Development and Characterization of Levodopa Loaded Pharmacosomes for Brain Targeting via Intranasal Route: Pharmacodynamic Evaluation in Rats

Yashaswini Kotha, Anil Goud Kandhula, Krishnaveni Janapareddi*

Department of Pharmaceutics, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Telangana, INDIA.

ABSTRACT

Parkinson's is the second most common progressive neurodegenerative disease and affects 1-2% people over the age 50. Levodopa is the drug of choice in the treatment of Parkinson's disease and exhibits low oral bioavailability (30%) and very low brain uptake. In an attempt to improve brain uptake and to avoid degradation of levodopa in peripheral circulation, brain targeting of levodopa loaded pharmacosomes via nasal route has been investigated. Pharmacosomes are colloidal dispersions of drug covalently bound to lipids and may exist as ultrafine vesicular, micellar or hexagonal aggregates, depending on the chemical structure of the drug-lipid complex. Pharmacosomes loaded with levodopa were prepared by solvent evaporation method and the optimized formulation contained levodopa and egg lecithin in the ratio of 1:3. The mean globule size, PDI, zeta potential, drug content, entrapment efficiency and drug release of formulation (F5) were 123.2nm, 0.211, -29.1mV, 96%, 99.97% and 62.1% respectively. Permeation enhancer, chitosan was incorporated at 0.5% concentration to optimal formulation (F7). The mean globule size, PDI, zeta potential, drug content, entrapment efficiency and drug release of optimised formulation (F7) were 125.5nm, 0.115, +33.7mV, 96.5%, 99.98% and 91.2% respectively. Formation of covalent bond between levodopa and lecithin was confirmed by FTIR spectra showing peak at 1639.9 cm^{-1}. *Ex-vivo* permeation studies using Franz diffusion cell on porcine nasal mucosa showed flux of 96.61 µg/cm²/h and 302µg/cm²/h. The steady state flux of F5 and F7 formulations was significantly high ($P<0.001$) and enhancement ratio was 2.26 and 3.12 times respectively compared to drug solution. Anti-parkinson's activity of optimized formulation (F7), drug solution via nasal route was compared with drug solution administered orally, in rotenone induced male wistar rats. Group treated with optimized pharmacosomes formulation (F7) showed significant recovery in rat weight (43%), locomotor activity by photo actometer (90%) and grip strength on retard (65%) compared to drug solution treated orally showed recovery in rat weight (32%), locomotor activity by photo actometer (57%) and grip strength on rotarod(30%). Biochemical parameters measured in brain homogenate showed significant reduction in induced levels of nitric oxide (82%), total protein (88%) and lipid peroxides (73%) and significant increase in reduced Glutathione (67%). Whereas drug solution treated via orally showed significantly less activity in the reduction of nitric oxide (28%), total protein (59%) and lipid peroxidation (33%) and reduced glutathione (10%).

Key words: Brain targeting, Levodopa, Nasal delivery, Parkinson's disease, Pharmacosomes.

Correspondence

Dr. Janapareddi Krishnaveni,

Assistant Professor, Department of Pharmaceutics, Kakatiya University, Warangal-506009. Telangana, INDIA.

Phone: +91 9247161127

Email: krishnaveni.janapareddi@gmail.com

INTRODUCTION

Parkinson's is the second most common progressive neurodegenerative disease and affects 1-2% people over the age 50. Levodopa is the drug of choice in the treatment of Parkinson's disease and exhibits low oral bioavailability (30%) and very low brain uptake.Currently, so many drugs and conventional drug delivery methods have been developed for CNS diseases but they failed in showing the concentration required for action at targeted site i.e. brain due to BBB (Blood Brain Barrier) and BCB (Blood Cerebrospinal fluid Barrier).[1,2] Two different approaches currently used to deliver therapeutics into the brain bypasses the BBB are: *Invasive and Non-Invasive methods* including BBB disruption, nasal delivery or colloidal drug carriers.[3,4] Researches reported a direct transport of drugs from the nose to the brain via olfactory region. Uptake by the olfactory epithelium and transfer via the olfactory bulb into brain parenchyma; the nerve cells of the olfactory epithelium project into the olfactory bulb of the brain, which provides a direct connections between brain and the external environment.[5-12]

Pharmacosomes, bearing unique advantages over liposome and niosomes could serve as a potential alternative to conventional vesicles. These are colloidal dispersions of drug covalently bound to lipids and may exist as ultrafine vesicular, micellar or hexagonal aggregates, depending on the chemical structure of the drug-lipid complex.[13] As the system is formed by linking a drug (pharmakon) to a carrier (soma), they are termed as "Pharmacosomes". Pharmacosomes are prepared out of natural lipids, which are interesting candidates for brain targeting, due to rapid uptake by the brain, bioacceptability, biodegradability and less toxicity compared to the polymeric nanoparticles. Any drug possessing an active hydrogen atom (-COOH, -OH, -NH², etc.) can be esterified to the lipid, with or without spacer chain that strongly result in an amphiphilic compound, which will facilitate membrane, tissue, or biological cell wall.[14,15]

Chitosan has been investigated for particulate delivery to brain for various drugs. Chitosan is a linear polysaccharide which is derived from naturally occurring chitin by deactivation with exposed amino group responsible for rendering the polymer cationic. This property in addition to its mucoadhesive nature and its influence on the permeability of epithelial membranes.[16-19]

In an attempt to improve brain uptake and to avoid degradation of levodopa in peripheral circulation, brain targeting of levodopa loaded pharmacosomes via nasal route has been investigated. The prepared

pharmacosomes were optimized for formulation parameters to obtain desired mean globule size, PDI, zeta potential, drug content, entrapment efficiency and drug release. *Ex-vivo* permeation studies using Franz diffusion cell on porcine nasal mucosa PBS buffer pH 6.4 were carried out. *In vivo* studies in rat model were carried out to account for the recovery of the drug in brain following intranasal administration of optimized formulation (F7), drug solution via nasal route was compared with drug solution administered orally, in rotenone induced male wistar rats. The results were determined and compared with the plain drug solution.

Materials

Levodopa was a gift sample from glochem private limited, egg lechitin (Tokyo chemical industry, Japan), tetrahydrofuran and low molecular weight chitosan were purchased from sigma Aldrich. Water used was ultrapure grade Millipore. All other reagents were of analytical reagent grade.

Animals

Male Wistar rats, weighing between 300 and 350 g were obtained from Sainadh Agencies, Hyderabad, India, after getting approval from "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA) and Institutional Animal Ethics Committee (IAEC), Kakatiya University (KU). The approval number IAEC/31/UCPSc/KU/2019. Animals were provided standard laboratory diet, water *ad libitum* and acclimatized to laboratory conditions (22 ± 2°C, 12-h light–dark cycle and 55–65% humidity) 1 week prior to initiation of experiments.

MATERIALS AND METHODS

Drug characterization by FT-IR

Pure drug, phospholipid and optimised formulation were analysed by FTIR (Bruker FT-IR Tensor 27) spectroscopy with KBr disc method. The spectrum was obtained at a resolution of 4 cm^{-1} between frequency range of 4000–400cm^{-1}.

Estimation by HPLC

The HPLC equiped with Pump, LC 20-AD Prominence; Detector, UV-Visible SPD-20A Prominence was used for the estimation. The column used was Reverse Phase C_{18} column, Phenomenix (250×4.6 mm, 5 μm size). The mobile phase was composed of methanol, 0.5% acetic acid in the ratio of 70:30 v/v. The injection volume was 20 μL. The detection wave length was 284 nm.[20]

Different dilutions were made with mobile phase to obtain 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5μg/ml with mobile phase. The solutions were injected into HPLC column. The standard graph was plotted based on peak area from the obtained chromatogram on Y-axis and Drug concentration on x-axis. (Peak area Vs concentration).

Preparation of pharmacosomes

Pharmacosomes were prepared by solvent evaporation method. Accurately weighed amounts of drug and lipids were dissolved in volatile organic solvent such as tetrahydrofuran in a round bottom flask. The organic solvent was evaporated at room temperature using a rotary flash evaporator which leaves a thin film of solid mixture deposited on walls of flask. The dried film was then hydrated with distilled water to form vesicles (pharmacosomes) embedded with levodopa.[21] The composition of various pharmacosomes of Levodopa was given in Table 1.

Table 1: Composition of various pharmacosomes of levodopa.

Formulations	Drug (mg)	Egg Lecithin (mg)	Tetrahydrofuran (ml)	Water (ml)	Chitosan (%)
F1	75	75	5	5	-
F2	75	112.5	5	5	-
F3	75	150	5	5	-
F4	75	187.5	5	5	-
F5	75	225	5	5	-
F6	75	225	5	5	0.25
F7	75	225	5	5	0.50
F8	75	225	5	5	0.75
F9	75	187.5	5	5	0.50

Characterization of pharmacosomes

Mean globule size, poly dispersity index (PDI) and zeta potential

The mean globule size analysis, PDI and zeta potential of formulation were determined using Zeta sizer (Nano-ZS 90, Malvern instruments Ltd.UK) on 100 times diluted sample.[22,23]

Drug content

Accurately weighed amount of pharmacosomes were suitably diluted with methanol, sonicated, filtered through 0.45μm filter and analyzed using UV-Visible spectroscopy.[23]

Determination of entrapment efficiency

The free drug in the aqueous phase was determined by separation by ultra-filtration using centrisort tubes. The amount of levodopa in the aqueous phase was estimated by HPLC method.

$$\text{Entrapment efficiency} = \text{Total drug} - \text{Free drug/ Total drug} * 100$$

In vitro drug release study of pharmacosomes

In vitro drug release studies were assessed using Franz diffusion cell. Pharmacosomes formulation equivalent to 15 mg of levodopa was placed in donor compartment separated from receptor compartment with dialysis membrane of molecular weight cutoff 1200-14000 daltons. Aliquots of samples were withdrawn from the receptor compartment at predetermined time intervals and replaced with fresh medium. The samples were then analyzed for drug content by UV-Visible Spectrophotometer at 280 nm.

Ex vivo permeation studies

Ex vivo permeation studies were performed on porcine nasalmucosa using Franz diffusion cells. The isolated porcine nasal mucosa was mounted on franz diffusion cells and allowed to equilibrate for 30 min in PBS pH 6.4 at room temperature. After equilibration the donor chamber was replaced with formulation/drug solution and the receptor was filled with fresh medium.[24,25] Samples were withdrawn at regular intervals up to 8 hrs. And replaced with fresh medium. The samples were analyzed by UV Visible spectrophotometer at 280 nm. The cumulative amount of drug permeated at different time points was calculated using the following formula

$$Q = \left[C_n V + \sum_{i=1}^{n-1} C_i S \right]$$

Where, **Q** = Cumulative amount of drug released; **C_n** = Concentration of drug (μg/mL) determined at n^{th} sampling interval; **V** = Volume of receptor compartment; $n-1\Sigma C_i S$ i =1 = Sum of concentration (μg/ml) determined at sampling points 1 to n-1 multiplied with sampling volume (S).

Determination of steady state flux (J_{ss}), Permeability coefficient (Kp), Enhancement ratio (ER)

The cumulative amount of levodopa permeated through excised porcine nasal mucosa was plotted as a function of time. Steady state flux (μg/cm²/h) was calculated from the linear portion of the plot by regression. The flux at steady-state was calculated as the slope divided by the effective nasal mucosa surface area (cm²). Permeability coefficient (K_p, cm/h) was calculated by dividing flux (J_{ss}/Cd) initial concentration of the drug in the donor cell. Enhancement ratio (ER) was calculated by dividing J_{ss} of the respective formulation by J_{ss} of the drug solution.

Nasal cilio toxicity studies

Nasal mucosa mounted on Franz diffusion cell was treated for 1h with formulation, Drug solution, isopropyl alcohol (positive control) and PBS pH 6.4 (negative control). The mucosa after treatment were rinsed with PBS pH 6.4 and preserved in 10% v/v formalin solution. The mucosal section obtained by microtome technique was stained with hematoxylin-eosin and observed under digital microscope to evaluate the damage to nasal mucosa.[26,27]

Scanning electron microscopy (SEM)

The morphology of optimized pharmacosomes was studied by scanning electron microscope (JSM-6510LA, JEOL and Indonesia). The sample was adhered on to the carbon-coated metallic stub, sputter coated with platinum coating machine (JFC-1600 Auto fine coater, JEOL). Image was carried out under high vaccum.[27]

Stability studies

Stability studies were conducted for optimized formulation at room temperature and refrigerator conditions for two months. Samples were withdrawn at monthly intervals and analyzed for mean globule size, PDI and Zeta potential.

In vivo studies

Male Wistar rats were weighed and randomly divided into five groups, each group consisting of six rats weighing between 300 and 350 g. Group -I: Normal Saline (0.9% w/v of NaCl) Intraperitonially (IP) (10ml/kg). Group II: Positive control -Rotenone - Intraperitonially (3mg/kg). Group III: Rotenone (3mg/kg, 1-11days) + drug solution (oral, 8.8 mg/kg, 12-21days). Group IV: Rotenone (3mg/kg, 1-11days) +drug solution (i.n, 2.5mg/kg, 12-21days). Group V: Rotenone (3mg/kg, 1-11days) + optimized Pharmacosome formulation F7 (i.n, 1.25mg/kg, 12-21days).

The behavioural parameters were recorded on 1st, 12th, 15th, 18th and 21st day. To assessed the anti-Parkinson activities of test formulations. PD induction and progression were assessed by recording changes in weight, locomotor activity by photoactometer and grip strength by rota rod test.

Standard calibration curve of levodopa was prepared in rat brain homogenate was calculated. Rats were sacrificed on 22nd day by cervical dislocation method and brains were isolated. Brain samples were homogenized and immediately centrifuged (10 000 rpm) for 10 min at ambient temperature. After centrifugation, supernatant plasma was transferred into clean, fresh Eppendorf's tube and stored in freezer at
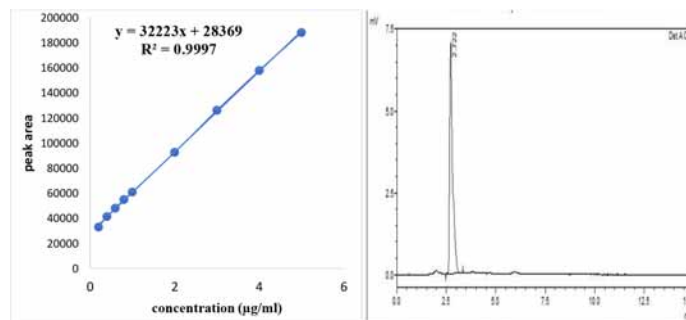

Figure 1: Chromatogram of levodopa showing retention time peak at 2.72 min.

Table 2: Mean globule size, PDI, Zeta Potential, Drug content and Entrapment efficiency of various formulations.

Formulation	Size (nm)	PDI	Zeta Potential (mV)	Drug content (%)	Entrapment Efficiency (%)
F1	237.5±2.7	0.46±0.05	-18.6±1.5	97.2	99.95
F2	216.2±4.3	0.340±0.11	-22.7±0.2	98.8	98.97
F3	199.8±2.8	0.334±0.10	-28.4±1.3	95.6	98.95
F4	145.1±3.6	0.259±0.13	-27.1±0.2	96.8	99.93
F5	123.2±2.9	0.211±0.06	-29.1±0.8	97.0	99.97
F6	128.6±4.3	0.297±0.07	+28.6±0.6	96.8	99.98
F7	125.5±5.2	0.115±0.15	+33.7±0.5	97.5	99.98
F8	126.3±2.7	0.124±0.06	+34.5±1.3	96.3	99.97
F9	140±1.9	0.212±0.11	+31±0.9	96.1	99.95

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

-20ºC to estimate biochemical parameters such as reduced glutathione (GSH), lipid peroxidation, nitric acid level and total protein.[28-32]

RESULTS AND DISCUSSION

Standard graphs of levodopa by HPLC

Dilutions were made as per procedure mentioned above and the chromatogram of levodopa was shown in Figure 1.

Characterization

All the formulations were characterized for mean globule size, poly disparity index and zeta potential, drug content, entrapment efficiency and drug release and results were shown in the Table 2. The mean globule size of formulations was in the range of 123nm to 237nm. The PDI of formulations was in the range of 0.1-0.4 indicating uniform dispersion of globules. Formulations F1 to F5 showed negative zeta potential values ranging -18 to -29mv. The zeta potential values of mucoadhesive pharmacosomes F6, F7, F8 and F9 were positive and in the range of +28.6 to +33.5mV due to the presence of chitosan, a cationic polymer, which imparts positive charge to the globules. The drug entrapment efficiency of all formulations was above 98%. The drug content of different formulations was found to be in the range of 98.92% to 99.99%. *In vitro* drug release was 63.37%.

In- vitro drug release studies

The drug release was measured over a period of 8 hrs in phosphate buffer pH 6.4 by Franz-diffusion cell method. The release profiles shown
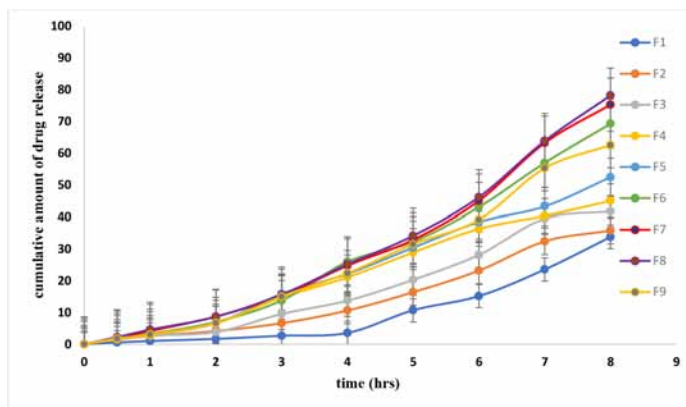
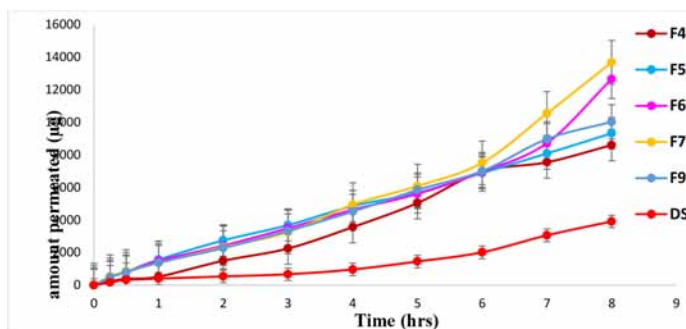**Figure 2:** *In vitro* drug release profiles of pharmacosomes.

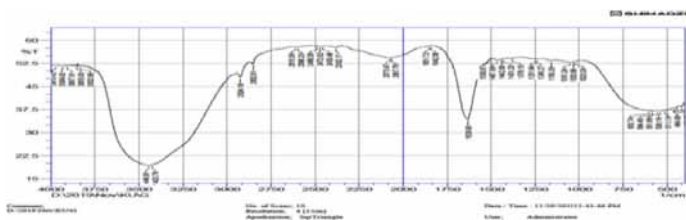**Figure 3:** Amount of drug permeated (µg) from pharmacosomal formulations and drug solution.

Figure 4: FT-IR of optimized formulation F7.

Table 3: Steady state Flux, K_p, ER values of selected formulations and drug solution.

S.no	Formulation Code	Flux (µg/cm²/h) Jss	Kp x10-3 (cm/h)	Enhancement Ratio
1.	F4	199.26± 1.23	13.16	2.06
2.	F5	239.15± 1.05	14.61	2.26
3.	F6	270.38± 0.78	18	2.79
4.	F7	302.13± 2.01	20.1	3.12
5.	F9	255.57±1.57	15.03	2.64
6.	DS	96.61 ± 1.49	6.44	1

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

Table 4: Effect of storage conditions on optimized pharmacosome formulation F7.

Day	Conditions	Mean globule size (nm)	PDI	Zeta potential (mV)
1	Room temperature (25°C)	125.5±5.2	0.115±0.15	+33.7±0.5
	Refrigerated temperature (4°C)	125.5±5.2	0.115±0.15	+33.7±0.5
30	Room temperature (25°C)	128±5.8	0.148±0.27	+33.4±0.84
	Refrigerated temperature (4°C)	127±4.4	0.120±0.21	+33.5±0.73
60	Room temperature (25°C)	133±4.8	0.26±0.37	+32.9±0.92
	Refrigerated temperature (4°C)	129±3.7	0.21±0.23	+33.1±0.48

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

(C=N). Formation of the imine functional group was confirmed by FTIR spectra.

Scanning Electron Microscopy (SEM)

Optimized formulation was subjected to SEM to study surface morphology. The SEM image of F7 formulation as shown in Figure 5 contained spherical shaped globules in group.
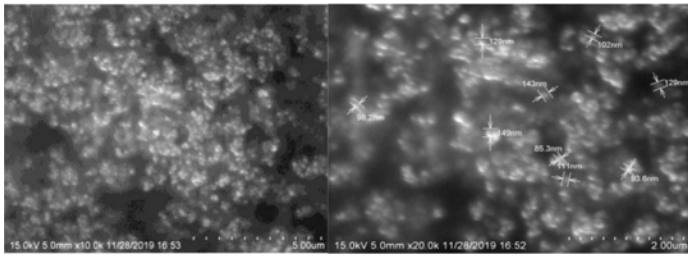
Stability studies

Optimized formulations was subjected for stability studies, stored at room temperature (25°C) and refrigerated temperature (4°C) for 2 months and results were Table 4. There was no significant difference observed in mean globule size and zeta potential but slight increase in PDI was observed.

Nasal toxicity studies

Nasal toxicity studies were conducted and the images of the mucosa stained with eosin and hematoxylin were shown in Figure 6. From the observations of the images, one can conclude that F7 and drug solution were safe without any significant toxicity. The mucosa treated with isopropyl alcohol (positive control) showed complete disruption of epithelial layer and damage of tissue.

in Figure 2. The *in-vitro* drug release showed higher drug release from F7 formulation in 8hrs (75.30%).

Ex vivo permeation studies

The *ex-vivo* permeation profiles of selected formulations and drug solution were shown in Figure 3 and flux values in Table 3. Flux value at steady state was observed between 199.26 to 302.13µg/cm²/hr. Formulation containing chitosan at 0.5%w/w concentration (F7) showed maximum flux 302.13µg/cm²/hr of which was significantly high compared to drug solution 96.61 ± 1.49 µg/cm²/hr. The enhancement ratio of F7 was 3.12 times when compared to drug solution and 2.26 times compared to F5 formulation.

FTIR Spectra of optimized formulation F7

FTIR spectra of optimized formulation F7 showing a peak at 1639.9 cm⁻¹ indicating C=N bond as shown in Figure 4. A covalent bond between levodopa and lecithin of pharmacosomes. Amine group of levodopa reacts with carbonyl groups of lecithin at C_{18} and C23 to give imine

Figure 5: SEM image of optimized formulation F7 (5.0mm x 20.0k magnification).

(a) pH 6.4 PBS (b) isopropyl alcohol

(c) drug solution (d) optimized pharmacosomes F7
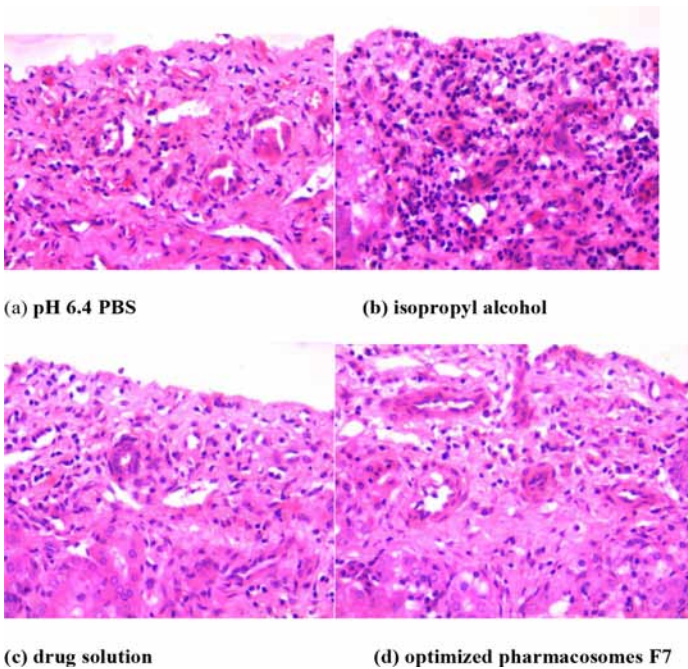
Figure 6: Histopathology images of porcine nasal mucosa (a) pH 6.4 PBS (b) isopropyl alcohol, (c) drug solution, (d) optimized pharmacosome F7.
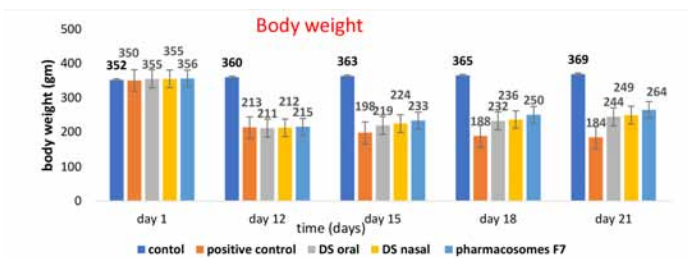

Figure 7: Comparison of body weights of rats administered with optimized formulation F7, DS oral and DS nasal.
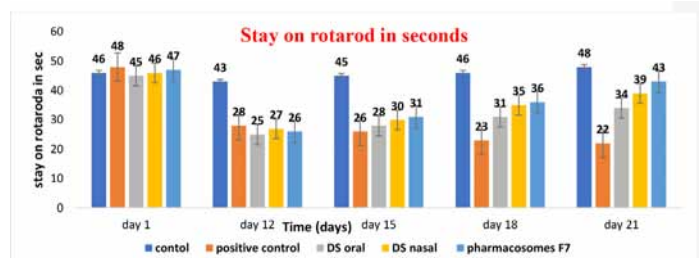Mean±SD (n =6)

PHARMACODYNAMIC STUDY

Estimation of Behavioral Parameter
Pharmacodynamic activity by Rotinone induced model

The results of body weight varation, locomotor activity and grip strength on rotarod were plotted as bar graphs and shown in Figure 7-9 respectively. In all behavioral parameters, control group was considered as 100%. Statistical comparisons were made using GraphPad Prism (viewer mode) 8.0.1.
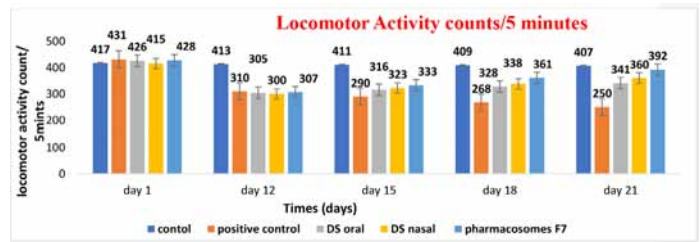
Figure 8: Comparison of locomotor activity of rats administered with optimized formulation F7, DS oral and DS nasal.
Mean±SD (n =6)

Figure 9: Comparison of grip strength of rats on rotarod administered with optimized formulation F7, DS oral and DS nasal.
Mean±SD (n =6)

Body weight

When the treated groups were compared to control, body weight in pharmacosome F7 was restored to 43.2%, DS nasal to 35% DS oral to 32.4%.

Locomotors activity

When all the treated groups were compared to control, locomotors activity in pharmacosome F7 was restored to 90%, DS nasal to 70%, DS oral to 57%.

Grip strength of rat on rotarod

When all the treated groups were compared to control, grip strength on rota rod in pharmacosomes F7 was restored to 65%, DS nasal to 50%, DS oral to 30%.
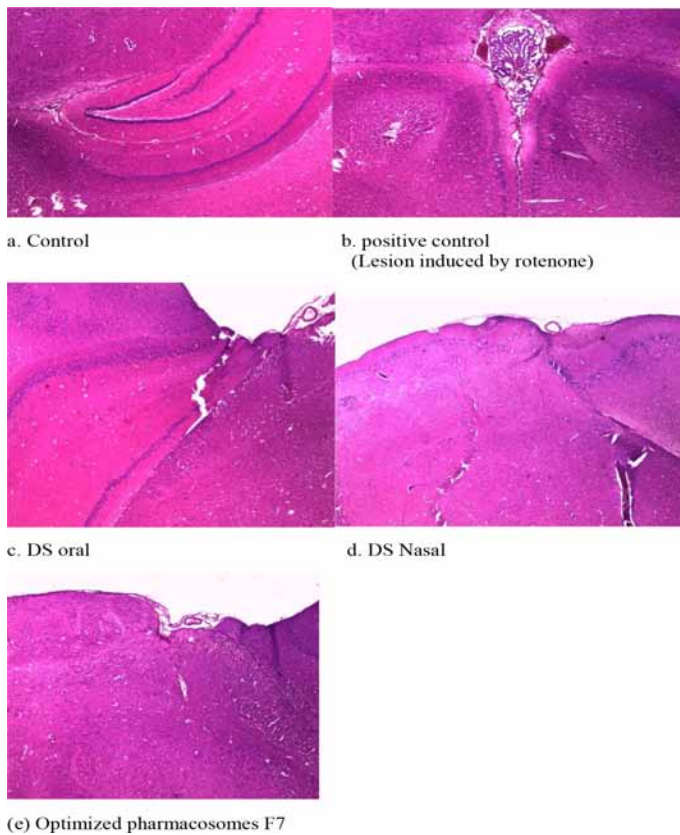
Histopathology of Mid brain of substantial nigra region of rat brain after exposure to 11days of treatment

Histopathology of stained sections from mid brain of substantia nigra region of rat brain after exposure to 11 days of treatment with Formulation, drug solution was Figure 10. The positive control group (rotenone induced) showed necrosis of neurons and lesions. The groups treated with DS oral and DS nasal showed mild lesions whereas optimised formulation F7 treated group not showed any lesions which indicates clear recovery from Parkinson's disease.

Biochemical Parameters

The results of levels of reduced glutathione, nitric oxide, total protein and lipid peroxidation were plotted as bar graphs and shown in Figure 11.

In all biochemical parameters, control group was considered as 100%. Statistical comparisons were made using Graph Pad Prism (viewer mode) 8.0.1.

Figure 10: Histopathology of Mid brain of substantia nigra region of rat brain after exposure to 11days of treatment.
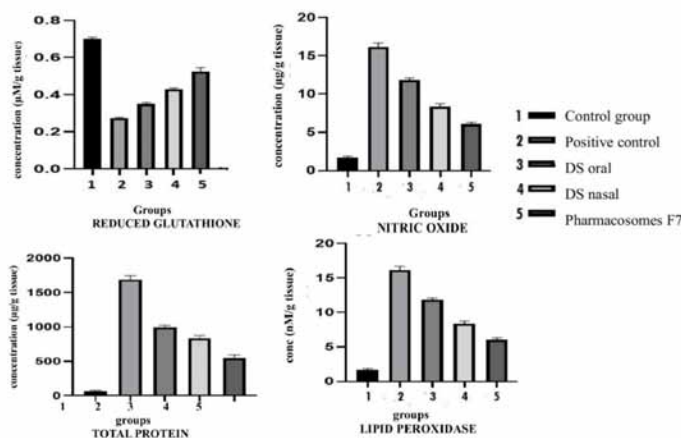
Figure 11: Comparison of levels of reduced glutathione, nitric oxide, total protein and lipid peroxidation.
Mean±SD (n =6)

Estimation of reduced Glutathione

When the treated groups were compared to control, the level of reduced glutathione in pharmacosomes F7 was restored to 67.4%, DS nasal to 30%, DS oral to 10%.

Estimation of Nitric Oxide

When all the treated groups were compared to positive control, the levels of nitric oxide decreased by 82.2% in pharmacosomes F7 treated group, 54% in DS nasal and 28% in oral group

Estimation of total protein

The total protein in pharmacosomes formulation nasal F7 treated group was significantly decreased by 88%, DS nasal by 74% and DS oral by 55.8%.

Estimation of lipid peroxidation

The levels of lipid peroxidation in pharmacosome F7 treated group was significantly decreased by 73%, DS nasal by 63.2% and DS oral by 33%.

Therefore, we conclude that optimized formulation F7 showed significantly high ($p<0.001$) pharmacodynamic activity and biochemical parameters when compared to nasal drug solution ($p<0.01$) and oral drug solution ($p<0.001$).

CONCLUSION

In the present study, pharmacosomes of levodopa were developed for delivery via intranasal route and evaluated for the pharmacodynamic activity in comparison to oral route. *In ex-vivo* permeation studies on porcine nasal mucosa, formulation F7 and F5 showed 3.12 times and 2.26 times enhancement in the steady state flux compared to drug solution respectively. Antiparkinson's activity in rats administered with Optimized pharmacosomes formulation showed significant increase in rat weight, locomotor activity by photoactometer and grip strength on rotarod. Biochemical parameters studied on homogenate of brain further showed significant reduction in levels of lipid peroxidase, nitric oxide, total protein and significant increase in reduced Glutathione ($P<0.001$) compared to drug solution treated via oral ($P<0.001$) and drug solution via nasal route ($P<0.01$). Also nasal drug solution showed significantly high ($P<0.001$) in pharmacodynamic activity compared to drug solution via oral route. Therefore, we conclude that intranasal pharmacosomes or solution administration could be potential benefit compared to oral route.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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