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PREPARATION AND EVALUATION OF ANTIPARKINSONISM DRUG LOADED POLYMERIC NANOPARTICLES FOR BRAIN TARGETING

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ABSTRACT

We have prepared and characterized the primaxazoledihydrochloride nanosuspension and showed potential neuroprotective effects in both *invitro* and *invivo*. The administration of nanosuspension significantly rescued the neuronal perturbations compared to the nonformulated pure drug which lend a hand in the enhancement of primaxazoledihydrochloride nanosuspension to cross the BBB for treatment in Parkinson's disease. The particle size was found to be 195 nm for formulation PPNP3. The entrapment efficiency was found to be 86.21% for formulation PPNP3. More than 85% drug release was observed in PPNP3. The zeta potential of PPNP3 was found to be 34.8mV. PPNP4 were rejected due to high content of polymer.

KEYWORDS: Nanosuspension, Blood Brain Barrier, Parkinson's disease, Neurons.

INTRODUCTION

BRAIN

The human brain is one of the complex and also a delicate organ in the central nervous system. It has a mass of pinkish grey tissues containing networks known as neurons. Interneurons mediate simple reflexes as well as it is responsible for the highest functions of the brain and gives a protective cover by blood-brain barrier (BBB).

Function of Blood-Brain Barrier

Blood-brain barrier encompasses a barrier functions. It includes physical transport and metabolic aspects. This also protects the Central Nervous System from abrupt changes in blood biochemistry that occur after meals, physical exercises or in pathological conditions.

The biological layer normally resists toxic agents and other xenobiotics to pass through the membrane. It also blocks the passage for endogenic compounds such as metabolites, hormones, mediators along with hydrophilic compounds to cross the barrier.

Neurodegenerative Diseases

It is a type of diseases where a extensive degeneration of neuron or neuro cell death. The nerveous systems are made with the help of neurons and there is a difficulties in regeneration of cells when the damage or death. This is due to the inability in generating of new cells by the body. Some of these are alzheimers, sclerosis, huntungtons and Parkinsons.

PARKINSON'S DISEASE

Parkinson's disease is a chronic progressive degenerative neurological disorder results from the destruction of brain nerve cells. This disorder results in motor and non-motor deficits. The signs and symptoms of the motor deficits are bradykinesia, rigidity and resting tremor. The non- motor signs and symptoms are insomnia, depression and cognitive dysfunction

Parkinson's disease is due to the progressive degeneration of the nigrostriatal dopaminergic system that leads to the loss of dopamine (DA). Parkinson's disease causes difficulty to perform coordinated movements during the early stage and as the disease progresses results in depression, cognitive impairment and neurogenic impairment resulting in the loss of bladder control.

DIAGNOSIS

There is no proper diagnosis test to identify the Parkinson diseases and the identification is based on the pathological findings in the neurons. There is no absolutely definitive diagnosis but at present it is achieved only by doing postmortem detection of Lewy bodies in brain tissue with neuron degeneration.

3 diagnostic steps as criteria of diagnosis are

- Patient's showing symptoms such as bradykinesia, rest tremor visual disturbance, vestibular, proprioceptive dysfunction.
- Emission of patients showing a spectrum of exclusion
- Adding the patients shows specific positive signs

MANAGEMENT

There is no drug or surgical approach has been shown unequivocally to slow the rate of progression of PD, but if any drug should be proved to delay the progression of the disease process, it should be incorporated in treatment early in the course of the disease. There is currently no cure for Parkinson's disease but medications can provide relief from symptoms. However, the mainstay of treatment is levodopa, the amino acid precursor of dopamine.

Drugs used to treat Parkinson's disease

- Dopamine precursors
  - Levodopa
- Peripheral decarboxylase Inhibitor
  - Carbidopa
  - Benserazide
- Catechol-O-methyltransferase inhibitors(COMT inhibitor)
  - Tolcapone
  - Entacapone
- Dopamine agonists
  - Bromocriptine
  - Pergolide
  - Pramipexole

MATERIALS AND METHOD

Materials used

- ✓ Pramipexole dihydrochloride - Sigma Aldrich, India
- ✓ PLGA - Sigma Aldrich, India
- ✓ MPEG-PCL - Sigma Aldrich, India
- ✓ Pluronic F 68 - Sigma Aldrich, India
- ✓ Acetone - Sisco Research Laboratories, India
- ✓ Potassium dihydrogen Phosphate - Sisco Research Laboratories, India
- ✓ Disodium Hydrogen Phosphate - Sisco Research Laboratories, India
- ✓ MTT - Sigma Aldrich, India
- ✓ MPP - Sigma Aldrich, India
- ✓ Rotenone - Sigma Aldrich, India

Preformulation studies

Preformulation is a part of Pharmaceutical science that utilizes biopharmaceutical principles in the determination of physicochemical properties of the drug substance that could effect drug performance and development of an efficacious stable and safe dosage form. Preformulation testing is the first step in the rational development of dosage forms of a drug substance. Preformulation commences when a newly synthesized drug shows a sufficient pharmacologic promise in an animal model to warrant evaluation in man. It will give the information of the nature of the drug substance and provide a frame work for the drug combination with pharmaceutical excipients in the dosage form. Hence, preformulation studies were performed for the obtained sample of drug for identification and compatibility studies.

1. Organoleptic properties
2. Solubility studies
3. Compatability studies

Organoleptic properties

The organoleptic characters of the drug like color, odor and taste play an important role in the identification of the sample and hence they were recorded in a descriptive terminology.

Solubility studies

The amount of substance that passes into solution in order to establish equilibrium at constant temperature and pressure to produce a saturated solution is known as solubility. Solubility is an important consideration in nanoparticle formulations as solubility of the solution is an essential requirement. The solubility of drug and polymer was carried out in various solvents such as distilled water, buffer solutions and organic solvents. The resulting solutions were filtered and analyzed for dissolved drug by measuring absorbance at 263 nm.

Compatibility studies

Fourier Transform Infra Red Spectroscopy (FTIR) studies

The Fourier transform infra red analysis was conducted for the structure characterization. FTIR spectra of the Pramipexole dihydrochloride, polymer and in combination were recorded. FTIR spectra were recorded on bruker alpha – T Spectrophotometer. Test samples were mixed with KBr, pressed into a pellet and scanned from 400 to 4000 cm^{-1}.

Differential Scanning Calorimetry (DSC)

Samples were analyzed by differential scanning calorimetry (DSC), with a shimadzu DSC T-60 using nitrogen gas. The sample was poured in an Al crucible, which was then sealed. The sample was kept at 25^{o}C for 10 min, and heated from 25 to 250^{o}C at a scan rate of 5^{o}C /min.

Preparation of standard plot of Pramipexole dihydrochloride

Accurately weighed 100mg of pramipexole dissolved in 100ml standard volumetric flask using distilled water to get the stock solution of 1mg/ml. From this stock solution 1ml of solution was withdrawn and made to 100ml, from these aliquots of 1, 2, 3, 4, and 5ml were withdrawn and further diluted to 10ml with water to obtain a concentration range of 10 - 50 µg/ml. The absorbance of the solutions was measured at 263nm by using UV-spectrophotometer. The graph was plotted.

Preparation of Pramipexoledihydrochloride nanosuspension

Preparation of Pramipexole dihydrochloride nanosuspension is carried by modified nano precipitation method.[98,99] In this method the external medium as phosphate buffer of pH 9.0 was used instead of aqueous phase. Various concentration ranging 10- 50 mg of PLGA and MPEG-PCL with 10 mg pramipexoledihydrochloride with correct weight is dissolved in acetone of 5ml. This organic solution was added slowly to Pluronic F 68 (1%) in buffer solution of phosphate with pH 9.0. The evaporation of the organic solvent is done in 1 to 2 hr by varying speed of 1000, 2000 rpm on a magnetic stirrer (Remi) on continuous stirring for optimizations studies. Optimizations are done by using following table -1.

Table: Optimization parameters of Pramipexole dihydrochloride loaded PLGA nanosuspension.

Sl. No.	Drug: polymer	RPM (Stirring Speed)	Stirring Time
1	A (1:1)	O Low (1000 rpm)	V Low (1hr)
2	B (1:2)		
3	C (1:3)	P High (2000 rpm)	X High (2hr)
4	D (1:4)		
5	E (1:5)		
6	A (1:1)	P High (2000 rpm)	X High (2hr)
7	B (1:2)		
8	C (1:3)	O Low (1000 rpm)	V Low (1hr)
9	D (1:4)		
10	E (1:5)		

Table: Various Formulations of Pramipexole dihydrochloride loaded PLGA nanosuspension.

Sl. No	Code (1:1)	Sl. No	Code (1:2)	Sl. No	Code (1:3)	Sl. No	Code (1:2)	Sl. No	Code (1:3)
1	PPAOY	5	PPBOY	9	PPCOY	13	PPDOY	17	PPEOY
2	PPAOX	6	PPBOX	10	PPCOX	14	PPDOX	18	PPEOX
3	PPAPV	7	PPBPV	11	PPCPV	15	PPDPV	19	PPEPV
4	PPAPX	8	PPBPX	12	PPCPX	16	PPDPX	20	PPEPX

Table Various Formulations of Pramipexole dihydrochloride loaded MPEG-PCL nanosuspension.

Sl. No	Code (1:1)	Sl. No	Code (1:M2)	Sl. No	Code (1:3)	Sl. No	Code (1:2)	Sl. No	Code (1:3)
1	PMPAOY	5	PMPBOY	9	PMPCOY	13	PMPDOY	17	PMPEOY
2	PMPAOX	6	PMPBOX	10	PMPCOX	14	PMPDOX	18	PMPEOX
3	PMPAPV	7	PMPBPV	11	PMPCPV	15	PMPDPV	19	PMPEPV
4	PMPAPX	8	PMPBPX	12	PMPCPX	16	PMPDPX	20	PMPEPX

The NP suspension was then centrifuged at 15,000 rpm for 30 min at 4^{o}C using high-speed centrifuge (Remi). Supernatant was taken for further evaluation.

Evaluation of nanoparticles

Particle size and zeta potential of Pramipexole dihydrochloride loaded with PLGA nanoparticles and also with MPEG-PCL nanoparticles were evaluated.

Particle size and Zeta potential

The size of the prepared nanoparticles was analyzed by using malvern apparatus. All samples were diluted with ultra purified water and the analysis was performed at a scattering angle of 90° and at a temperature of 25^{o}C. The mean diameter for each sample and mean hydrodynamic diameter was generated by cumulative analysis in triplicate. Nanoparticles were characterized with Zeta potential using a Zeta Sizer. The measurements were performed using an aqueous dip cell in an automatic mode by placing diluted samples in the capillary measurement cell and cell position is adjusted.

Optimized formulation of Pramipexole dihydrochloride loaded with PLGA and also with MPEG-PCL is taken for further characterization studies.

Scanning Electron Microscopy (SEM)

The surface morphology of the particles were studied using Scanning Electron Microscopy

Quanta 200 FEG scanning electron microscope (FEI Quanta FEG 200} set at 200 kV by placing an air dried nanoparticle suspension on copper electron microscopy grids and the image was captured at desired magnification.

Transmission electron microscopy (TEM)

TEM analysis where done for the optimized formulations to study the morphology of particles. The nanosuspension with 0.01% of phosphotungstic acid was placed on a copper grid which was already coated with carbon film and the sample where placed in a single drop which was performed at t80 kV .after placing sample on the grid, then it is placed into the sample holder of transmission microscope where the chamber is vacuum. Observe the image under low vacuum which was then recorded.

Drug content

Drug content was determined by taking 1ml of the PLGA Nanoparticles loaded with Pramipexole dihydrochloride. To this formulation 1ml of aqueous potassium dihydrogen phosphate solution (30mM) was added and the mixture was centrifuged at 33,000 xg at 15^0C. The clear supernatant was removed and analysed spectrophotomertically and drug content was calculated.

Drug entrapment efficiency

The drug loaded nanoparticles are centrifuged at 13000xg for 30 min and the supernatant is assayed for non-bound drug concentration by spectrophotometer. Entrapment efficiency was calculated as follows:

$$DEE\% = \frac{\text{Amount of drug actually present}}{\text{Theoretical drug load expected}} \times 100$$

***In vitro* release studies**

In vitro release studies were performed using diffusion test apparatus USP-II at 50rpm[100], in this nanoformulation measuring 10ml is placed on a dialysis membrane of molecular weight 12000 to 14000 dl. Then the membrane is soaked in buffer of phosphate saline for 12 hours prior the procedure starts. Pramipexoledihydrochloride nanoformulation which is already placed on the membrane is placed in the bowl containing 100 ml of buffer of phosphate saline pH 7.4. at the fixed time intervals 1 ml of the sample is withdrawn with the addition of equal quantity of fresh saline buffer of phosphate 7.4 of pH is refilled to maintain the equal volume.

In- Vitro Studies

Analysis of Cell Viability

To evaluate the activity of Pramipexole dihydrochloride nano-suspension protection against Parkinson's disease MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay was performed as an *in vitro* model.[101] The MTT assay where performed by using SH-SY5Y cells lines, the cells are plated separately in The MTT assay where performed by using SH-SY5Y cells lines, the cells are plated separately in tissue culture plates of 96 wells in concentration of 1×10^5 cells/well. The cells are taken after 24hrs then they are washed with serum free medium of 100ml and stored at 37^0C for 30 mins. After the time the test solution is added and kept for 24 hours. After the completion of the treatment the medium is removed and fresh serum free medium containing MTT (0.5 mg/ml) is added. Then the plates are incubated for 4 hrs at 37^0C in a CO_2 incubator. After the incubation period discard the medium containing MTT by washing the cells with PBS (200 µl). then 100 µl of DMSO is added to dissolve the crystals, it should be mixed properly by pipetting up and down.

Stability Studies

The optimized trials of prepared nanoformulations were selected for stability studies. They are subjected to long term and accelerated stability studies. Long term stability studies were carried out at 5^0C ± 3^0C and 30^0C ± 2^0C, 65% ± 5% RH. The samples were stored at the above said condition for minimum one year and their drug content, *invitro* release were determined for every 3 months. Similarly an accelerated stability study was carried out by storing the selected preparation at 40^0C ± 2^0C, 75% ± 5% RH for about six months. The drug content and *invitro* drug release studies were determined for every three months (ICH guidelines).

In- Vivo Studies

Adult male Wistar rats, approximately weighing 150-200g were housed in a temperature controlled room and maintained on 12:12-h light/dark cycles, with free access to food and water. The animals were handled according to the principles of laboratory care framed by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, Govt. of India. The experimental protocol was reviewed and approved animal Ethics Committee (IAEC) has approved for animal studies and the approval no is **XV/VELS/PCOL/10/2000/CPCSEA/IAEC/30.10.13.** Rotenone was purchased from Sigma Aldrich Chemical Company.

DOSING AND TREATMENT

Animal are separated into 5 different groups and six animals in every group.

Group A: Rats received saline (2 ml/kg body weight by per oral route) for 10 days.

Group B: Rats received rotenone (2.5 mg/ml /day/kg body weight) in saline by ip for 10

G**roup C**: Rats are receiving Pramipexoledihydrochloride nano-suspension (PPNP3) (drug equivalent to 0.8 mg/ml /day/kg body weight) was administered by oral gavage once daily 30 min. before rotenone (2.5 mg/kg) in saline for 10 days

Group D: Rats are receiving Pramipexoledihydrochloride alone (1mg/ml /day/kg body weight) was administered by oral gavage once daily 30 min. before rotenone (2.5 mg/kg) in saline for 10 days

Group E: Rats are receiving Pramipexole dihydrochloride nano-suspension (PMPNP2) (drug equivalent to 0.8 mg/ml /day/kg body weight) was administered by oral gavage once daily 30 min. before rotenone (2.5 mg/kg body weight) dissolved in saline for 10 days

Behavioral Studies

The behavioural studies such as locomotor plus maze activities were determined.

Elevated Plus Maze

In this method a maze having two black open arms in opposite of 50 X 10 cm which is crossed with closed arms of two in same dimensions containing 40 cm height walls. They all are connected with a central square having a dimension of 10 cm. The total maze was elevated to a height of 50 cm from the floor. The memories of the animals are tested on day 1. Individual animal are placed on the open arm facing far from the central square. The time taken by the animal to move from the open arm to the closed arm is recorded as an initial time and animals are returned to cage after allowing them for 20 sec in the maze. This was repeated for the day 5 and day 10, the time taken in second is recorded to assess the memory of control and other treated animals.

Locomotor activity

Locomotor activity where performed with experimental animals which receives nano preparation, pure drug and rotenone groups.

Preparation of tissue homogenates

All the animals in each groups are anesthetized and sacrificed by decapitation. After the brain is removed then it is washed in ice cold physiological saline Brain tissue is separated in

ice cold condition according to the method Glowinshi and iversan (1966), tissue (brain) were instantly processed and used for various biochemical evaluations.

The tissue (brain) is immediately homogenized to a known volume of 0.1 M Tris HCl buffer (pH 7.4), by a motor driven teflon-glass tissue homogenizer. The homogenate was centrifuged in refrigerated centrifuge at 300 x *g* for 15 min and aliquots of this homogenate were used for the assays.

BIOCHEMICAL STUDIES

a. Enzymatic Antioxidant Estimation

i. Estimation of superoxide dismutase (SOD)

SOD activity is determined by the method of Durak *et al*., 1996.

Briefly, 2.8 ml of reactive mixture [xanthine 0.3 mM, EDTA (ethylenediaminetetraacetic acid) 0.67 mM, 150 µM nitrotetrazolium blue chloride (NBT), sodium carbonate 0.4 M, bovine albumin 30 mg/30 ml] is added to 0.1 ml tissue homogenate and 50 µl xanthine oxidase (10 µl in 2M ammonium sulphate), incubated at 25°C for 20 min. and mixed with 0.1 ml 8 M copper chloride. The colour reaction was measured at 560 nm.

ii. Estimation of Catalase (CAT)

Catalase activity is estimated by following the procedure of Aebi, 1984, at room temperature.

A total of 100 µl of tissue homogenate was placed in an ice bath for 30 min and then for another 30 min at room temperature. A total of 10 µl Triton-X 100 was added to each tube. In a cuvette containing 200 µl phosphate buffer and 50µl of tissue homogenate, 250 µl of 0.066 M H_2O_2 was added (in phosphate buffer) and a decrease in optical density was measured at 240 nm for 60s. The molar extinction coefficient of 43.6 M/Cm was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2 degraded/min per mg protein.

iii. Estimation of Glutathione perodiase (GPx)

GPx was determined by the modified procedure of Flohe and Gunzler, 1984.

A reaction mixture consisting of 700µl phosphate buffer (0.05 M containing 0.01 mM EDTA, PH 7.0), 100 µl 0.01 M GSH (reduced form), 100µl 1 mM NADPH, and 100 µl glutathione reductase (GR) (0.24 units) was used. tissue homogenate (50 µl) was added to the reaction mixture and incubated at 37°C for 10 min. Then 50 µl of 12 mM cumene hydroperoxide was added to the reaction mixture and the absorbance was measured at 340 nm for 180s on a spectrophotometer. The molar extinction coefficient of 6.22×10^3cm/l was used to determine

the activity of GPx. One unit of enzyme activity is equivalent to 1 mM of NADPH oxidized/min/mg protein.

b. Non-Enzymatic Antioxidant Estimation

i. Estimation of Glutathione (GSH)

The assessment of GSH is done according to the procedure reported by Griffith 1980.

The tissue homogenate was taken and centrifuged at 1700 rpm for 10 min. the clear supernatant of 100 µl in cuvette containing 800 µl of 0.3 mM reduced nicotinamide adenine dinucleotide phosphate along with freshly prepared 6 mM 5,5-dithiobis-2-nitrobenzoic acid of 100 µl and 10 µl of 50 units/ml GR. The absorbance was recoreded for a period of 4 min. at 412nm at 30°C. The determined GSH levels were compared the test solution and standard GSH where the changes in absorbance is recorded.

Lipid Peroxidation (LPO)

LPO was estimated by the method of Ohkawa, *etal*.

The homogenate was added to 0.01 mol/l Tris–HCl buffer, pH 7.4 to make 10% solution. From this 0.1ml of homogenate is taken in the test tube containing 0.2ml of 8.1% sodium dodecyl sulphate (SDS). To this 1.5 ml of 20% acetic acid solution of pH 3.5 and 1.5 ml of 0.8% TBA (thiobarbituric acid) solution where added. Add 4.0 ml of distilled water to dilute the mixture then heat it for 60 min at 95°C. Then cool the solution in ice and it was extracted with 4.0 ml of mixture of n-butanol and pyridine (15:1, v/ v). The organic phase is collected for the measurement at 532 nm the absorbance was recorded. The results were expressed as nanomoles of MDA released per milligram protein.

Statistical analysis

All data were expressed as mean ± SD number of experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 13.0 (SPSS) and the individual comparison were obtained. A value of $p<0.05$ was considered to indicate a significant difference between groups.

Histopatholigical Studies

For histopathology studies, the brain tissue fixed in 10% formalin for 4 days and dehydrated in 50, 70, 95 and 100% ethanol, 20 min. each time, and then submerged in xylene twice, 10 min. each time. Then sections was taken and stained with Hematoxylin-eosin. Digital microphotographs were taken at 400X magnification.

Pharmacokinetic studies

An adult male wistar rat, approximately weighing 150-200g was used for the experiment and 3 animals were used in the experiment for every trial done in the formulation. Nanoformulation nearly 1 mg drug (equal to 0.45 milligram/kilogram BW) was given through IV. The different parameters are determined by software which includes AUMC0–24, Cmax, MRT, AUC0–24 (Microsoft Excel of PK Functions).Sampling of drug was done through cardiac puncture at the rate of 0,0.25, 0.5, 1, 2, 4, 6, 8, 20 and one day in centrifuge tubes. The mixture is added with 8 milligram of anticoagulant EDTA. The sample of blood was then added along with the Heparin and centrifuged at 4000 rpm for 20 minutes. After this process the blood plasma is isolated and kept in -21 C till the experimental analysis was completed by LC/MS/MS. The brain of the animals was washed by using NS and the different quantity is homogenized of 25mM buffer (pH 7.4). As above it is centrifuged and the aliquots was isolated and kept in -21^0C until the experimental analysis was completed by LC/MS/MS.

RESULTS AND DISCUSSION

Drug content and entrapment efficiency

The drug content and entrapment efficiency of the prepared nanoformulations where determined which was shown in table-14. The results shows 0.57 mg/ml has 86.21% entrapment efficiency in PPNP3 with higher drug content having more entrapment efficiency, since other formulation shows low drug content when compare to PPNP3 with varying entrapment efficiency. Thus based upon result PPNP3 may be best formulation among the remaining nano suspension.

Table: Drug content and entrapment on efficiency of pramipexole dihydrochloride loaded PLGA nanosuspension.

Formulation	Average drug content mg/ml*	Average entrapment efficiency (%)*
PPNP1	0.35±0.03	67.28% ± 2.2
PPNP2	0.49±0.05	75.53% ± 1.2
PPNP3	0.57±0.02	86.21% ± 2.6
PPNP4	0.55±0.04	86.13% ± 1.8
PPNP5	0.54±0.03	85.89% ± 2.1

* Values indicated are in the results ± s.e.m of triplicate trials

The drug content of the prepared nanoformulations where determined and the results shows 0.59 mg/ml with 88.53% entrapment efficiency in PMPNP2 with higher drug content having

more entrapment efficiency which was shown in table-15, since other formulation shows low drugs content when compare to PMPNP2 with varying entrapment efficiency. Thus based upon result PMPNP2 may be best formulation among the remaining nanosuspension.

Table: Drug content and entrapment on efficiency of pramipexole dihydrochloride loaded MPEG-PCL nanosuspension.

Formulation	Ratio	Average drug content mg/ml*	Average entrapment efficiency (%)*
PMPNP1	1:1	0.45 ±0.04	57.28% ±2.14
PMPNP2	1:2	0.59 ±0.10	88.53% ±1.42
PMPNP3	1:3	0.47 ±0.06	68.21% ±1.72
PMPNP4	1:4	0.43 ±0.09	69.13% ±2.04
PMPNP5	1:5	0.35 ±0.14	80.27% ± 1.24

* Values indicated are in the results ± s.e.m of triplicate trials

However, the percentage of entrapment efficiency of the drug was dependent on the polymer ratio, stirring speed and stirring rpm. The entrapment efficiency of nanosuspension was found to be increased up to drug: polymer ratio. This may be due to increased adsorption of the pramipexoledihydrochloride on to the surface of the polymeric matrices. However, a further increase of polymeric concentration had not indicated increase in entrapment efficiency.

***In vitro* release studies**

The table and fig shows *in vitro* drug release of all formulations; in that, PPNP1, PPNP2 shows the highest drug release but was rejected. PPNP 4 and PPNP 5 were not selected despite low particle size because they act as release retardants due to a high concentration of polymer. So PPNP 3 is selected as the best formulation due to its optimum drug release 95.2 in 24hrs which is an controlled release than 16 hrs in PPNP1, 20 hrs in PPNP2 and 76.8%, 7.2.1% in PPNP4, PPNP5 both formulation even after 24hrs the drug was no released, hence based on results we obtained PPNP3 may be best formulation. The selected formulation was taken for further cell viability studies and *in vivo* studies.

Table: *In vitro* release studies of pramipexoledihydrochloride loaded PLGA nanosuspension.

S. No.	TIME (HOURS)	% CUMULATIVE DRUG RELEASE*				
		PPNP1	PPNP2	PPNP3	PPNP4	PPNP5
1.	0	0	0	0	0	0
2.	1	12.3± 1.1	11.2± 1.1	7.2± 1.5	6.7± 0.7	6.1± 0.6
3.	2	36.1± 1.8	28.7± 2.2	10.8± 1.3	11.1± 1.2	9.2± 1.2
4.	4	58.2± 3.3	45.1± 2.4	21.2± 2.5	22.7± 3.2	19.9± 2.6

5.	6	75.9± 2.2	59.5± 3.3	33.5± 3.3	35.4± 2.6	30.7± 2.1
6.	8	83.4± 3.1	74.3± 3.2	46.1± 3.4	52.1± 2.3	43.4± 3.1
7.	12	99.9± 1.1	88.4± 2.3	62.6± 1.2	61.3± 3.4	59.3± 2.9
8.	16	100± 0.3	97.9± 3.4	71.3± 3.6	73.8± 2.8	60.5± 2.7
9.	20	-	100± 0.5	85.7± 1.5	75.3± 3.1	65.7± 2.2
10.	24	-	-	89.2± 2.4	76.8± 2.6	72.1± 1.8

* Values indicated are in the results ± s.e.m of triplicate trials

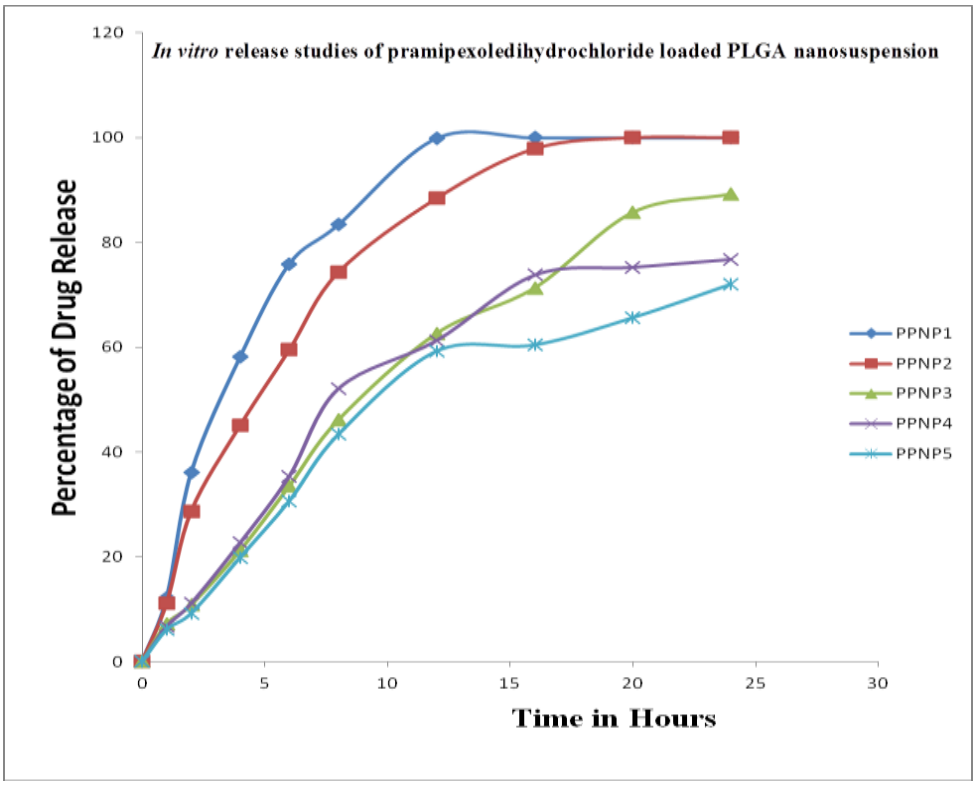


Fig: *In vitro* release studies of pramipexoledihydrochloride loaded PLGA nanosuspension.

The table and fig shows *in vitro* drug release of all formulations; in that, PMPNP1 shows the highest drug release but was rejected. PMPNP 3, PMPNP 4 and PMPNP 5 were not selected despite low particle size because they act as release retardants due to a high concentration of polymer. So PMPNP 2 is selected as the best formulation due to its optimum drug release 96.53 in 24hrs which is an controlled release than 16 hrs in PMPNP1 and 76.77%,68.98%, 58.33% in PMPNP3, PMPNP4, PMPNP5 formulation even after 24hrs the drug was no released, hence based on results we obtained PMPNP2 may be best formulation. The selected formulation was taken for further cell viability studies and *in vivo* studies.

Table: *In vitro* release studies of pramipexoledihydrochloride loaded MPEG-PCL nanosuspension.

S. No.	TIME (HOURS)	% CUMULATIVE DRUG RELEASE*				
		PMPNP1	PMPNP2	PMPNP3	PMPNP4	PMPNP5
1.	0	0	0	0	0	0
2.	1	7.22± 0.2	7.12± 0.3	5.99± 0.4	6.32± 1.2	5.87± 1.0
3.	2	21.85± 1.4	11.91± 1.2	14.90± 1.4	12.62± 1.3	10.40± 1.3
4.	4	35.67± 1.6	25.43± 1.6	37.65± 2.4	24.68± 1.1	19.39± 1.1
5	6	58.52± 1.8	38.66± 1.5	37.65± 2.4	36.52± 1.2	25.26± 1.2
6	8	74.06± 1.3	43.88± 0.5	37.65± 2.4	45.67± 1.4	30.29± 1.2
7	12	92.56± 0.6	62.13± 1.2	53.74± 1.6	52.17± 1.3	41.38± 1.3
8	16	100± 0.4	69.84± 1.0	58.84± 1.2	60.16± 1.1	48.32± 1.4
9	20	-	87.90± 1.2	65.38± 1.4	64.45± 1.3	52.43± 1.3
10	24	-	96.53± 1.3	76.77± 1.1	68.98± 1.2	58.33± 1.4

* Values indicated are in the results ± s.e.m of triplicate trials

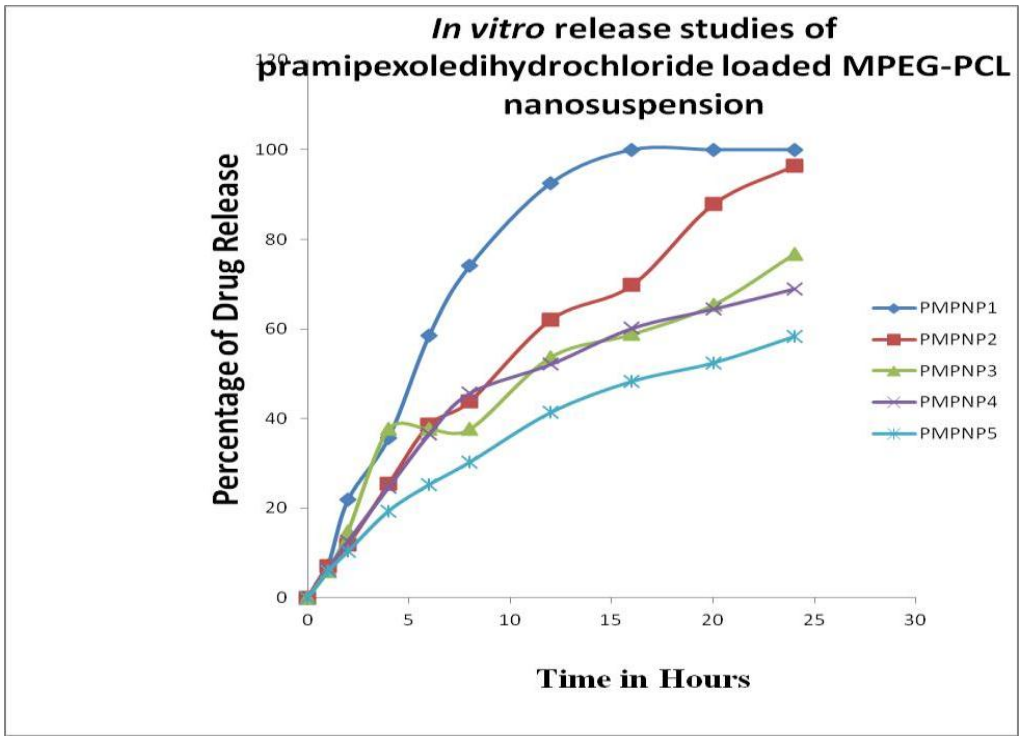


Fig. *In vitro* release studies of pramipexoledihydrochloride loaded MPEG-PCL nanosuspension.

The comparison of the results such as the particle size, zeta potential, drug content, entrapment efficiency and invitro drug release, the formulation PPNP3 and PMPNP2 was indicated the least particle size, higher zeta potential, drug content, entrapment efficiency and drug release. Hence among the various trials of pramipexoledihydrochloride nanosuspension PPNP3 and PMPNP2 have been identified to carry out further studies.

***In vitro* studies**

Cell Viability studies

Cell viability studies where performed by MTT assay. It is depends on mitochondrial dehydrogenases for MTT conversion and these enzymes are also inhibited by MPP. As shown in Figure- 19, MPP significantly decreased cell viability.

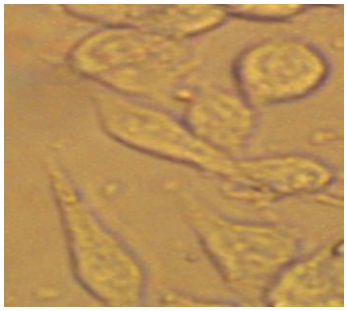


Fig: The plates of MTT assay of control.

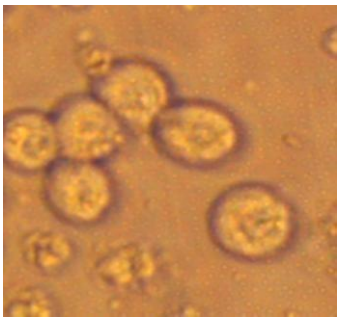
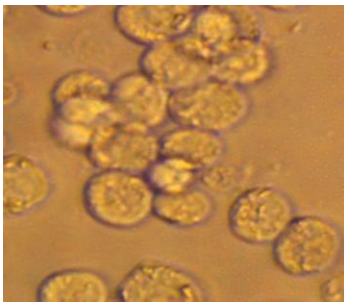
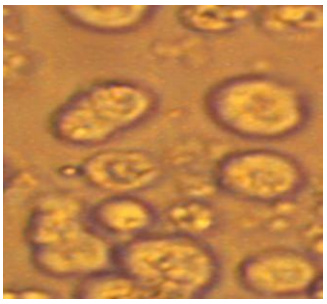


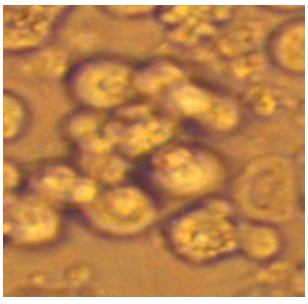
Fig: The plates of MTT assay of MPP.



a MPP+ PUREDRUG 1 µg/ml



b MPP+ PUREDRUG 2 µg/ml



c MPP+ PUREDRUG 5 µg /ml

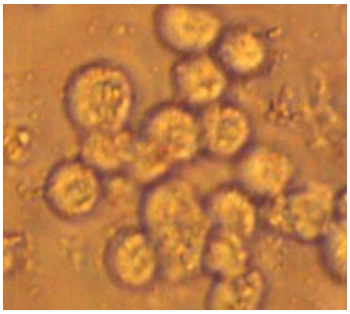
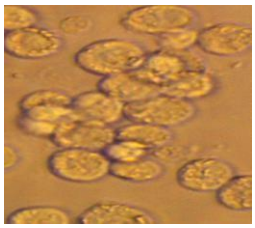
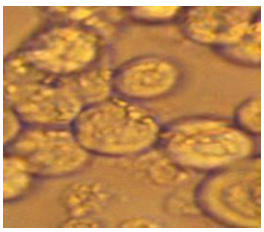


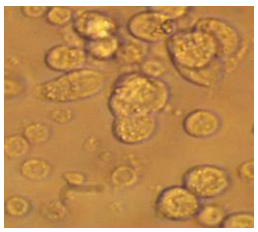
Fig: The plates of MTT assay of MPP+ Plain PLGA Nanosuspension in 50µl concentration.



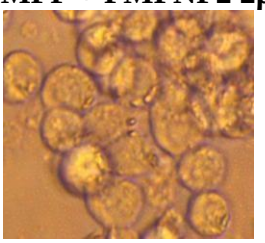
PMPNP2 1µl



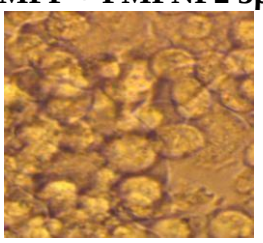
b.MPP + PMPNP2 2µl



c.MPP + PMPNP2 5µl



d.MPP + PMPNP2 10µl



e.MPP + PMPNP2 50µl

Fig: The plates of MTT assay of MPP and pramipexole dihydrochloride nanosuspension loaded MPEG-PCL in different concentration.

Stability Studies

The prepared pramipexoledihydrochloride loaded PLGA and MPEG-PCL nanoformulation were stored at the following conditions i.e., 5± 3^0C, 30 ± 2^0C, 65% ± 5% RH (long term stability), 40± 2^0C, 75%± 5%(accelerated stability). Every three months the drug content and *invitro* studies release were determined for the nanoformulation subjected for long term stability studies and the results were shown in the following table.

The drug content of PPNP3 stored at 5± 3^0C for a period of 12 months showed a slight decrease in the drug content when compared to the initial drug content of the nanoformulation after storing the sample for 12 months.

Table: Stability studies- *invitro* drug release of PPNP3 stored at 5± 3^{0}C (After 0, 3, 6, 9 & 12 months as per QA1 (R)).

Time (Hrs)	0 Month	3 Months*	6 Months*	9 Months*	12 Months*
0	0	0	0	0	0
1	7.2± 1.5	7.8± 1.8	7.4± 1.2	6.8± 1.5	7.3± 1.3
2	10.8± 1.3	11.6± 1.6	10.4± 1.5	11.2± 1.5	11.0± 1.2
4	21.2± 2.5	20.8± 2.2	21.5± 2.0	20.7± 1.8	21.0± 2.1
6	33.5± 3.3	33.4± 3.0	33.2± 2.8	32.8± 3.1	33.0± 2.7
8	46.1± 3.4	46.5± 3.2	45.7± 3.5	46.1± 3.4	46.3± 3.2
12	62.6± 1.2	62.2± 1.5	61.8± 0.9	62.4± 1.4	61.2± 1.7
16	71.3± 3.6	70.7± 3.2	71.5± 3.2	70.8± 2.8	69.8± 2.5
20	85.7± 1.5	84.5± 1.7	83.8± 1.2	85.2± 1.0	84.6± 0.8
24	89.2± 2.4	87.6± 1.4	88.4± 1.7	89.5± 1.8	88.8± 2.6

* Values indicated are in the results ± s.e.m of triplicate trials

The PPNP3 shows less difference in the *invitro* drug release when compared to the initial formulation after storing the sample at 5± 3^{0}C (after 0, 3, 6, 9 & 12 months as per QA1 (R)).

The drug content of PMPNP 2 stored at 5± 3^{0}C for a period of 12 months showed a slight decrease in the drug content when compared to the initial drug content of the nanoformulation after storing the sample for 12 months.

Table: Stability studies- *invitro* drug release of PMPNP 2 stored at 5± 3^{0}C (After 0, 3, 6, 9 & 12 months as per QA1 (R)).

Time (Hrs)	0 Month	3 Months*	6 Months*	9 Months*	12 Months*
0	0	0	0	0	0
1	7.12± 0.3	5.42± 1.3	6.44± 1.0	7.24± 0.8	7.42± 0.6
2	11.91± 1.2	10.82± 1.4	9.44± 1.7	11.31± 1.5	10.88± 1.8
4	25.43± 1.6	24.32± 1.2	23.84± 1.8	25.24± 1.1	24.82± 1.3
6	38.66± 1.5	37.84± 1.3	36.72± 1.8	38.54 ± 1.8	37.75± 1.2
8	43.88± 0.5	42.68± 1.5	42.66± 1.2	43.45± 1.2	42.79± 1.0
12	62.13± 1.2	63.43± 1.7	61.82± 1.5	60.17± 1.8	62.58± 1.6
16	69.84± 1.0	68.52± 1.6	70.24± 1.4	68.75± 1.7	69.42± 1.2
20	87.90± 1.2	88.43± 1.5	88.21± 1.7	86.98± 1.4	87.52± 1.1
24	96.53± 1.3	95.45± 1.7	95.72± 1.6	96.22± 1.8	96.18± 1.4

* Values indicated are in the results ± s.e.m of triplicate trials

The PMPNP 2 shows less difference in the *invitro* drug release when compared to the initial formulation after storing the sample at 5± 3^{0}C (after 0, 3, 6, 9 & 12 months as per QA1 (R)). The nanoparticles stored at 30± 2^{0}C, 65 ± 5 % RH after 0, 3, 6, 9 & 12 months storage showed only slight decrease in the drug content and *invito* drug release studies when compared to the initial drug content of the nanoformulation.

Table: Stability studies- *invitro* drug release of PPNP3 stored at 30± 2^{0}C, 65 ± 5 % RH (after 0, 3, 6, 9 & 12 months as per QA1 (R)).

Time (Hrs)	0 Month	3 Months*	6 Months*	9 Months*	12 Months*
0	0	0	0	0	0
1	7.2± 1.5	8.4± 1.2	7.6± 1.3	8.1± 1.4	7.8 ± 1.0
2	10.8± 1.3	12.4± 1.4	11.6± 1.7	12.2± 1.\6	11.2± 1.2
4	21.2± 2.5	19.5± 1.8	20.5± 1.5	21.4± 2.2	20.8± 1.6
6	33.5± 3.3	35.4± 2.5	34.2± 2.2	33.2± 2.7	32.8± 2.5
8	46.1± 3.4	45.4± 2.2	44.5± 2.8	45.8± 3.2	45.8± 3.0
12	62.6± 1.2	63.5± 1.8	60.8± 1.5	62.4± 1.4	62.1± 1.0
16	71.3± 3.6	71.3± 3.6	71.3± 3.6	71.3± 3.6	71.3± 3.6
20	85.7± 1.5	87.2± 1.9	86.4± 1.6	85.2± 1.1	86.5± 1.4
24	89.2± 2.4	90.4± 2.7	91.6± 1.8	89.8± 2.8	90.4± 1.6

* Values indicated are in the results ± s.e.m of triplicate trials

The PPNP 3 show less difference in the *invitro* drug release after storing the sample at 30 ± 2^{0}C, 65 ± 5% RH after 0, 3, 6, 9 & 12 months when compared with initial drug release of the nanoformulation.

Table; Stability studies- *invitro* drug release of PMPNP 2 stored at 30± 2^{0}C, 65 ± 5 % RH (after 0, 3, 6, 9 & 12 months as per QA1 (R)).

Time (Hrs)	0 Month	3 Months*	6 Months*	9 Months*	12 Months*
0	0	0	0	0	0
1	7.12± 0.3	9.22± 1.3	8.34± 1.4	7.57± 1.0	7.64± 1.1
2	11.91± 1.2	14.21± 1.7	12.96± 1.6	12.22± 1.6	12.45± 1.8
4	25.43± 1.6	28.33± 1.3	27.22± 1.2	25.75± 1.8	26.15± 1.3
6	38.66± 1.5	40.22± 1.2	39.46± 1.8	40.34± 1.7	39.24± 1.1
8	43.88± 0.5	45.52± 1.3	44.64± 1.2	45.28± 1.7	44.26± 1.2
12	62.13± 1.2	67.26± 2.1	64.32± 1.4	64.82± 2.2	63.25± 1.8
16	69.84± 1.0	71.24± 1.4	70.54± 1.8	68.12± 1.8	69.24± 1.4
20	87.90± 1.2	89.35± 1.8	90.52± 1.4	86.25 ± 1.8	88.20± 1.6
24	96.53± 1.3	98.22± 1.0	97.86± 1.4	96.32± 1.7	97.42± 1.5

Values indicated are in the results ± s.e.m of triplicate trials

The PMPNP 2 show less difference in the *invitro* drug release after storing the sample at 30 ± 2^{0}C, 65 ± 5% RH after 0, 3, 6, 9 & 12 months when compared with initial drug release of the nanoformulation.

Table Stability studies- *invitro* drug release of PPNP3 stored at 40± 2^{0}C, 75 ± 5 % RH (after 0, 6, & 12 months as per QA1 (R))

Time (hrs)	0 Month*	6 Months*	12 Months*
0	0	0	0
1	7.2± 1.5	4.1± 0.5	2.5± 0.8
2	10.8± 1.3	7.2± 1.4	5.2± 1.2
4	21.2± 2.5	11.4± 1.8	8.6± 1.2
6	33.5± 3.3	22.4± 2.4	15.2± 1.6
8	46.1± 3.4	30.2± 2.2	23.1± 1.7
12	62.6± 1.2	44.3± 1.5	32.5± 1.8
16	71.3± 3.6	58.5± 2.3	44.6± 2.4
20	85.7± 1.5	68.2± 2.1	55.2± 1.7
24	89.2± 2.4	76.7± 1.8	64.3± 1.4

*Values indicated are in the results ± s.e.m of triplicate trials

The optimized nanoformulation PPNP 3 at 40± 2^{0}C, 75 ± 5 % RH after 0, 6, 12 months storage showed significant decrease in cumulative drug release when compared to the initial cumulative drug release of the same nanoformulation.

The optimized nanoformulation PMPNP 2 at 40± 2^{0}C, 75 ± 5 % RH after 0, 6, 12 months storage showed significant decrease in cumulative drug release when compared to the initial cumulative drug release of the same nanoformulation.

Table; Stability studies- *invitro* drug release of PMPNP 2 stored at 40± 2^{0}C, 75 ± 5 % RH (after 0, 6, & 12 months as per QA1 (R)).

Time (hrs)	0 Month*	6 Months*	12 Months*
0	0	0	0
1	7.12± 0.3	3.30± 0.4	1.14± 0.5
2	11.91± 1.2	6.82± 0.5	3.22± 0.8
4	25.43± 1.6	10.51± 1.2	7.21± 1.1
6	38.66± 1.5	17.45± 1.3	9.21± 1.1
8	43.88± 0.5	28.62± 1.4	18.32± 1.4
12	62.13± 1.2	37.41± 1.6	26.22± 1.4
16	69.84± 1.0	49.34± 1.6	34.24± 1.7
20	87.90± 1.2	55.33± 1.5	42.42± 1.4
24	96.53± 1.3	67.12± 1.6	55.45± 1.6

*Values indicated are in the results ± s.e.m of triplicate trials

Table Mean pharmacokinetic parameters of pramipexoledihydrochloride, PPNP3 nanoformulation and PMPNP2 nanoformulation in brain and plasma of experimental animals.

Samples Tested		C_{max} (ng/mL) *	T_{max} (hr) *	AUC_{0-24} (ng*h/mL) *	AUC_{0-24} (ng*h/mL) *	CL (mL/min/ Kg) *	Vd (L/Kg) *	MRT (hr) *
PP	**Blood**	524.45±18.24	0.082	1153.20±6.4	1210.2±27.23	1.62	1.4	5.74±4.8
	Brain	16.42±10.4	3.6	364.33±26.24	452±7.56	1.12	3.41	12.32±1.44
PPNP 3	**Blood**	651.32±15.32	0.090	2142.21±8.4	15234.5±25.41	1.2	1.45	175.41±3.26
	Brain	121.11±12.11	5.00	2452±22.12	7102±9.21	1.42	6.55	52.55±1.02
PMP NP2	**Blood**	725.26±18.12	0.091	2212.21±8.2	16354.2±19.32	1.5	1.52	182.32±3.93
	Brain	128.32±13.15	6.00	2521±24.32	7324±7.25	1.23	6.25	56.33±1.11

SUMMARY AND CONCLUSION

There was no incompatibility observed between drug and polymer. The particle size was found to be 195 nm for formulation PPNP3. The entrapment efficiency was found to be 86.21% for formulation PPNP3. More than 85% drug release was observed in PPNP3. The zeta potential of PPNP3 was found to be 34.8mV. PPNP4 were rejected due to high content of polymer. Therefore, PPNP3 were chosen as the best formulation as they has better release than other formulations. The particle size was found to be 145 nm for formulation PMPNP2. The entrapment efficiency was found to be 75.53% for formulation PMPNP2. More than 85% drug release was observed in PMPNP2. The zeta potential of PMPNP2 was found to be 33.4mV. The cell viability in SH-SY5Y cells were performed in that the protections of cells at 50 µl/ml of pramipexole diHydrochloride NPs suspension was nearly equal to control. MPP reduce the cell viability and NPs suspension showed dose-dependent protection which was supported by Histopathological evaluation of rat brain in male Wistar rats. The different pharmacokinetics parameters where examined for pramipexoledihydrochloride nanoformulation and the drug solution to execute the concentration of drug in plasma as well as brain tissue homogenate. The pharmacokinetic parameters of pramipexoledihydrochloride nanoformulation were comparedwith that of drug solution administered by intravenous route. Thus, the biodegradable polymers which help in production of more controlled release dosage form for treatment in Parkinson's disease. The nanosupension using MPEGPLA shows better activity than PLGA usedpreparations.

We have prepared and characterized the primaxazoledihydrochloride nanosuspension and showed potential neuroprotective effects in both *invitro* and *invivo*. The administration of nanosuspension significantly rescued the neuronal perturbations compared to the

nonformulated pure drug which lend a hand in the enhancement of primaxazoledihydrochloride nanosuspension to cross the BBB for treatment in Parkinson's disease.

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