction with 2 ml acetonitrile. After centrifugation, the organic layer was combined with the first organic layer and the combined extract was evaporated to dryness under nitrogen gas at room temperature. Dried samples were dissolved in 0.1 ml acetonitrile and centrifuged at 5000 rpm for 10 min. Then 20 μl clear supernatants were injected into the HPLC.

2.7.2. Pharmacokinetic analysis

Plasma concentration–time profiles of VLF after i.v. and i.n. delivery were evaluated by pharmacokinetic software (PK Functions for Microsoft Excel, Pharsight Corporation, Mountain View, CA). The maximum plasma concentration of VLF (C_{max}) and the time required to reach the maximum concentration (T_{max}) were obtained directly from the actual plasma profiles. The area under the curve between 0 and 480 min was calculated by the linear trapezoidal method. Brain targeting efficiency was calculated using two equations mentioned below (Kumar et al., 2008b).

Drug targeting efficiency (DTE%) that represents time average partitioning ratio was calculated as follows:

$$\text{Drug targeting efficiency (DTE\%)} = \frac{(\text{AUC}_{\text{brain}}/\text{AUC}_{\text{blood}})\text{i.n.}}{(\text{AUC}_{\text{brain}}/\text{AUC}_{\text{blood}})\text{i.v.}} \times 100$$

Nose to brain direct transport percentage (DTP%) was calculated as follows:

$$\text{Direct transport percentage (DTP\%)} = \frac{B_{\text{i.n.}} - B_{\text{x}}}{B_{\text{i.n.}}} \times 100$$

where $B_x = (B_{i.v.}/P_{i.v.}) \times P_{i.n.}$, B_x is the brain AUC fraction contributed by systemic circulation through the BBB following intranasal administration; $B_{i.v.}$ is the AUC_{0-480} (brain) following intravenous administration; $P_{i.v.}$ is the AUC_{0-480} (blood) following intravenous administration; $B_{i.n.}$ is the AUC_{0-480} (brain) following intranasal administration; $P_{i.n.}$ is the AUC_{0-480} (blood) following intranasal administration.

2.8. Statistical analysis

All data are reported as mean ± S.E.M. and the differences between the groups were tested using Dunnett test at the level of $P < 0.05$ and $P < 0.01$. More than two groups were compared using ANOVA and differences of $P < 0.05$ were considered significant and differences of $P < 0.01$ were considered highly significant.

3. Results and discussion

3.1. Preparation and characterization

Preliminary studies were performed to identify the experimental conditions for the formation of chitosan NPs. It was found that the preparation of chitosan NPs was only possible for some specific concentrations of chitosan and TPP. The chitosan and TPP mass ratio was optimized on the basis of particle size and particle size distribution. Chitosan NPs were obtained using a chitosan and TPP concentration of 1.75 mg/ml and 2.0 mg/ml with a chitosan/TPP mass ratio of 2.18/1. S.E.M. analysis revealed that VLF chitosan NPs had a solid structure and a round shape. Chitosan NPs displayed an average particle size in the range of 167 ± 6.5 nm with a positive zeta potential in range of +23.83 ± 1.76 mV (Table 1). Its polydispersity index (PDI) of 0.367 ± 0.045 indicated monodisperse stable system whereas its final pH of 5.12 indicated it to be well within nasal pH range. The chitosan NPs had an average loading content from 32.25 ± 1.63% and average encapsulation efficiency up to 79.3 ± 2.6%. The release profile of VLF from chitosan NPs under sink conditions in phosphate buffer (pH 7.4) was found to be 89.56 ± 3.58%. Results showed two phases of VLF release, the initial phase accounted for release of 44.3% of VLF from the NPs in 2 h, followed by very slow drug release over 24 h.

3.2. Evaluation of ex vivo permeation studies

The *ex vivo* permeation studies through porcine nasal mucosa were performed for both the VLF chitosan NPs and VLF solution. The cumulative amount of drug permeated (CADP) per cm^2 was found to be 408.04 and 142.67 μg/cm^2 after 24 h from VLF chitosan NPs and VLF solution, respectively. Chitosan NPs enhanced the cumulative amount of drug permeated by almost three times as compared to VLF solution. This augmentation in the permeation of VLF could be attributed to an interaction of a positively charged amino group on the C-2 position of chitosan with negatively charged sites on the cell membranes and tight junctions of the mucosal epithelial cells to allow opening of the tight junctions. It has been demonstrated earlier that chitosan when applied to confluent cell cultures, is able to transiently open the tight junctions between the cells (Artursson and Lindmark, 1994; Dyer and Hinchcliffe, 2002). This effect is maximal when chitosan is fully protonated (i.e. in acidic condition); however literature reports that even at pH 6.4, 25% of the amino groups still remain protonated to interact with negatively charge sialic acid residue in the mucosa (Chenite, Gori, Shive, Desrosiers, & Buschmann, 2006). VLF is a basic drug having pKa 9.4, thus at nasal mucosa (5.5–6.5) pH, much of the drug remains in protonated form and thus is transported through tight junctions opened by chitosan. Therefore, increase in permeation through nasal mucosa is due to contribution of paracellular transport through tight junctions.

3.3. Evaluation of antidepressant effect of VLF CS NPs

The FST is the most widely used pharmacological model for assessing antidepressant activity. The development of immobility when the rodents are placed in an inescapable cylinder of water reflects the cessation of persistent escape-directed behavior. The test was performed by modification of the traditional FST method described by Porsolt et al. All the four parameters of pharmacodynamic tests were evaluated for their significance (Table 2). The control group was found to differ significantly in all the four parameters than untreated naive (group 1) ($P < 0.01$). VLF chitosan NPs increased the total swimming and reduced the immobility time more significantly ($P < 0.01$) than climbing time ($P < 0.05$) when compared with depressed control. VLF (oral) though increased the total swimming time significantly but it reduced the total immobility period only to a small extent and failed to improve climbing time to a significant level ($P > 0.05$). Similar results were obtained with VLF (i.n.). VLF chitosan NPs (i.n.) maintained a prolonged concentration of VLF in brain in comparison to VLF (oral) and VLF (i.n.), thus showing greater antidepressant effect than both of them. Evaluation of locomotor activity showed maximum activity in naive animals and the least activity in control groups due to inducement of depression and disturbances in their monoaminergic transmission in brain. While the groups administered VLF chitosan NPs exhibited significant increase ($P < 0.01$) in locomotor activity. VLF solution (i.n.) also showed significant improvement in locomotor activity when compared to control group. VLF (oral) displayed much lower locomotor counts as compared to VLF chitosan NPs (i.n.) proving the later to be superior in terms of brain uptake (Table 2). The pharmacodynamic results of better brain uptake of VLF by i.n. route were confirmed by drug concentration measurement in brain tissues.

3.4. Evaluation of qualitative localization and biodistribution studies

For qualitative localization and biodistribution studies the ROD-123 loaded chitosan NPs displayed an average particle size between 181.4 and 190.6 nm with a positive zeta potential of 28.4–34.6 mV. These NPs had an average encapsulation efficiency up to 90% and further demonstrated a biphasic ROD-123 release pattern quite

Table 1
Formula for optimized VLF loaded chitosan NPs.

Drug: polymer ratio	Conc. of CS (mg/ml)	Conc. of TPP (mg/ml)	Mean particle size[a] (nm) ± S.D.	Mean PDI[a] ± S.D.	Percent yield[a] ± S.D.	LC[a] (%) ± S.D.	Zeta potential[a] (mV) ± S.D.	EE[a] (%) ± S.D.
1:1	1.75	2	167 ± 6.5	0.367 ± 0.045	71.42 ± 3.24	32.25 ± 1.63	+23.83 ± 1.76	79.3 ± 2.6

[a] $n = 3$.

Table 2
Effect of VLF chitosan NPs on modified FST and locomotor activity in depressed rats.

Group no.	Treatment	Parameters			
		Swimming time (s)	Climbing time (s)	Immobility time (s)	Locomotor counts
1	Naive (No treatment)	102.38 ± 1.319**	123.48 ± 2.263**	74.14 ± 4.868**	310.33 ± 2.103**
2	Control (i.n.)	42.49 ± 1.611##	68.74 ± 1.845##	188.78 ± 2.284##	82.66 ± 3.383##
3	VLF tablets (Oral)	88.93 ± 1.586**	71.95 ± 2.342 (ns)	139.12 ± 1.718*	92.33 ± 3.480 (ns)
4	VLF solution (i.n.)	82.67 ± 2.079**	73.37 ± 2.057 (ns)	143.96 ± 3.75*	103.67 ± 4.485**
5	VLF chitosan NPs (i.n.)	99.81 ± 1.546**	78.21 ± 1.923*	121.98 ± 0.456**	136.33 ± 6.11**

Values are mean ± standard error (S.E.M.), *$P < 0.05$ vs control, **$P < 0.01$ vs control. ##$P < 0.01$ vs naive, *$P < 0.05$ results are significant, **$P < 0.01$ results are highly significant and $P > 0.05$ results are non significant (ns).

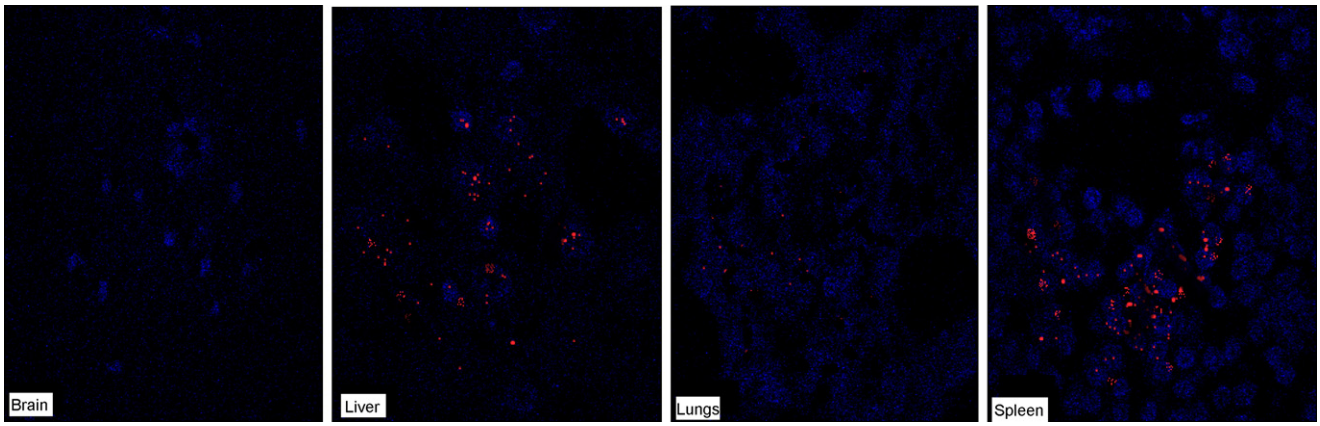

Fig. 1. CLSM images of brain, lungs, liver and spleen at 20 min after administration of VLF chitosan NPs (i.v.). (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

similar to VLF chitosan NPs accounting for 28.6% drug release in 2 h followed by slow release for over 24 h in phosphate buffer (pH 7.4). After the *in vivo* administration of these NPs, the brain without the arachnoid membrane and the meningeal vessels, the liver, the spleen and the lungs of the rats were removed. Fig. 1 shows the biodistribution of ROD-123 loaded chitosan NPs at 20 min after the i.v. administration. The red spots shown in Fig. 1 clearly indicate a regional distribution of the ROD-123 embedded NPs, which is confined mainly to the hepatic and the splenic parenchyma.

In fact, after 20 min, ROD-123 chitosan NPs (i.v.) was confined to a great extent in the liver and the spleen, confirming the results previously obtained (Moghimi, Hunter, & Murray, 2001). On the other hand ROD-123 loaded chitosan NPs when administered by i.n. route showed little florescence in lungs and no florescence in hepatic and the splenic parenchyma at 20 min (Fig. 2). In order to determine the distribution and localization of ROD-123 chitosan NPs qualitatively in the brain regions, several CLSM studies were carried out on microtomed sections for the evaluation of 3D images. At 20 min ROD-123 loaded NPs were present not only in both the right and left encephalon but also in the frontal section and in the cerebellum, when administered by i.n. route. The numbers of red spots were comparatively greater at 60 and 120 min as ROD-123 chitosan NPs (i.n.) were found to be strongly present in the different macro areas of brain parenchyma (Fig. 3). The CLSM images displayed the presence of ROD-123 NPs very close to the cerebral nuclei of brain cells (blue fluorescence due to DAPI) and, in some cases, located into the cytoplasm. CLSM studies revealed no florescence activity in brain macroareas at 20 min after administration of ROD-123 loaded CS NPs by i.v. route (Fig. 1). However at 120 min florescence activity were observed in microtomes brain tissues (Fig. 4). Comparing the florescence activities in lungs, it was found that the florescence activity were greater at all time

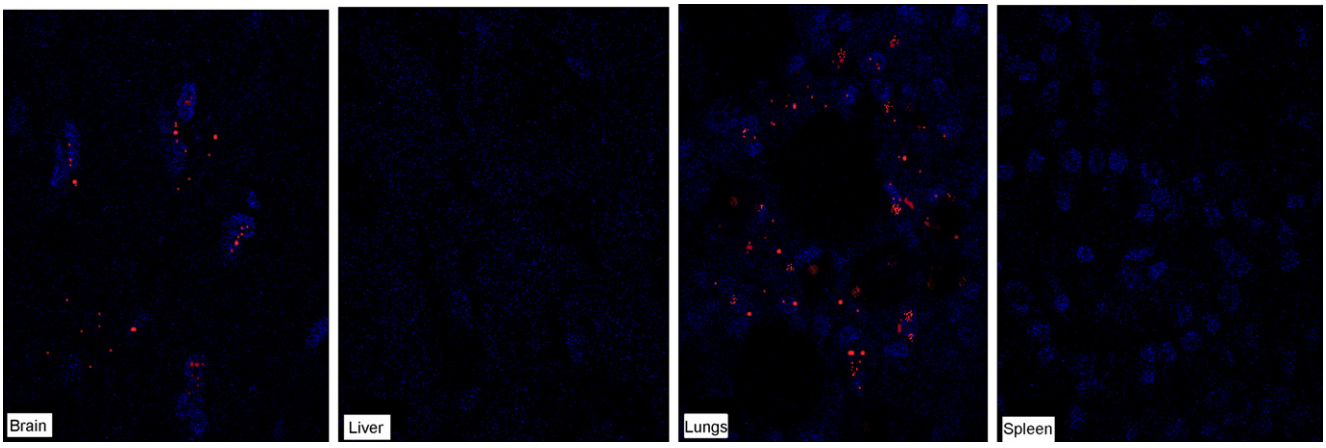

Fig. 2. CLSM images of brain, lungs, liver and spleen at 20 min after administration of VLF chitosan NPs (i.n.).

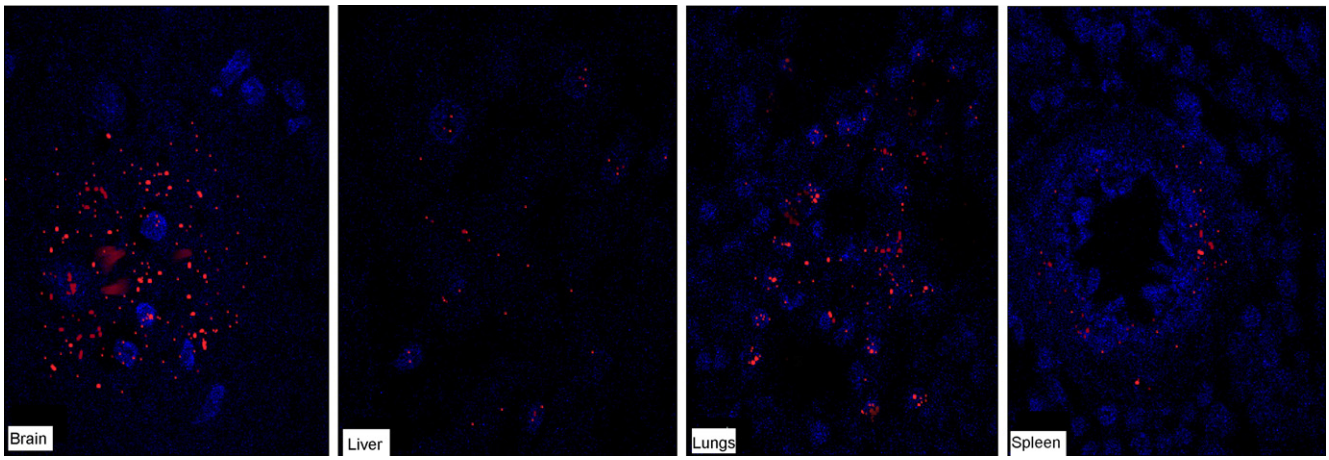

Fig. 3. CLSM images of brain, lungs, liver and spleen at 120 min after administration of VLF chitosan NPs (i.n.). (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

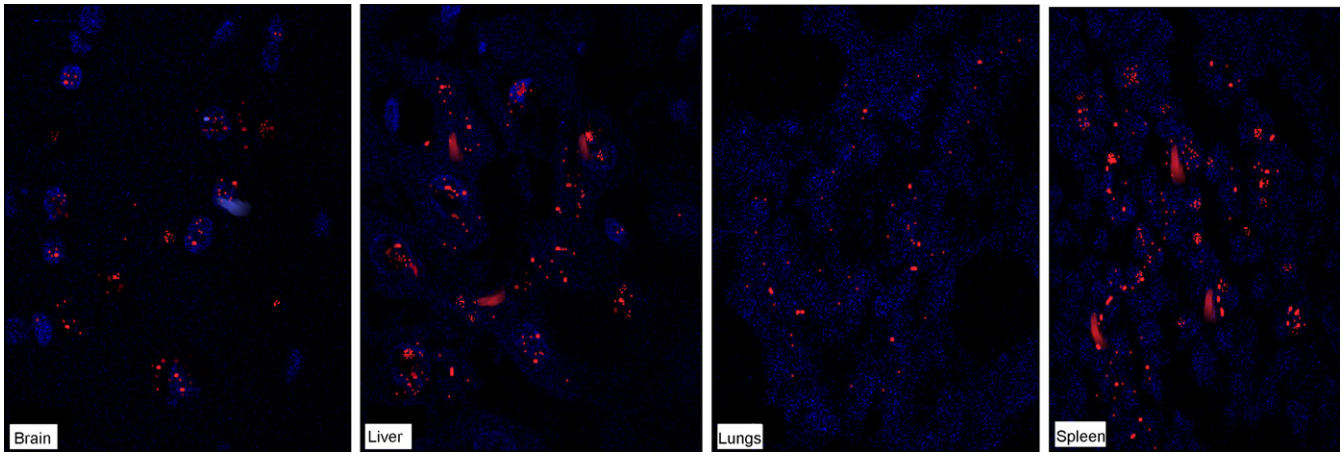

Fig. 4. CLSM images of brain, lungs, liver and spleen at 120 min after administration of VLF chitosan NPs (i.v.).

points in lungs for ROD-123 chitosan NPs when given by i.n. route than i.v. route (Figs. 1–4). This confirms the previous reported research that intranasal route not only impart direct nose to brain transportation but also provides for systemic delivery of drugs. Nose to systemic transportation can be prevented by the use of vasoconstrictor like phenylephrine hydrochloride which enhances intranasal drug delivery to the CNS by limiting absorption into the systemic circulation and increasing the amount of drug available for direct transport into the CNS by olfactory pathways (Dhuria, Hanson, & Frey II, 2009). Nose to brain transportation can also be enhanced by fabrication of some special delivery devices which deliver the mucoadhesive formulation to olfactory region, present on roof of nasal cavity, rather than the respiratory regions of nasal cavity which transport the drug to systemic circulation. Free ROD-123 is not able to cross the BBB (Lange et al., 1998) and also ROD 123 is a substrate for P-gp efflux transporters which are strongly present at BBB (Werle & Hoffer, 2006; Schmitz, Hombach, & Bernkop-Schnürch, 2008). These transporters actively pump out ROD-123 and prevent its entry across BBB to brain. However ROD-123 encapsulated in CS NPs enters the brain, evident from the florescence activity recorded in different macroareas of brain from 20 min onwards. The possible reason behind the presence of florescence in brain is the chitosan NPs, which modulated the P-gp efflux transporters and carried the encapsulated ROD-123 across the BBB. This reason is further validated by the fact that even after 2 h 71% of ROD-123 was still encapsulated in chitosan NPs as demonstrated by its *in vitro* release studies. ROD-123 chitosan NPs (i.v.) were not able to bypass the liver and spleen uptake, therefore the brain macroareas showed no florescence at first 20 min. Also, the florescence activities after administration of ROD-123 chitosan NPs (i.v.) were comparatively less at 60 and 120 min than ROD 123 chitosan NPs (i.n.) as the majority of ROD-123 NPs were confined to liver and spleen. ROD-123 chitosan NPs (i.n.) reached the brain by unique olfactory pathway circumventing the BBB. There are three likely mechanisms underlying the direct nose-to-brain drug delivery, two extracellular transport-mediated routes and one intracellular transport-mediated route (Illum, 2000; Jin, Xie, & Childs, 2003; Thorne et al., 2004). These two likely extracellular transport-based routes can underlie the rapid entrance of drugs into the brain, which can occur within minutes of intranasal drug administration (Dhanda et al., 2005; Thorne et al., 2004). In the first extracellular transport-based route, intranasally administered substances first cross the gaps between the olfactory neurons in the olfactory epithelium, which are subsequently transported into the olfactory bulb. In the second extracellular transport-based route, intranasally administered substances are transported along the trigeminal nerve to bypass the BBB (Dhanda et al., 2005; Thorne et al., 2004). After reaching the olfactory bulb or trigeminal region the substances may enter into other brain regions by diffusion, which is also facilitated by a 'perivascular pump' that is driven by arterial pulsation. Intracellular transport-based route is a relatively slow process, taking hours for

Table 3
Compartmental distribution of VLF chitosan NPs (i.n.), VLF solution (i.n.) and VLF solution i.v. at different time intervals in normal Male Wistar rats.

Formulation	Organ/tissue*	15 min (ng/ml)	30 min (ng/ml)	60 min (ng/ml)	120 min (ng/ml)	480 min (ng/ml)
VLF (i.v.)	Brain	187.62 ± 21.93	382.91 ± 28.54	302.22 ± 29.02	243.73 ± 23.33	88.81 ± 19.33
	Blood	16265.67 ± 2445.13	13035.34 ± 2000.73	10840.76 ± 2126.34	6980 ± 1772.76	4171.12 ± 2047.67
VLF (i.n.)	Brain	98.34 ± 22.80	273.82 ± 34.98	396.97 ± 29.17	264.5 ± 24.88	105.88 ± 23.03
	Blood	1920.33 ± 261.36	3909.67 ± 118.83	3168.34 ± 183.64	3016.82 ± 153.75	2183.33 ± 156.68
VLF chitosan NPs (i.n.)	Brain	200.79 ± 25.25	383.37 ± 26.95	926.05 ± 32.79	783.56 ± 30.75	449.49 ± 26.63
	Blood	1948 ± 174.69	2377.62 ± 195.79	4283.72 ± 195.52	5781.33 ± 200.24	2960 ± 196.10
VLF (i.v.)	Brain/blood	0.0115 ± 0.0023	0.0293 ± 0.0033	0.0278 ± 0.0057	0.0349 ± 0.0024	0.0212 ± 0.0047
VLF (i.n.)	Brain/blood	0.0512 ± 0.0056	0.0700 ± 0.0067	0.1252 ± 0.035	0.0876 ± 0.0016	0.0484 ± 0.0051
VLF chitosan NPs (i.n.)	Brain/blood	0.1030 ± 0.037	0.1612 ± 0.062	0.2161 ± 0.042	0.1356 ± 0.034	0.1516 ± 0.0029

* $n = 3$.

Table 4
Pharmacokinetic parameters of VLF (i.v.), VLF (i.n.) and VLF chitosan NPs (i.n.).

Formulation	Organ/tissue*	C_{max} (ng/ml)	T_{max} (min)	$AUC_{0-480min}$ (ng/ml min)	K_e (h^{-1})	$T_{1/2}$ (min)
VLF (i.v.)	Brain	382.91 ± 28.54	30	90791.63 ± 10491.23	0.1523 ± 0.016	275.1733 ± 30.161
	Blood	16265.67 ± 2445.13	15	3119723 ± 899900.4	0.1654 ± 0.050	266.9334 ± 77.233
VLF (i.n.)	Brain	396.97 ± 29.17	60	99365.55 ± 11640.9	0.0898 ± 0.009	466.1726 ± 47.345
	Blood	3909.67 ± 118.83	30	1271477 ± 73387.58	0.1387 ± 0.180	1223.186 ± 29.713
VLF chitosan NPs (i.n.)	Brain	926.05 ± 32.79	60	297259.8 ± 13522.2	0.0198 ± 0.003	2134.04 ± 315.422
	Blood	5781.33 ± 200.24	120	2007753 ± 91862.25	0.0134 ± 0.0009	3145.8 ± 229.286

* $n = 3$.

intranasally administered substances to reach the olfactory bulb by processes such as endocytosis. Appearance of florescence activity in frontal, left and right encephalon as well as cerebellum within 20 min substantiates the fact that extracellular delivery (time frame 10–15 min) is the main route for intranasal therapeutics to reach the brain from the nasal mucosa (Jin et al., 2003).

3.5. Evaluation of brain uptake and in vivo pharmacokinetic studies

Brain uptake studies following VLF (i.v.), VLF chitosan NPs (i.n.) and VLF (i.n.) on male Wistar rats were performed and the drug concentration in blood and brain were estimated at different intervals up to 8 h (Table 3). Following i.v. administration, VLF attained a high concentration of 16 ± 2 μg/ml in blood plasma, and then declined quickly for 120 min, after which the decline became slow. In the first 15 min drug plasma concentrations obtained with VLF chitosan NPs (i.n.) and VLF (i.n.) were lower than those of VLF (i.v.). The elimination half life of VLF (i.v.) was found to be 266.93 and 275.17 min, respectively, in blood and brain. VLF CS NPs (i.n.) were found to enhance the elimination half life of VLF significantly ($P < 0.05$) in both blood and brain. The rate of elimination of VLF chitosan NPs (i.n.) was much lower than VLF (i.n.) and VLF (i.v.) (Table 4). Following i.n. administration, VLF chitosan NPs (i.n.) enhanced the nasal absorption of VLF to a greater extent than VLF (i.n.) ($P < 0.05$). Table 4 shows the calculated pharmacokinetic parameters for the VLF (i.v), VLF (i.n) and VLF chitosan NPs (i.n). The peak plasma concentrations of VLF in brain after VLF (i.v.), VLF (i.n.) and VLF chitosan NPs were found to be 382.91 ± 28.54, 396.97 ± 29.17 and 926.05 ± 32.79 ng/ml with T_{max} of 30, 60 and 60 min, respectively (Table 4). The lower T_{max} values for brain (1 h) when compared to blood (2 h) may also be attributed to preferential nose to brain transport following i.n. administration of VLF chitosan NPs. The brain–blood ratio of the drug at all sampling time points for different formulations was also calculated and is recorded in Table 3. The concentrations of VLF in brain following the VLF chitosan NPs (i.n.) were found to be significantly higher at almost all time points than VLF (i.n.) and VLF (i.v.) ($P < 0.05$). The brain/blood ratios of VLF for VLF (i.v.), VLF (i.n.), VLF chitosan NPs (i.n.) were 0.0293, 0.0700 and 0.1612, respectively, at 0.5 h are indicative of direct nose to brain transport bypassing the BBB, hence proving the superiority of VLF chitosan NPs for nose to brain delivery of VLF. When the C_{max} and AUC of brain concentration of the VLF (i.v), VLF (i.n) and VLF chitosan NPs (i.n) were compared, the C_{max} (926.05 ng/ml) and AUC (297.2 μg/ml min) of VLF chitosan NPs (i.n) were found to be significantly higher than VLF (i.v.) and VLF (i.n.) ($P < 0.05$). The nasal administration of VLF CS NPs significantly enhanced the VLF concentration in brain tissue when compared with same dose by VLF (i.n.) or VLF (i.v.). The possible reason could be the contribution of paracellular transport through tight junctions as chitosan NPs not only open the tight junctions between the cells but also decrease the mucociliary clearance, which under normal circumstances rapidly clears the instilled formulation (Artursson and Lindmark, 1994; Dyer and Hinchcliffe, 2002). Reported studies have proved that the chitosan NPs transiently open the tight junctions because of an interaction of CS with the Protein Kinase C pathway (Smith & Dornish, 2005). The DTP% and DTE% represent the percentage of drug directly transported to the brain via the olfactory pathway. DTP% and DTE% were calculated using tissue/organ distribution data following intranasal and intravenous administration. VLF loaded chitosan NPs showed the highest DTE% (508.59) and DTP% (80.34) amongst the three tested formulations. Nearly two-fold higher DTE% of VLF chitosan NPs (i.n.) compared to VLF (i.n.) proves the benefit of mucoadhesive nanoparticulate formulation. The higher DTE% and DTP% substantiate that VLF chitosan NPs have better brain targeting efficiency. These findings are also in concurrence with previous reported results that mucoadhesive nano-formulations increase nose to brain uptake of drugs (Al-Ghananeem et al., 2010; Kumar et al., 2008a).

4. Conclusion

Mucoadhesive venlafaxine loaded chitosan NPs enhanced the uptake of venlafaxine to the brain when delivered by intranasal route. Significant quantity of venlafaxine was quickly and effectively delivered to the brain by intranasal administration of mucoadhesive NPs of venlafaxine. On the basis of these research findings, it was concluded that venlafaxine loaded chitosan NPs could be an efficacious drug delivery system for the treatment of depression. The study, however, still requires significant clinical data to evaluate its efficacy in human and its risk/benefit ratio.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

The authors are grateful to All India Council for Technical Education (AICTE) Government of India for providing fellowship to Shadabul Haque as financial assistance and, are also grateful to Department of Science and Technology (DST), New Delhi for providing DST INSPIRE Fellowship to Shadab Md as financial assistance.

References

Al-Ghananeem, A. M., Saeed, H., Florence, R., Yokel, R. A., & Malkawi, A. H. (2010). Intranasal drug delivery of didanosine-loaded chitosan NPs for brain targeting; an attractive route against infections caused by aids viruses. *Journal of Drug Targeting*, *18*, 381–388.

Ali, J., Ali, M., & Baboota, S. (2010). Potential of nanoparticulate drug delivery systems by intranasal administration. *Current Pharmaceutical Design*, *10*, 1644–1653.

Artursson, P., & Lindmark, T. (1994). Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharmaceutical Research*, *11*(9), 1358–1361.

Calvo, P., Remunan-Lopez, C., Vila-Jata, J. L., & Alonso, M. J. (1997). Chitosan and chitosan: Ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. *Pharmaceutical Research*, *14*, 1431–1436.

Chenite, A., Gori, S., Shive, M., Desrosiers, E., & Buschmann, D. (2006). Monolithic gelation of chitosan solutions via enzymatic hydrolysis of urea. *Carbohydrate Polymers*, *64*, 419–424.

Cryan, J. F., Markou, A., & Lucki, I. (2002). Assessing antidepressant activity in rodents: Recent developments and future needs. *Trends in Pharmacological Science*, *23*, 238–245.

Davis, S. S. (1997). Biomedical applications of nanotechnology – Implications for drug targeting and gene therapy. *Trends in Biotechnology*, *15*, 217–224.

Dhanda, D., Frey, W. H., II, Leopold, D., & Kompella, U. B. (2005). Nose to brain delivery: Approaches for drug deposition in the human olfactory epithelium. *Drug Delivery Technology*, *5*(4), 64–72.

Dhuria, S. V., Hanson, L. R., & Frey, W. H., II. (2009). Novel vasoconstrictor formulation to enhance intranasal targeting of neuropeptide therapeutics to the central nervous system. *The Journal of Pharmacology and Experimental Therapeutics*, *328*(1), 312–320.

Dyer, A. M., & Hinchcliffe, M. (2002). Nasal delivery of insulin using novel chitosan based formulations: A comparative study in two animal models between simple chitosan formulations and chitosan NPs. *Pharmaceutical Research*, *19*(7), 998–1008.

Genta, I., Perugini, P., Modena, T., Pavanetto, F., Castelli, F., Muzzarelli, R. A. A., et al. (2003). Myconazole-loaded 6-oxychitin-chitosan microcapsules. *Carbohydrate Polymers*, *52*, 11–18.

Illum, L. (2000). Transport of drugs from the nasal cavity to central nervous system. *European Journal of Pharmaceutical Sciences*, *11*, 1–18.

Jin, K., Xie, L., & Childs, J. (2003). Cerebral neurogenesis is induced by intranasal administration of growth factors. *Annals of Neurology*, *53*, 405–409.

Khan, S., Patil, K., Bobade, N., Yeole, P., & Gaikwad, R. (2010). Formulation of intranasal mucoadhesive temperature-mediated in situ gel containing ropinirole and evaluation of brain targeting efficiency in rats. *Journal of Drug Targeting*, *18*, 223–234.

Khan, S., Patil, K., Yeole, P., & Gaikwad, R. (2009). Brain targeting studies on buspirone hydrochloride after intranasal administration of mucoadhesive formulation in rats. *Journal of Pharmacy and Pharmacology*, *61*, 669–675.

Kilts, C. D. (2003). Potential new drug delivery system for antidepressants: An overview. *Journal of Clinical Psychiatry*, *64*, 31–33.

Kumar, M., Misra, A., Babbar, A. K., Mishra, A. K., Mishra, P., & Pathak, K. (2008). Intranasal nanoemulsion based brain targeting drug delivery system of risperidone. *International Journal of Pharmaceutics*, *358*, 285–291.

Kumar, M., Misra, A., Mishra, A. K., Mishra, P., & Pathak, K. (2008). Mucoadhesive nanoemulsion-based intranasal drug delivery system of olanzapine for brain targeting. *Journal of Drug Targeting*, *16*, 806–814.

Lange, E. C., Bock, G. D., Shinkel, A. H., Boer, A. G., & Breimer, D. D. (1998). BBB transport using MDR 1A (−/−) and wild-type mice. Total brain versus microdialysis concentration profiles of Rhodamine-123. *Pharmaceutical Research*, *15*, 1657–1665.

Moghimi, S. M., Hunter, A. C., & Murray, J. C. (2001). Long-circulating and target specific NPs: Theory to practice. *Pharmacological Reviews*, *53*, 283–318.

Pires, A., Fortuna, A., & Alves, G. (2009). Intranasal drug delivery: How, why and what for. *Journal of Pharmacy and Pharmaceutical Science*, *12*, 288–311.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: A primary screening tests for antidepressants. *Archives Internationales de Pharmacodynamie et de Thérapie*, *229*, 327–336.

Schmitz, T., Hombach, J., & Bernkop-Schnürch, A. (2008). Chitosan-N-acetyl cysteine conjugates: *In vitro* evaluation of permeation enhancing and P-glycoprotein inhibiting properties. *Drug Delivery*, *15*, 245–252.

Sintov, A. C., Levy, H. V., & Botner, S. (2010). Systemic delivery of insulin via the nasal route using a new microemulsion system: *In vitro* and *in vivo* studies. *Journal of Controlled Release*, *148*, 168–176.

Smith, J. M., & Dornish, M. (2005). Involvement of protein kinase C in chitosan glutamate-mediated tight junction disruption. *Biomaterials*, *26*(16), 3269–3276.

Subramanium, G. S., Arumugam, K., Reddy, M. S., & Udupa, N. (2008). A study of rivastigmine liposomes for delivery into the brain through intransal route. *Acta Pharmaceutica*, *58*, 287–297.

Stahl, S. M., Grady, M. M., Moret, C., & Briley, M. (2005). SNRIs: Their pharmacology clinical efficacy and tolerability in comparison with other classes of antidepressants. *CNS Spectrum*, *10*, 732–747.

Thorne, R. G., Pronk, G. J., Padmanavan, V., & Frey, W. H., II. (2004). Delivery of insulin like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intransal administration. *Neuroscience*, *127*, 481–496.

Tosi, G., Costantino, L., Rivasi, F., Ruozi, B., Leo, E., Vergoni, A. V., et al. (2007). Targeting the central nervous system: *In vivo* experiments with peptide-derivatized NPs loaded with loperamide and rhodamine-123. *Journal of Controlled Release*, *122*, 1–9.

Troy, S. M., Parekar, V. D., Fruncillo, R. J., & Chiang, S. T. (1995). The pharmacokinetics of venlafaxine when given in a twice daily regimen. *Journal of Clinical Pharmacology*, *35*, 404–409.

Vergoni, A. N., Tosi, G., Tacchi, R., Vandelli, M. A., Bertolini, A., & Costantino, L. (2009). NPs as drug delivery agents specific for CNS: *In vivo* biodistribution. *Nanomedicine: Nanotechnology, Biology and Medicine*, *5*, 369–377.

Vllasaliu, D., Harris, R. E., Heras, A., Casettaria, L., Garnetta, M., Illum, L., et al. (2010). Tight junction modulation by chitosan NPs: Comparison with chitosan solution. *International Journal of Pharmaceutics*, *400*, 183–193.

Werle, M., & Hoffer, M. (2006). Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine. *Journal of Controlled Release*, *111*, 41–46.

Wilson, A. D., Howell, C., & Waring, W. S. (2007). Venlafaxine ingestion is associated with rhabdomyolysis in adults: A case series. *Journal of Toxicological Sciences*, *32*, 97–101.