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Oil based nanocarrier system for transdermal delivery of ropinirole: A mechanistic, pharmacokinetic and biochemical investigation

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ABSTRACT

Ropinirole, a recent introduction in the clinical treatment of Parkinson's disease, suffers with the problems of low oral bioavailability and frequent dosing. An effective transdermal nano-emulsion drug delivery system can however resolve these issues effectively with greater therapeutic benefits and clinical significance. Therefore, the present work focuses precisely on pharmacokinetic, biochemical and mechanistic assessment of transdermal nanoemulsion gel in rats induced with Parkinson lesioned brain by 6-OHDA. DSC and FT-IR studies showed that NEG affects the normal lipid packing of stratum corneum to enhance the drug permeation. Study of pharmacokinetic parameters (AUC, C_{max}, and T_{max}) revealed a greater and more extended release of ropinirole from nanoemulsion gel compared to that from a conventional gel (RPG) and oral marketed tablet (Ropitor®). The $AUC_{0\rightarrow\infty}$ for RPCNG and RPTNG was found to be 928.07 ± 206.5 and 1055.99 ± 251.7 ng h/mL, respectively in comparison to 137.25 ± 31.3 and 467.15 ± 106.1 ng h/mL for RPG and oral tablet, respectively. The relative bioavailability of ropinirole has been enhanced more than two fold by RPTNG. Furthermore, antiparkinson activity was evaluated in terms of estimating the level of thiobarbituric acid reactive substances, glutathione antioxidant enzymes and catalase in lesioned brain of rats. Formulations were also found to be non-toxic and non-irritant by histological investigations.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized typically by motor features of tremor, rigidity and bradykinesia due to the depletion of dopaminergic nigrostriatal neurons. It is the second most common neurodegenerative disease after Alzheimer's disease. Non-motor disorder symptoms such as dementia, depression and falls emerge with the progression of the disease (Hely et al., 2005). Parkinson disease results in a significant decline in quality of life (Schrag et al., 2000) for both patients and family and contributes to significant economic and institutional costs on family and society (Findley et al., 2003). Because of the ageing of the world population, the importance of Parkinson's disease as a public health issue is expected to increase.

Drugs with short half lives necessitate frequent administration which causes great inconvenience to the Parkinson's patients. When symptoms like slowness of movement, tremor and rigidity return due to wearing off of the patient's medication, it can be problematic, causing difficulty with simple activities and movement in patients with Parkinson disease. Reducing the period of "off" time would allow patients to carry on their normal daily activities for a longer period of time, making it an attractive option for Parkinson's sufferers. Therapies that provide more continuous stimulation of dopamine receptors have been found to reduce motor complications in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, treated monkeys and in patients with Parkinson disease (Olanow et al., 2005). Maximizing bioavailability enhances delivery of the drug to the active site and results in greater consistency in population pharmacokinetics. Optimal bioavailability may lower the variability in clinical response and minimize the long-term development of motor fluctuations (Nyholm, 2006).

Ropinirole is a recently introduced dopamine agonist, which stimulates striatal dopamine receptors to produce dopamine, for the treatment of PD. It is effective both as monotherapy as well as combination therapy with levodopa whereby it helps in reduction of the dose of levodopa. The drug has low bioavailability of only about 50% by oral route (Dollery, 1999; Kaye and Niholls, 2000). The usual dose is 3–9 mg daily and has to be taken in three divided doses owing to short half life of the drug. This causes great inconvenience to the patients. The continuous delivery of ropinirole from the transdermal system may delay or prevent the onset of levodopa related motor complications due to continuous dopaminergic

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stimulation (Chase, 1998). It could be anticipated that once daily regimen shall significantly increase the patient compliance (Grosset et al., 2005) while at the same time reducing the burden of the care giver. Therefore in the present study transdermal route was selected as an alternative choice of route of administration for such drugs. Transdermal therapeutic system (TTS) is a self-contained discrete system which when applied to the intact skin, delivers the drugs through the skin at a controlled rate to the systemic circulation thereby providing sustained release of the drugs. Moreover, hepatic degradation and frequency of ropinirole could be avoided by this route.

However, by far this route is not fully explored for the achievement of therapeutic levels of the drug systemically. The most crucial reason for this is the inability of most of the drugs to pass the barrier nature of stratum corneum (Blank and Scheuplein, 1969). Many penetration enhancement strategies like use of surfactants, chemical modifications, surfactant vesicles; micro-needles, microwaves, etc. are suggested for making drugs available to the blood in higher concentrations. Nevertheless, these methods are associated with skin toxicity issues and lack of patient acceptability. Nanoemulsion systems have been invigorated keen interest in the recent years for the transdermal permeation enhancement of drugs (Rizwan et al., 2010; Talegaonkar et al., 2008). Nanoemulsion is a versatile technology which can enhance the percutaneous absorption of both hydrophilic and lipophilic drugs as compared to the conventional formulations. The dispersed phase, lipophilic or hydrophilic (o/w or w/o nanoemulsions) can behave as a potential reservoir of lipophilic or hydrophilic drugs, respectively. The drug partitions between dispersed and continuous phase, and when the system comes into contact with the skin, the drug can be transported through the barrier. Drug release with pseudo zero order kinetics can be obtained, depending on the volume of the dispersed phase, the partition of the drug and the transport of the drug.

Our previous work dealt extensively with the formulation development and in vitro evaluation of NEG of ropinirole for transdermal permeation enhancement and to alleviate the problem of low and variable oral bioavailability for the better management of the Parkinson patients (Azeem et al., 2009a,b). The optimized gel comprised of Capryol 90 as the oil phase, Tween 20 as the surfactant and Carbitol as the cosurfactant, A 7.5 fold increase in skin permeation rate was observed when compared with the conventional gel in vitro (Azeem et al., 2009a).

The principal aim of the present investigation is to evaluate the in vivo efficacy of the developed formulation wherein relative bioavailability of the drug from the transdermal nanoemulsion gel was assessed in comparison to the oral administration of marketed formulation. Moreover, biochemical tests such as lipid peroxidation, reduced glutathione content and catalase activity were carried out to further corroborate the obtained pharmacokinetic data.

2. Materials and methods

Ropinirole was a gift sample from USV (Mumbai, India), while propylene glycol monocaprylate (Capryol 90) was a courtesy from Colorcon Asia (Mumbai, India). Diethylene glycol monoethyl ether (Carbitol) and polyoxyethylene sorbitan monolaurate (Tween 20), 6-hydroxydopamine, glutathione reductase, 5-5′-dithio-bis-2-nitrobenzoic acid, thiobarbituric acid, ethylene-diamine tetraacetic acid (EDTA) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetonitrile and ammonium acetate were procured from E-Merck (Mumbai, India). Water was obtained from Milli-Q water purification system (Millipore, MA, USA). All other chemicals and solvents were of analytical grade.

Table 1
Composition of the nanoemulsion gels (RPTNG and RPCNG) and conventional ropinirole gels (RPG) and relative permeability coefficient values.

Ingredients (%)	NEG		RPG
	RPTNG	RPCNG	
Ropinirole	0.66	0.66	0.66
Capryol 90	5	5	–
Tween 20:Carbitol (2:1)	35	–	–
Cremophor:Carbitol (1:1)	–	20	–
Water	58.06	73.09	98.06
Carbopol 934	0.75	0.75	0.75
Triethanolamine	0.5	0.5	0.5
Permeability coefficients K_p ($\times 10^{-2}$ cm/h)	1.036 ± 0.152	0.942 ± 0.011	0.041 ± 0.021

2.1. Preparation of nanoemulsion gel and conventional gel

The detailed description of the preparation and optimization of the ropinirole nanoemulsion gels (RPTNG and RPCNG), and the conventional gel (RPG) is provided in our previously published article (Azeem et al., 2009a,b). The nanoemulsion system for the optimized NEG was chosen from two different nanoemulsion systems: nanoemulsion containing Tween 20 (RPTNG) and nanoemulsion containing cremphore EL (RPCNG) as a surfactant, respectively. However, the optimized nanoemulsion gel comprised of Capryol 90 as the oil phase, Carbitol as the cosurfactant and Carbopol 934 was used as a gelling agent in both the types of the gel (Table 1).

2.2. Skin permeation study

2.2.1. Preparation of stratum corneum (SC)

Abdominal skins were obtained from male albino Wistar rats weighing 200 ± 30 g after sacrificing by diethyl ether aspiration. The skin was carefully excised and the subcutaneous tissue was removed surgically and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The hairs on the skin were removed with depilatory. Then the skin was washed with water, wrapped in aluminium foil and stored in freezer at $-20\,^{\circ}$C before further use.

Two samples of rat epidermis, one is control and another one is RPTNG treated were incubated in a petridish over filter paper imbibed with 0.1% trypsin solution in PBS (pH 7.4) at $37\,^{\circ}$C for 4 h (Vaddi et al., 2002). The SC was removed, thoroughly washed, and dried in a vacuum desiccator and used in the FT-IR and DSC studies.

2.2.2. FT-IR spectroscopy

The SC was cut into small circular discs with approximate diameter of 1.5 cm (Shakeel et al., 2008). Isotonic solution of sodium chloride (0.9%, w/v) was prepared containing 0.01% (w/v) sodium azide which acted as an antibacterial and antimycotic agent (Jain et al., 2001). Equal volume of 0.9% (w/w) of sodium chloride was placed in different conical flasks and SC was floated over it for 3 days. After 3 days of hydration, these discs were thoroughly blotted over filter paper and FT-IR spectra (Perkin Elmer, Germany) of each SC disc were recorded. After recording the FT-IR spectra, the same discs were treated with RPTNG for 24 h (equivalent to the permeation studies). Each SC disc after treatment was washed, blotted dry and then air dried for 2 h. Samples were kept under vacuum in desiccators to remove the traces of solvent completely. The FT-IR spectra of treated SC discs were recorded again. Each sample served as its own control (Fig. 1).

2.2.3. DSC studies

Approximately 20 mg SC was taken and hydrated over saturated potassium solution for 3 days. Then the SC was blotted to get hydration between 20 and 25%. The SC was treated with the prepared nanoemulsion system (RPTNG) and conventional gel (REG) separately for 24 h. After treatment, SC was washed with water and

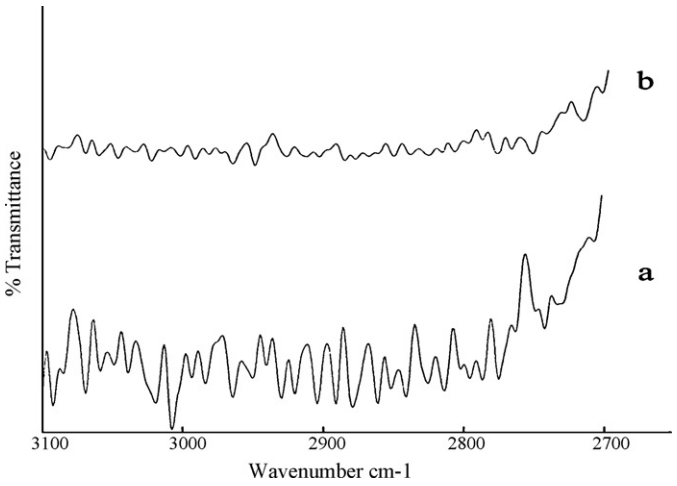

Fig. 1. FT-IR spectra of rat SC showing (A) asymmetric and symmetric C–H stretching absorbances with: (a) control; (b) RPTNG.

blotted dry. It was cut (to obtain weight about 5 mg) and sealed in aluminium hermetic pans. The changes in the structure of SC were assessed by DSC. The SC samples were scanned on a DSC6 Differential Scanning Calorimeter (Perkin-Elmer) at a scanning rate of 5 °C/min over the temperature range of 30–150 °C (Panchagnula et al., 2001) (Fig. 2).

$$\text{percentage of hydration} = \frac{\text{weight of hydrated SC} - \text{weight of dry SC}}{\text{weight of dry SC}}$$

2.2.4. Determination of activation energy

Ex vivo studies of ropinirole across rat skin was carried out at various temperatures (27, 37 and 47 °C of receptor medium). Receptor medium comprised of 30% PEG400-water and RPTNG was taken in donor compartment. Permeability coefficients (K_p) were calculated at each temperature and activation energies of ropinirole were then calculated from Arrhenius relationship (Shakeel et al., 2008; Narishetty and Panchagnula, 2004) (Fig. 3).

$$P = P_o e^{-Ea/RT}$$

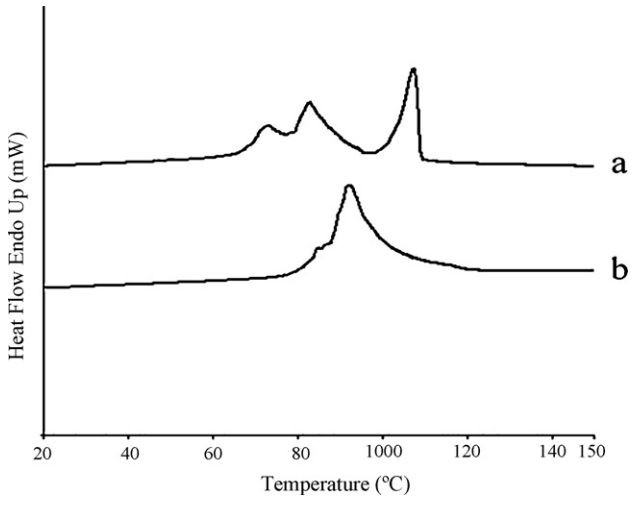

Fig. 2. DSC thermogram of SC: (a) control; (b) treated with RPTNG for 24 h.

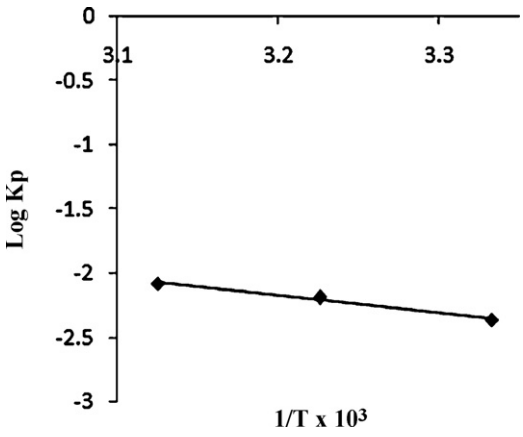

Fig. 3. Arrhenius plot of RPTNG permeation across rat skin.

or

$$\log P = \frac{E_a}{2.303RT} + \log P_o$$

where E_a is the activation energy, R is the gas constant (1.987 kcal/mol), T is the absolute temperature, P is the permeability coefficient, and P_o is the Arrhenius factor.

2.3. Histological evaluation

Histological studies were undertaken to evaluate any irritation evoked in vivo on rat skin after application of RPTNG The *albino wistar* rats were prepared for the drug administration a day before by manually trimming the hair of abdominal region of the rats to the length of 2 mm maximally with a pair of scissors followed by careful shaving with electrical shaver. After application of the nanoemulsion gel on the abdominal skin for a period of 24 h, the rats were sacrificed and specimens of the exposed areas and of adjacent untreated skin area were taken for histological examination. The skin pieces were immediately fixed in 10% formalin. Subsequently, each tissue was rinsed with running water, dehydrated using a graded series of alcohols and embedded in paraffin wax and sections of 5 μm thickness were cut from each sample. The sections were then stained with haematoxylin–eosin for microscopic observation (Motic, Tokyo, Japan). Skin not treated with the formulation served as a control. The skin specimens were evaluated for the elucidation of the mechanism of penetration enhancement (Fig. 4A–D).

2.4. Pharmacokinetic studies

2.4.1. Animals

The animal study protocol to carry out in vivo studies was reviewed and approved by the Institutional Animal Ethics Committee (Approval No. 435, 2008). The male Wistar rats were kept under standard laboratory conditions (temperature 25 ± 2 °C and relative humidity 55 ± 5%) and were housed in polypropylene cages, six per cage, with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water ad libitum.

2.4.2. Experimental

The nanoemulsion gel formulations (RPTNG and RPCNG), ropinirole conventional gel (RPG) and marketed tablet (Ropitor®) were taken for in vivo pharmacokinetic (PK) studies. The dose calculation for the rats was based on the body weight of the rats according to the surface area ratio and was determined to be 1.1 mg/kg (Ghosh, 2005). The skin on the abdominal side of rats was shaved in each group except the group treated with marketed tablet (Ropitor®).

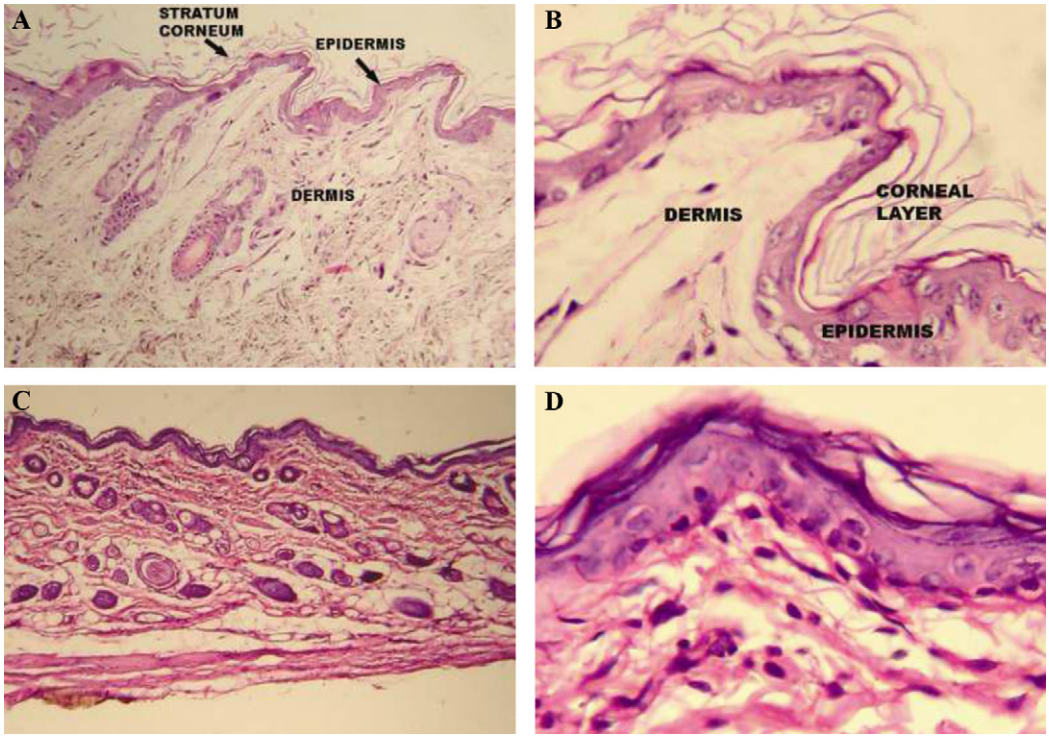

Fig. 4. Photomicrographs of rat skin sample: (A) control group showing normal epidermis, dermis and subcutaneous tissues at low power view (HE 100×); (B) control group at high power view (HE 400×); (C) skin sample from RPTNG treated animal at low power view (HE 100×); (D) RPTNG treated group at high power view (HE 400×).

Before application of the formulations, the rats were kept under observation for 12 h for any untoward effects of shaving and were fasted over this period. The ropinirole formulations RPTNG, RPCNG and RPG were given transdermally while the tablet was administered as suspension orally using oral feeding sonde. The rats were divided into 4 groups ($n = 6$). Group I received RPTNG, Group II received RPCNG, Group III received RPG and Group III received marketed tablet. The rats were anaesthetized using ether and blood samples (0.3 mL) were withdrawn from the tail vein of the rats at 0.5, 1, 2, 3, 6, 12, 24 and 36 h in microcentrifuge tubes in which EDTA was added as an anticoagulant. The blood collected was mixed with the anticoagulant vigorously and centrifuged at 4000 rpm for 15 min. The plasma was separated and stored at −21 °C until drug analysis was carried out using a reported HPLC method with slight modifications (Luzardo-Alvarez et al., 2003).

2.4.3. HPLC analysis of ropinirole in plasma

A Shimadzu model HPLC equipped with quaternary LC-10A VP pumps, variable wavelength programmable UV/VIS detector SPD-10AVP column oven (Shimadzu), SCL 10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 μL loop and Class-VP 5.032 software was used. The drug was extracted from the plasma by using tert-butyl-methyl-ether. Briefly, this solvent was added to the plasma samples, stirred for 1 min, and then centrifuged (4000 rpm × 15 min). The supernatant organic layer was removed. This process was repeated twice. Finally, the organic phase was dried and the residue obtained was dissolved in the mobile phase. Analysis was performed on a LiChrospher 100 C_{18} column (5 μm, 250 mm × 4.6 mm i.d.). The mobile phase consisting of acetonitrile: 0.05 M ammonium acetate buffer pH 2.5 (25:75, v/v) was pumped through the column at a flow rate of 1 mL/min. Aliquots of 20 μL from each sample were injected into the system via the manual injector. All the samples were filtered through 0.22 μm membrane filter prior to injection. Detection was performed at 254 nm (Luzardo-Alvarez et al., 2003).

2.5. Pharmacokinetic and statistical analysis

The plasma concentration of ropinirole at different time intervals was subjected to PK analysis to calculate various parameters: maximum plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), and area under the plasma concentration–time curve ($AUC_{0\to t}$ and $AUC_{0\to\infty}$). The values of C_{max} and T_{max} were read directly from the arithmetic plot of time vs plasma concentration of ropinirole (Fig. 5). The AUC was calculated by using the trapezoidal method (Table 2, Fig. 5). The relative bioavailability (F) of ropinirole after the transdermal administration versus the oral administration was calculated as follows:

$$\%F = \frac{\text{AUC}_{\text{sample}} \times \text{dose}_{\text{oral}}}{\text{AUC}_{\text{oral}} \times \text{dose}_{\text{sample}}} \times 100$$

Data were expressed as mean ± SD. The PK data of different formulations was compared for statistical significance by one

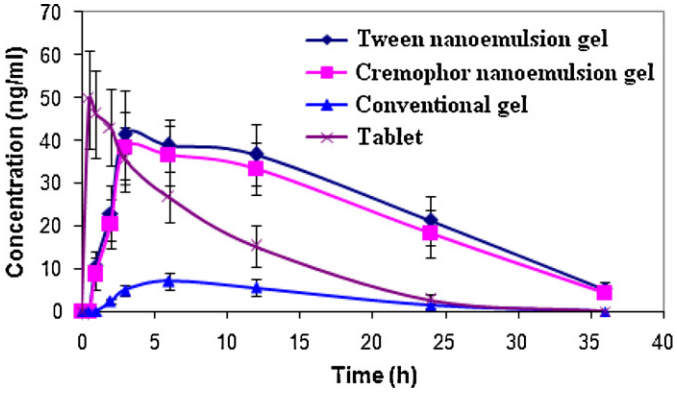

Fig. 5. Plasma concentration time profile of ropinirole from Tween nanoemulsion gel (RPTNG), Cremophor nanoemulsion gel (RPCNG), conventional gel (RPG) and tablet (mean ± SD; $n = 6$).

Table 2
Relative bioavailability and mean pharmacokinetic parameters of ropinirole obtained from RPTNG, RPCNG, RPG and tablet (mean ± S.D; $n = 6$).

Formulation	[a]T_{max} (h)	[b]C_{max} (ng/mL)	[c]$AUC_{0\to t}$ (ng h/mL)	[d]$AUC_{0\to\infty}$ (ng h/mL)	[e]F
RPTNG	3.0* ± 0.8	41.36* ± 7.33	905.78* ± 216.2	1055.99* ± 251.7	7.69#, 2.26##
RPCNG	3.0* ± 0.7	38.18* ± 8.44	811.15* ± 183.4	928.07* ± 206.5	6.76#, 1.99##
RPG	6.0 ± 1.1	7.02 ± 2.04	101.85 ± 22.9	137.25 ± 31.3	–
Tablet	0.5 ± 0.2	49.63 ± 11.56	445.98 ± 98.6	467.15 ± 106.1	–

[a] Time of peak concentration.
[b] Peak of maximum concentration.
[c] Area under the concentration time profile curve until last observation.
[d] Area under the concentration time profile curve from time 0 to infinity.
[e] Relative bioavailability.
[#] Relative bioavailability when compared with RPG.
[##] Relative bioavailability when compared with tablet.
[*] $p < 0.05$ when compared with conventional RPG gel and tablet formulation using one way ANOVA followed by Tukey Kramer multiple comparison test.

way ANOVA followed by Tukey–Kramer multiple comparisons test using GraphPad Instat software (GraphPad Software Inc., CA, USA).

2.6. Biochemical studies

Although the exact cause of Parkinson's disease still remains a mystery, evidences suggest that immense oxidative stress, free radical formation, genetic susceptibility, and programmed cell death all have a role in the development of Parkinson's disease. The neuropathology of the disease is based on cell loss in the dopaminergic nigrostriatal tract of the brain, with the corresponding decrease in the striatal dopamine (DA) concentrations. Following transdermal nanoemulsion gel formulations (RPTNG and RPCNG) treatment, lipid peroxidation, reduced glutathione and catalase activity were determined in the rat striatum and compared with the control and Parkinson induced rats.

2.6.1. Induction of Parkinson brain lesioning

Male Wistar rats obtained from Central Animal House of Jamia Hamdard, weighing 220 ± 10 g and aged 80–90 days at the start of the experiment were used. Rats were housed in groups of three animals per cage and had free access to food and water ad libitum. The animals were anaesthetized with 35 mg/kg sodium pentobarbitone intraperitoneally (i.p.). Each animal was mounted on a stereotaxic stand (Fig. 6) and the skin overlying the skull was cut to expose it, and the coordinates for the striatum (Paxinos and Watson, 1982) were measured accurately as anterio-posterior 0.5 mm, lateral 2.5 mm, dorso-ventral 4.5 mm relative to bragma and ventral from dura with the tooth bar set at 0 mm. Thereafter, all the animals in the experimental groups were lesioned by injecting 10 μg 6-hydroxydopamine (6-OHDA)/2 μL in 0.1% in ascorbic acid-saline into the right striatum, whereas the sham operated group (control) received 2 μL of the vehicle. The injections were made manually with the help of a Hamilton syringe through the burr holes made for the purpose (Fig. 7). The injection rate was 1.0 μL/min and the needle was kept in place for an additional 1.0 min before being slowly retracted. The experiments were in accordance with the guidelines of the Animal Ethics Committee of Jamia Hamdard, New Delhi.

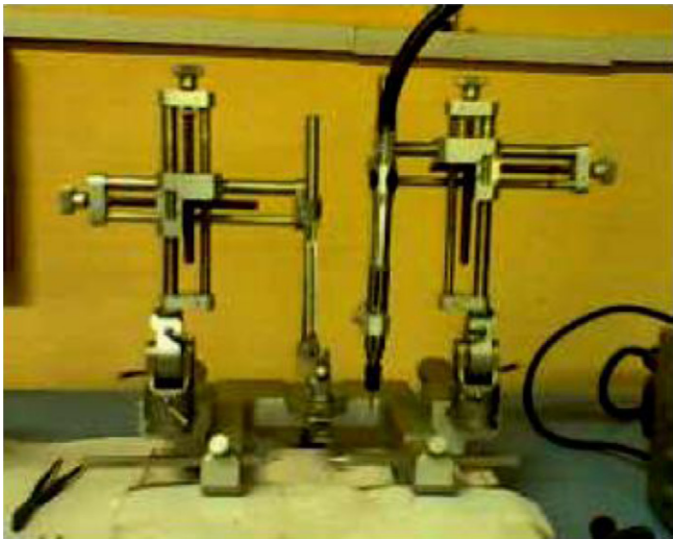

Fig. 6. Stereotaxic apparatus.

2.6.2. Post-operative care

Recovery of anaesthesia took approximately 4–5 h. The rats were kept in a well-ventilated room at 25 ± 3 °C in individual cages till they gained full consciousness and then were housed together in a group of three animals per cage. Food and water were kept inside the cages for the first week so that animals could easily access it without any physical trauma due to overhead injury. Then the animals were treated normally; food, water, and the bedding of the cages were changed every day as usual.

2.6.3. Tissue preparation for antioxidant enzymes and glutathione assays

After 6 weeks (time sufficient for Parkinson induction) the animals were sacrificed and their brains were taken out for harvesting striatum and substantia nigra by cutting acoronal section of 1 mm thickness using rat brain matrix in the light of rat brain atlas. For enzymatic assays, striatum was homogenized (10%, w/v) in 0.01 M phosphate buffer (pH 7.0) and centrifuged at 10,500 × g for 20 min at 4 °C to get post-mitochondrial supernatant (PMS). This was used for the estimation of TBARS (thiobarbituric acid reactive substances) and GSH (glutathione, antioxidant enzyme) and catalase activity.

2.6.4. Lipid per oxidation

The method of Utley et al. (1967) with slight modification was used for the estimation of lipid peroxidation (LPO). Briefly, 0.2 mL PMS was pipetted in an Eppendorf tube and incubated at 37 ± 1 °C in a metabolic water bath shaker for 60 min at 120 strokes up and down; another 0.2 mL was pipetted in an Eppendorf tube and placed at 0 °C incubation. After 1 h of incubation, 0.4 mL of 5% trichloroacetic acid and 0.4 mL of 0.67% TBA was added in both samples (i.e., 0 °C and 37 °C). The reaction mixture from the vial was transferred to the tube and centrifuged at 1125 × g for 15 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was measured at 535 nm. The rate of

Fig. 7. Injection of 6-hydroxydopamine (6-OHDA) into rat striatum for Parkinson's disease induction.

lipid peroxidation was expressed as nmol of TBARS reactive substance formed/(h mg protein) (Fig. 8).

2.6.5. Assay for reduced glutathione content (GSH)

GSH was determined by the method of Jollow et al. (1974). 0.2 mL of PMS (10%, w/v) was precipitated with 0.2 mL of sulfosalicylic acid (4%). The sample were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 × *g* for 15 min at 4 °C. The assay mixture contained 0.1 mL of filtered aliquot (10%, w/v), 1.7 mL phosphate buffer (0.1 M, pH 7.4) and 0.2 mL DTNB (5-5′-didithiobis-2-nitrobenzoic acid) (4 mg/1 mL of phosphate buffer, 0.1 M, pH 7.4) in a total volume of 2 mL. The yellow color developed was read immediately at 412 nm (Fig. 9).

2.6.6. Determination of catalase activity

Catalase activity was assayed by the method of Coliborne (1985). Briefly, the assay mixture consisted of 2 mL phosphate buffer (0.1 M, pH 7.4), 0.95 mL hydrogen peroxide (0.019 M) and 0.05 mL of PMS in a total volume of 3.0 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/(min mg protein) (Fig. 10).

3. Results and discussion

3.1. FT-IR spectral analysis of nanoemulsion formulation-treated and untreated rat skin

IR spectra were recorded in the frequency range 4000–10,000 cm^{-1}. Fig. 1 shows the typical FT-IR spectra of control (untreated) and nanoemulsion gel treated SC over the wavenumber range 3100–2700 cm^{-1}. Many of the IR spectra bands of SC can be attributed to lipid and protein molecular vibrations. The molecular vibrations of lipids and proteins are related to various peaks in the IR spectrum of the SC. The bands at 2920 and 2850 cm^{-1} were due to the asymmetric CH_2 and symmetric CH_2 vibrations of long chain hydrocarbons of lipids. The height and area of these two bands are proportional to the amount of the lipids present. Therefore, any extraction of SC results in a decrease of peak height and area. The shift of CH_2 stretching peaks to a higher wave number (trans to gauche conformation) and increase in their peak widths indicate fluidization of the SC. The bands at 1650 cm^{-1} and 1550 cm^{-1} are due to the amide I and amide II bands from C=O stretching and N–H bending vibration. The frequencies of these two bands, especially amide I band, are

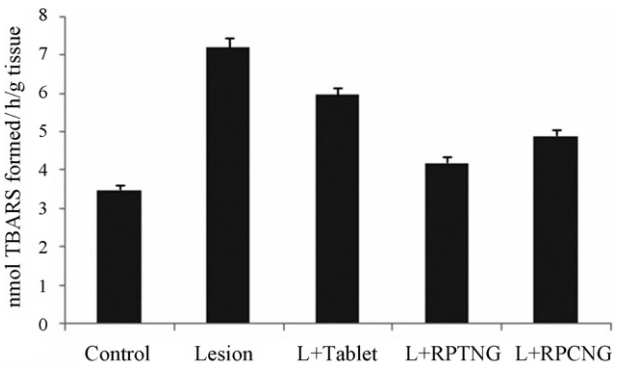

Fig. 8. Effect of nanoemulsion formulations on thiobarbituric acid reactive substances (TBARS) content in rats lesioned by 6-OHDA ($n = 6$).

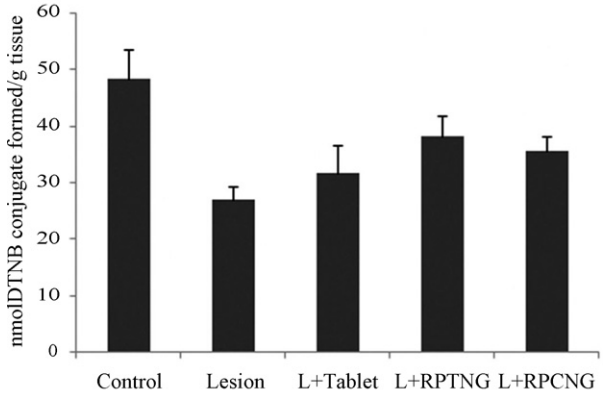

Fig. 9. Effect of nanoemulsion formulations on GSH content in lesioned rats ($n = 6$).

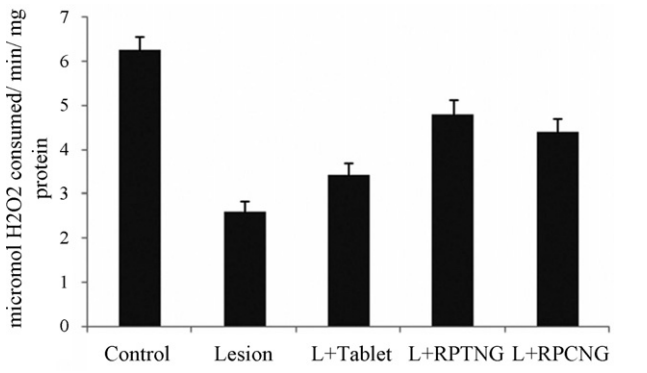

Fig. 10. Effect of nanoemulsion formulations on catalase activityin lesioned rats ($n = 6$).

sensitive and shift to higher or lower frequencies according to the change in protein conformation (Tori and Tasumi, 1996).

There was a marked difference in the FT-IR spectra of untreated and nanoemulsion gel (RPTNG) treated SC with prominent decrease in the height and area of asymmetric and symmetric C–H stretching (Fig. 3). The decrease in peak height suggested an overall extraction of the lipids from the SC. This disruption might have led to the formation of microcavities in the bilayer and, which may in turn, had increased the volume available for drug diffusion. Hence, extraction of the lipids from SC might have led to enhanced percutaneous absorption of the drug. As the peak shift to a higher wavenumber was not observed, it was inferred the RPTNG formulation did not fluidize the lipids.

3.2. DSC studies

The mechanism of skin permeation enhancement was further elucidated by DSC studies. DSC provides another method to probe changes in SC structure, based on its thermal properties. Thermogram of the skin specimen treated with RPTNG was compared with the control sample. DSC thermogram of untreated rat epidermis presented three endotherms which were obtained at 68 °C (T1), 79 °C (T2) and 104 °C (T3) (Fig. 2a). The first and second endotherm T1 and T2 appeared due to the melting of the SC lipids and the third endotherm was attributed to the keratin denaturation. It was observed that both T2 and T3 endotherms shifted to lower melting points or disappeared in thermogram of the treated samples (Fig. 2). This indicated that the components of RPTNG enhanced skin permeation of drugs through extraction of SC lipids.

Another lipid peak was reported at 32 °C was not observed in our experiments which might be attributable to the low enthalpy of this peak (Naik and Guy, 1997) and it has not been detected by many investigators. This endotherm was attributed to sebaceous secretion (Lee et al., 2005) or surface contamination and to minor structural rearrangement within the lipid bilayers. Due to the very small enthalpy and absence from thermograms of many samples, the importance of this endotherm while investigating the mechanism of action of penetration enhancers remains obscure.

3.3. Activation energy determination

The magnitude of activation energy (E_a) for diffusion of a drug molecule across skin depends on its route of diffusion and physicochemical properties of substance. It is affected by change in skin composition or phase behavior of lipids. Therefore, nanoemulsions by their action on SC lipid bilayers may change the E_a. Permeation studies were carried out at 27, 37 and 47 °C and K_p were determined at all temperatures. Subsequently, log K_p values were plotted against $1/T$ as depicted in Fig. 3. The Arrhenius plot was found to be linear in the temperature range studied, indicating no significant structural or phase transition changes had occurred within the skin membrane. The activation energy for ion transport has been reported as10.7 kcal/mol across rat phosphatidylcholine bilayer (Monti et al., 1995) and 4.1 kcal/mol across human epidermis (Pagano and Thompson, 1968). The E_a value for the permeation of ropinirole from RPTNG across rat skin was calculated from the slope of the Arrhenius plot and it was found to be 1.379 kcal/mol. The decrease in E_a for ropinirole permeation across rat skin indicated that the SC lipid bilayers were significantly disrupted ($p < 0.05$).

3.4. Histological studies

The skin specimen was histologically examined to evaluate the cutaneous irritation potential of the optimized formulation (RPTNG). The photomicrographs of untreated rat skin (control) showed normal skin with well defined epidermal and dermal layers (Fig. 4A and B). Uniformly layered SC and loosely textured collagen in the dermis could be observed. Dermis was also devoid of any inflammatory cells. In contrast to this definite changes were observed in the skin morphology when it was treated with RPTNG. The treated section (Fig. 4C and D) showed a clear disruption of SC organization confirming the penetration enhancing capacity of these vesicular carriers. Dermis did not show any edema or inflammatory cell infiltration. There were no apparent signs of skin irritation (erythema and edema) observed on visual examination of the skin specimens treated with RPTNG formulation indicating that developed formulation is nonirritating. Also in the present study optimized NEG components (oil, surfactant and cosurfactant) were fall under GRAS (Generally regarded as safe) category and hence the formulation appeared to be safe for transdermal delivery.

3.5. PK studies

PK analysis was performed by non-compartmental (model independent) method. The total drug exposure was estimated by determining area under the curve (AUC) by using trapezoidal method. The comparative pharmacokinetic profiles of RPG, RPTNG, RPCNG and tablet are depicted in Fig. 5 while the calculated PK parameters are given in Table 1. T_{max} was found to be 3 ± 0.8 h and 3 ± 0.7 h for RPTNG and RPCNG, respectively while it was about 6 ± 1.1 h for RPG. On the other hand, T_{max} of ropinirole tablet was only 0.5 ± 0.2 h. The difference between T_{max} of nanoemulsion gel formulations and RPG was highly significant ($p < 0.05$) when compared to that of tablet and evaluated statistically. The difference in C_{max} of RPTNG and RPCNG formulations were also significant when compared with the tablet and RPG ($p < 0.05$).

The C_{max} attained with RPTNG, RPCNG, RPG and tablet were 41.36 ± 7.33, 38.18 ± 8.44, 7.02 ± 2.04 and 49.63 ± 11.56 ng/mL, respectively. The low C_{max} and prolonged T_{max} observed with the transdermal formulations in contrast to the oral administration can be attributed to the efficient barrier properties of the skin.

The PK studies revealed greater extent of absorption from nanoemulsion gel formulations (RPTNG and RPCNG) than the tablet and conventional gel of ropinirole. The $AUC_{0\to\infty}$ for RPTNG, RPCNG, RPG and tablet were found to be 1055.99 ± 251.7, 928.07 ± 206.5, 137.25 ± 31.3 and 467.15 ± 106.1 ng h/mL, respectively (Table 2). The observed difference in $AUC_{0\to t}$ and $AUC_{0\to\infty}$ of RPTNG and RPCNG formulation were found to be significant as compared to tablet and RPG formulations ($p < 0.05$). The significantly high AUC value observed with nanoemulsion gel formulations (RPTNG and RPCNG) indicated increased bioavailability of the drug as compared to oral administration. The absorption of ropinirole from RPTNG resulted in 2.26-fold increased in bioavailability as compared to conventional tablet formulation and 7.69 times with reference to the RPG gel formulation. Similarly the absorption of

Table 3
Treatmant groups for biochemical tests.

Group no.	Group	Administration route	No. of rats
I	Control	–	6
II	Parkinson induced [lesioned group (L)]	–	6
III	L + tablet	Oral	6
IV	L + RPTNG	Transdermal	6
V	L + RPCNG	Transdermal	6

ropinirole from RPCNG resulted in 1.99-fold increased in bioavailability as compared to conventional tablet formulation and 6.76 times with reference to the RPG gel formulation. The higher absorption achieved with respect to the nanoemulsion gel formulations might be due to the presence of efficient permeation enhancers in the form of surfactant and cosurfactant in them. Also, the drug was present in the solubilized form which resulted in better permeation when compared with conventional gel where the drug was in dispersed form. Increased bioavailability from RPTNG and RPCNG formulations in comparison to oral tablet formulation might be due to the avoidance of the hepatic first pass metabolism.

3.6. Biochemical studies

Although the etiology of Parkinson disease has not been fully elucidated, the generation of reactive oxygen species (ROS), leading to oxidative stress, together with a relative paucity of antioxidant defenses in the substantia nigra and nigrostriatal dopaminergic pathway, is widely considered as the final biochemical cause of neuronal death (Gille et al., 2004). The brain is thought to be vulnerable to oxidative damage due to its high oxygen consumption, presence of high levels of polyunsaturated fatty acids, and the non-regenerative nature of neurons (Floyd and Carney, 1992). A major consequence of oxidative stress is damage to cellular macromolecules. Auto-oxidation of DA and 6-OHDA produces H_2O_2, which is subsequently converted to hydroxyl radical by Fe^{2+}, causing fragmentation of the lipid or alteration of its chemical structure (Floor, 2000). Fatty aldehydes, such as 4-hydroxynonenal, that are generated as a result of lipid peroxidation can react with free thiol groups, such as cysteines on proteins, to produce thioesters, which may affect protein function and stability (Halliwell, 1992). This results in the damage of DNA, membrane lipid, carbohydrate, proteins and finally damage. Thus, it can be predicted that 6-OHDA potentiates lipid peroxidation in the nigrostriatal system. The rat striatum was taken out 24 h and divided into five different treatment groups (Table 3) post transdermal administration of ropinirole nanoemulsion gels (RPTNG, RPCNG) and oral administration of ropinirole tablet suspension in case of drug treated lesioned animals. Various biochemical estimations were carried out subsequently. The content of TBARS in striatum was elevated (106.6%) significantly ($p < 0.001$) in the lesioned group (L) as compared to the control group (Fig. 8). The increased TBARS level was significantly restored (86.28% and 66.28%) in L + RPTNG (Group IV) and L + RPCNG groups, respectively while restoration was only 35.71%. in case of lesioned group treated with the tablet (Group III). On the other hand, the content of GSH was depleted (44.42%) significantly ($p < 0.001$) in the L group as compared to the control group (Fig. 9). The depleted level was restored significantly (23.44% and 18%) in L + RPTNG (Group IV) and L + RPCNG group (Group V) respectively and only 9% with tablet.

An inverse relationship has also been previously reported between LPO and GSH activities and its related enzymes in Parkinsonism (Zafar et al., 2003; Cohen, 1984). A reduction in GSH may impair H_2O_2 clearance and promote OH formation, thus increasing the free radical load, which triggers oxidative stress and consequently disrupts homeostasis. It is reasonable to infer that depletion in GSH triggers LPO, leading to the degeneration of nigrostriatal neurons, which, in turn, would deplete DA. The increase in the content of GSH and decrease in the extent of LPO showed the beneficial effects of the developed nanoemulsion formulation in Parkinsonism.

The activity of catalase in striatum was significantly ($p < 0.001$) decreased (58.49%) in the L group as compared to the control group (Fig. 10). The depleted level was restored significantly (35.09% and 28.84%) in L + RPTNG group (Group IV) and L + RPCNG (Group V) respectively. In contrast to this, restoration was only 13.30% with the tablet suspension treated group (Group III).

It was observed that the 6-OHDA-induced lesions produce the characteristic alterations in the behavioral pattern of our experimental model, which was further corroborated by the biochemical alterations in the various classical parameters used in characterizing Parkinson and Parkinson-related disorders. It can be concluded on the basis of above biochemical studies that ropinirole nanoemulsion gel afforded better activity as compared to conventional tablet in case of Parkinson induced rats.

4. Conclusions

The extract of our investigation, suggested a sufficient manipulation of stratum corneum barrier induced by nanoemulsion formulation. However, lack of histological injuries supported the safety prospective of the formulation. The studies also showed that nanoemulsion gel formulations of ropinirole had good pharmacokinetic features which can replace oral dosage form for the same. Furthermore nanoemulsion gel of ropinirole proved its mettle in restoration of biochemical changes in Parkinson's disease model in rats. Hence, pharmacokinetic superiority as well as better biochemical recovery of brain using the transdermal nanoemulsion gel of ropinirole inferred to its significant value in clinical treatment of Parkinson's disease.

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References

Azeem, A., Rizwan, M., Ahmad, F.J., Iqbal, Z., Aqil, M., Khar, R.K., Talegaonkar, S., 2009a. Nanoemulsion components screening and selection: a technical note. AAPS PharmSciTech 10, 69–76.
Azeem, A., Rizwan, M., Ahmad, F.J., Khar, R.K., Iqbal, Z., Talegaonkar, S., 2009b. Components screening and influence of surfactant and cosurfactant on nanoemulsion formation. Curr. Nanosci. 5, 220–226.
Blank, I.H., Scheuplein, R.J., 1969. Transport into and within the skin. Br. J. Dermatol. 81, 4–10.
Chase, T.N., 1998. The significance of continuous dopaminergic stimulation in the treatment of Parkinson's disease. Drugs 55, 1–9.
Cohen, G., 1984. Oxyradical toxicity in catecholamine neurons. Neurotoxicology 5, 77–82.
Coliborne, A., 1985. Catalase activity. In: Green Wald, R.A. (Ed.), CRC Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, FL, pp. 283–284.
Dollery, C., 1999. Therapeutic Drugs, second ed. Churchill Livingstone, Edinburgh.
Findley, L.J., Aujla, M., Bain, P., Baker, M., Beech, C., Bowman, C., Holmes, J., Kingdom, W.K., MacMohan, D.G., Peto, V., Playfer, J.R., 2003. Direct economic impact of Parkinson's disease: a research study in the United Kingdom. Mov. Disord. 18, 1139–1145.
Floor, E., 2000. Iron as a vulnerability factor in nigrostriatal degeneration in aging and Parkinson's disease. Cell. Mol. Biol. 46, 709–720.
Floyd, R.A., Carney, J.M., 1992. Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. Ann. Neurol. 32, S22–S27.
Ghosh, M.N., 2005. Fundamentals of Experimental Pharmacology, third ed. Hilton and Company, Kolkata.

Gille, G., Hung, S.T., Reichmann, H., Rausch, W.D., 2004. Oxidative stress to dopaminergic neurons as models of Parkinson's disease. Ann. N.Y. Acad. Sci. 1018, 533–540.
Grosset, K.A., Bone, I., Grosset, D.G., 2005. Suboptimal medication adherence in Parkinson's disease. Mov. Disord. 20, 1502–1507.
Halliwell, B., 1992. Reactive oxygen species and the central nervous sytem. J. Neurochem. 59, 1609–1623.
Hely, M.A., Morris, J., Reid, W.G.J., 2005. Sydney multicenter study of Parkinson's disease: non-motor problems dominate at 15 years. Mov. Disord. 20, 190–199.
Jain, A.K., Thomas, N.S., Panchagnula, R., 2001. Transdermal drug delivery of imipramine hydrochloride. I. Effect of terpenes. J. Control. Release 79, 93–101.
Jollow, D.J., Mitchell, J.R., Zampagloine, N., Gillete, J.R., 1974. Bromo-benzene induced liver necrosis: protective role of glutathione and evidence for 3,4-bromobenzeneoxide as the hepatotoxic intermediate. Pharmacology 11, 151–169.
Kaye, C.M., Niholls, B., 2000. Clinical pharmacokinetics of ropinirole. Clin. Pharmacokinet. 39, 243–254.
Lee, J., Lee, Y., Kim, J., Yoon, M., Choi, Y.K., 2005. Formulation of microemulsion systems for transdermal delivery of aceclofenac. Arch. Pharm. Res. 28, 1097–1102.
Luzardo-Alvarez, A., Delgado-Charro, M.B., Blanco-Mendez, L., 2003. In vivo iontophoretic administration of ropinirole hydrochloride. J. Pharm. Sci. 92, 2441–2448.
Monti, D., Saettone, M.F., Giannaccini, B., Galli-Angeli, D., 1995. Enhancement of transdermal penetration of dapiprazole through hairless mouse skin. J. Control. Release 33, 71–77.
Naik, A., Guy, R.H., 1997. Infrared spectroscopic and differential scanning calorimetric investigations of the stratum corneum barrier function. In: Potts, R.O., Guy, R.H. (Eds.), Mechanisms of Transdermal Drug Delivery. Marcel Dekker, New York, pp. 87–162.
Narishetty, S.T.K., Panchagnula, R., 2004. Transdermal delivery of zidovudine: effect of terpenes and their mechanism of action. J. Control. Release 95, 367–379.
Nyholm, D., 2006. Pharmacokinetic optimization in the treatment of Parkinson's disease: an update. Clin. Pharmacokinet. 45, 109–136.
Olanow, C.W., Agid, Y., Mizuno, Y., Albanese, A., Bonucelli, U., Damier, P., Yebenes, J.D., Gershanik, O., Guttman, M., Grandas, F., Hallett, M., Hornykiewicz, O., Jenner, P., Katzenschlager, R., Langston, W.J., Lewitt, P., Melamed, E., Mena, M.A., Michel, P.P., Mytilineou, C., Obeso, J.A., Poewe, W., Quinn, N., Raisman-Vozari, R., Rajput, A.H., Rascol, O., Sampaio, C., Stocchi, F., 2005. Levodopa in the treatment of Parkinson's disease: current controversies. Mov. Disord. 20, 645.
Pagano, R., Thompson, T.E., 1968. Spherical bilayer membranes: electrical and isotopic studies of ion permeability. J. Mol. Biol. 38, 41–57.
Panchagnula, R., Salve, P.S., Thomas, N.S., Jain, A.K., Ramarao, P., 2001. Transdermal delivery of naloxone: effect of water, propylene glycol, ethanol and their binary combinations on permeation through rat skin. Int. J. Pharm. 219, 95–105.
Paxinos, G., Watson, C., 1982. The Rat Brain Sterotaxic Coordinates. Academic Press, Sydney.
Rizwan, M., Aqil, M., Azeem, A., Talegaonkar, S., Sultana, Y., Ali, A., 2010. Enhanced transdermal delivery of carvedilol using nanoemulsion as a vehicle. J. Exp. Nanosci. 5, 390–411.
Shakeel, F., Baboota, S., Ahuja, A., Ali, J., Shafiq, S., 2008. Skin permeation mechanism of aceclofenac using novel nanoemulsion formulation. Pharmazie 63, 580–584.
Schrag, A., Jahanshahi, M., Quinn, N.P., 2000. What contributes to quality of life in patients with Parkinson's disease. J. Neurol. Neurosurg. Psychiatry 69, 308–312.
Talegaonkar, S., Akhter, S., Jain, G.K., Ahmad, F.J., Khar, R.K., Jain, N., Khan, Z.I., 2008. Investigation of nanoemulsion system for transdermal delivery of domperidone: ex-vivo and in vivo studies. Curr. Nanosci. 4, 381–390.
Tori, H., Tasumi, M., 1996. Theoretical analyses of the amide I infrared bands of globular proteins. In: Mantsch, H.H., Chapman, D. (Eds.), Infrared Spectroscopy of Biomolecules. Wiley-Liss, New York, pp. 1–18.
Utley, H.C., Bernheim, F., Hochslein, P., 1967. Effect of sulfhydryl reagent on peroxidation in microsome. Arch. Biochem. Biophys. 260, 521–531.
Vaddi, H.K., Ho, P.C., Chan, S.Y., 2002. Terpenes in propylene glycol as skin penetration enhancers: permeation and partition of haloperidol, fourier transform infrared spectroscopy and differential scanning calorimetry. J. Pharm. Sci. 91, 1639–1651.
Zafar, K.S., Siddiqui, A., Sayeed, I., Ahmad, M., Saleem, S., Islam, F., 2003. Protective effect of adenosine in rat model of Parkinson's disease: neurobehavioral and neurochemical evidences. J. Chem. Neuroanat. 26, 143–151.