TURKEY - FRANCE
HACETTEPE UNIVERSITY - FRANÇOIS RABELAIS DE TOURS UNIVERSITY
INSTITUTE of HEALTH SCIENCES - UMR INSERM U 930

THE PREPARATION OF THERAGNOSTIC IMMUNOLIPOSOMES/IMMUNONIOSOMES FOR THE DIAGNOSIS AND THERAPY OF PARKINSON'S DISEASE

Pharm. Mine SILINDIR GUNAY (M.Sc.)

Radiopharmacy Programme - UMR Inserm U 930, Team 3, Molecular Imaging and Brain

DOCTORAL THESIS

ANKARA – TOURS
2016

TURKEY - FRANCE
HACETTEPE UNIVERSITY - FRANÇOIS RABELAIS DE TOURS UNIVERSITY
INSTITUTE of HEALTH SCIENCES - UMR INSERM U 930

THE PREPARATION OF THERAGNOSTIC IMMUNOLIPOSOMES/IMMUNONIOSOMES FOR THE DIAGNOSIS AND THERAPY OF PARKINSON'S DISEASE

Pharm. Mine SILINDIR GUNAY (M.Sc.)

Radiopharmacy Programme - UMR Inserm U 930, Team 3, Molecular Imaging and Brain

DOCTORAL THESIS

THESIS ADVISOR
Prof.Dr. A. Yekta ÖZER

THESIS CO-ADVISORS
Dr. Sylvie CHALON
Prof.Dr. Denis GUILLOTEAU

ANKARA - TOURS
2016

UNIVERSITÉ FRANÇOIS – RABELAIS DE TOURS

ÉCOLE DOCTORALE SSBCV

UMR INSERM U930, « Imagerie et Cerveau »

Equipe « Imagerie moleculaire du cerveau »

THÈSE présentée par :

Mine SILINDIR GUNAY

soutenue le : **8 Septembre 2016**

pour obtenir le grade de : **Docteur de l'université François-Rabelais de Tours**

Discipline/ Spécialité : Sciences de la Vie et de la Sante/ Neurosciences, Radiopharmacie

THE PREPARATION OF THERAGNOSTIC IMMUNOLIPOSOMES/IMMUNONIOSOMES FOR THE DIAGNOSIS AND THERAPY OF PARKINSON'S DISEASE

THÈSE dirigée par :

[OZER A. Yekta]	Prof.Dr., Hacettepe University
[CHALON Sylvie]	Dr., Université François-Rabelais de Tours
[GUILLOTEAU Denis]	Prof.Dr., Université François-Rabelais de Tours

RAPPORTEURS :

[LAGARCE Frederic]	Prof.Dr., Angers University
[ASIKOGLU Makbule]	Prof.Dr., Ege University

JURY :

[OZER A. Yekta]	Prof.Dr., Hacettepe University
[CHALON Sylvie]	Dr., Université François-Rabelais de Tours
[GUILLOTEAU Denis]	Prof.Dr., Université François-Rabelais de Tours
[SAKA TOPCUOGLU Esen]	Prof.Dr., Hacettepe University
[VURAL Imran]	Prof.Dr., Hacettepe University
[ERDOGAN Suna]	Assoc.Prof.Dr., Hacettepe University

ALTERNATE MEMBER :

[ARICA YEGIN Betul]	Prof.Dr., Hacettepe University

Department	:Radiopharmacy
Program	:Radiopharmacy Doctoral Program
Dissertation Title	:The Preparation of Theragnostic Immunoliposomes/Immunoniosomes for the Diagnosis and Therapy of Parkinson's Disease
Name of the Student	:Mine Silindir Günay
Date of Dissertation Defence	:08.09.2016

This study has been accepted and approved as a Master / PhD dissertation by the examining committee, whose members are listed below.

Chair of the Committee and Advisor of the Dissertation:	Prof.Dr. A. Yekta Özer (Hacettepe University)	(Signature)
Member:	Dr. Sylvie Chalon (Tours University)	(Signature)
Member:	Prof.Dr. Denis Guilloteau (Tours University)	(Signature)
Member:	Prof.Dr. Esen Saka Topçuoğlu (Hacettepe University)	(Signature)
Member:	Assoc.Prof.Dr. Suna Erdoğan (Hacettepe University)	(Signature)
Member:	Prof.Dr. Betül Arıca Yegin (Hacettepe University)	(Signature)
External Reviewer:	Prof.Dr. Makbule Aşıkoğlu (Ege University)	(Signature)
External Reviewer:	Prof.Dr. Frederic Lagarce (Angers University)	UFR SANTÉ Directeur du Département Pharmacie Frédéric LAGARCE

APPROVAL

This dissertation has been approved by the committee above in conformity to the regulations and bylaws of Hacettepe University Graduate Programs andhas been accepted by the Board of Directors of the Institute of Health Sciences.

(Signature)

Prof. Diclehan ORHAN M.D.,Ph.D

Institute Director

ACKNOWLEDGEMENT

I want to deeply thank;

My supervisor, Prof.Dr. A. Yekta ÖZER, for her great effords and support during thesis, preparation of protocols between Hacettepe Univ. and François Rabelais de Tours Univ. and obtaining my scholarschip.

My co-supervisors, Dr. Sylvie CHALON and Prof.Dr. Denis GUILLOTEAU for their effords and understanding during thesis, preparation of protocols and proceeding my experiments at Tours in the lab of UFR, UMR Inserm U930, Equipe 3, Imagerie Moléculaire du cerveau, Imagerie et cerveau.

To Assoc.Prof.Dr. Suna ERDOĞAN for her efforts, support and understanding within thesis period and Prof.Dr. Esen SAKA TOPÇUOĞLU for her leading knowledge.

Mrs. Sylvie BODARD for her assistance during my animal experiments at Tours in the lab of Equipe 3 Imagerie Moléculaire du cerveau, Inserm U930, UFR. Mrs. Zuhal GULAN for autoradiography studies and Dr. İpek BAYSAL and Prof.Dr. İmran VURAL, Ecz. Naile ÖZTÜRK for cell culture studies.

TUBITAK-SBAG for project grant (Project No:112S244), Dr. Maria BONNAFOUS BOUCHER, Dr. Alexis MİCHEL and Sibel ERDOĞAN YALÇIN at French Embassy-Service of Cooperation and Cultural Actions and Campus France for grant.

Dr. Ferhad Farsi from Abdi İbrahim Pharmaceutical Industry for generous gifts for Pramipexole, Lipoid for phospholipids, Ibrahim Ethem Pharmaceutical Industry, Dr. Özlem ATİK for the help of transport of phospholipids.

All members of Hacettepe University Faculty of Pharmacy Department of Radiopharmacy and Tours University UFR, UMR Inserm U930, Team 3 and Hacettepe University Pharmaceutical Technology Department and Dr. Tugba GÜLSÜN İNAL for their great support.

MY HUSBAND Necati GÜNAY for his great support and tolerance in my life during the thesis period.

MY FAMILY for giving me a great love and support during thesis period as well as every stage of my life.

ABSTRACT

M.Sci.Pharm. Mine Silindir Gunay, The Preparation of Theragnostic Immunoliposomes/Immunoniosomes for the Diagnosis and Therapy of Parkinson's Disease, Hacettepe Üniversitesi – François Rabelais de Tours University, Health Sciences Institute, Radiopharmacy Doctoral Programme, UMR Inserm U 930, Team 3, Molecular Imaging and Brain Programme, Doctoral Thesis, Ankara-Tours, 2016. Parkinson's Disease (PD) is degeneration of dopamine producing cells in substantia nigra. Blood-brain barrier (BBB) is a strong obstacle in PD therapy. More penetration and accumulation in the target tissue can be obtained by preventing RES uptake via "stealth effect". Liposomes and niosomes are the promising systems for being biodegredable, bioavailable, non-toxic and targetable. Although CNS disorders are the first to endorse at their research in the diagnosis and therapy with several framework projects in Europe and over the world, there is still a huge gap in CNS drug delivery and the success of PD therapy. Although different studies have performed with pramipexole, evaluation of penetration and antiparkinsonian effect of pramipexole encapsulated liposomes and niosomes has never been studied before.

Among this thesis, nanosized, polyethylene glycol (PEG) coated, neutral and positively charged, pramipexole encapsulated liposomes and noisomes were formulated, characterized and release kinetics of the systems were evaluated. In vitro penetration of all formulations was evaluated in BBB cell co-culture model. Therapeutic efficacy of neutral, pramipexole encapsulated liposomes and niosomes were evaluated in 6-hydroxydopamine (6-OHDA) lesioned rats by rotometer test and autoradiography.

All formulations have approximately 10% encapsulation efficiency, around 100 nm particle sizes and fitted to first-order release kinetics. All formulations were found BBB permeable at in vitro cell culture studies. Nanosized, neutral niosomes designated similar but slightly better effect than pramipexole solution in autoradiograhy studies in 6-OHDA lesioned rats. This pramipexole dose is approximately 9 times lesser doses applied with conventional pramipexole tablets for humans in Neurology clinics. Nanosized, pramipexole encapsulated, neutral niosomes showed potential PD therapeutic effect in PD animal model depending on non-ionic surfactant properties of niosomes.

Key Words: Pramipexole Liposomes, Pramipexole Niosomes, Brain Targeting, Parkinson's Disease Therapy, Dopamine Transporter Autoradiography.

Supporting Organizations: TUBITAK-SBAG (Project No: 112S244).
French Embassy-Service of Cooperation and Cultural Actions, Campus France.

ÖZET

Uzm. Ecz. Mine Silindir Gunay, Parkinson Hastalığı'nın Teşhis ve Tedavisi İçin Kullanılacak Nanoboyutlu Teragnostik İmmünolipozom/İmmunoniozomlar Üzerine İn Vitro İn Vivo Çalışmalar, Hacettepe Üniversitesi – François Rabelais de Tours University, Sağlık Bilimleri Enstitüsü, Radyofarmasi Programı, UMR Inserm U 930, Ekip 3, Moleküler Görüntüleme ve Beyin Programı, Doktora Tezi, Ankara-Tours, 2016. Parkinson Hastalığı (PH) substantia nigra'daki dopamin üreten hücrelerin dejenerasyonundan kaynaklanmaktadır. Kan-beyin bariyeri (KBB) PH'nın tedavisinin önünde kuvvetli bir engeldir. Hedef dokudaki yüksek penetrasyon ve tutulum "*stealth etki*" ile RES tutulumunun engellenmesi ile sağlanabilir. Lipozom ve niozomlar biyoparçalanırlıkları, biyouyumlulukları, non-toksik ve hedeflendirilebilir olmaları nedeniyle en çok tercih edilen sistemlerdendir. Santral sinir sistemi hastalıklarının araştırılması Avrupa ve tüm dünyada yapılan pekçok çerçeve projelerinde ilk sırada olmasına rağmen, halen beyne ilaç taşınması ve PH'nin tedavi başarısı konusunda büyük boşluklar bulunmaktadır. Pramipeksol ile pek çok çalışma yapılmasına karşılık, bizim çalışmamız pramipeksol enkapsüle edilmiş lipozom ve niozomların beyin penetrasyonunun ve antiparkinson etkisinin değerlendirilmesi konusunda yenidir.

Tez kapsamında, nanoboyutlu, PEG kaplı, nötral ve pozitif yüklü lipozom ve niozomların formüle edilmiş, karakterizasyon ve salım kinetikleri değerlendirilmiştir. Tüm formülasyonların KBB geçirgenliği, hücre KBB ko-kültürü çalışmalarında incelenmiştir. Nötral, pramipeksol enkapsüle edilen lipozom ve niozomların tedavi etkinliği in vivo olarak 6-hidroksidopamin (6-OHDA) ile lezyon yapılarak PH modeli oluşturulan sıçanlarda rotametre ve otoradyografi çalışmaları ile incelenmiştir.

Tüm formülasyonlar yaklaşık %10 enkapsülasyon etkinliği ve 100 nm civarında partikül boyutu dağılımı ve birinci derece salım kinetiği göstermiştir. Hücre kültürü çalışmalarında, tüm formülasyonların KBB'nden penetre olabildiği saptamıştır.

6-OHDA lezyonlu sıçanlarda Parkinson hastalığının tedavisinde nanoboyutlu, nötral, pramipeksol enkapsüle edilen niozomlar, aynı dozdaki pramipeksol çözeltisi ile benzer hatta biraz daha iyi sonuçlar göstermiştir. Bu doz Nöroloji kliniklerinde Parkinson tedavisinde rutin olarak kullanılan konvansiyonel pramipeksol tabletlerindeki dozun yaklaşık olarak 9 kat düşük dozlarıdır. Nanoboyutlu, pramipeksol enkapsüle edilen, nötral niozomlar, niozomların non-iyonik sürfaktan özellikleri nedeniyle PH modeli sıçanlarda potansiyel bir antiparkinson terapötik etki göstermiştir.

Anahtar Sözcükler: Pramipeksol Hapsedilmiş Lipozomlar, Pramipeksol Hapsedilmiş Niozomlar, Beyne Hedeflendirme, Parkinson Hastalığı'nın Tedavisi, Dopamin Transporter Otoradyografi.

Destekleyen Kuruluşlar: TUBITAK-SBAG (Project No: 112S244).
Fransa Büyükelçiliği-Kültür Ataşeliği, Campus France.

RÉSUMÉ

M.Sci.Pharm. Mine Silindir Gunay, Les teragnostiques immunolipozom/immuniozom en nanocristallin pour utiliser dans les recherches in vitro in vivo de pronostic et traitement de la maladie de Parkinson, L'université de Hacettepe – François Rabelais de L'université de Tours, L'institut Scientifique de Santé, Programme de Radiopharmacie, Programme de UMR Inserm U 930, Equipe 3, Imagerie Moleculaire du Cerveau, Thèse de Doctorat, Ankara –Tours, 2016.

La maladie du Parkinson (PD) provient de la dégénération des cellules du locus niger produisant de la dopamine. La barrière sang-cerveau (BBB) est un véritable obstacle devant le traitement du PH. Cela peut se provoquer par “l’effet stealth” c’est-a-dire en bloquant l’absoption du RES par la haute pénétration et l’absorbtion dans le tissu cible. Les liposomes et les niosomes sont les systhèmes les plus préférés en raison de leurs biocompatibilité, bio fragmentation, non-toxique et de leurs capacités de ciblage. İl existe toujours de grands vides dans la conduite de médicaments dans le cerveau et dans la réussite du traitement du PD, malgrès le placement au premier rang dans de nombreux projets de recherche en Europe et dans le monde sur les maladie du système nerveux centrales et malgrès que beaucoup de travaux ont été réalisé avec le pramipexole, notre étude représente une nouveauté dans la pénétration dans le cerveau des pramipexoles encapsulés des liposomes et des niosomes et dans la valorisation des effets antiparkinson. Dans le cadre de la thèse, la caractérisation et la diffusion cinétique des liposomes et niosomes formulés neutre et positif, couvert de PEG, nanocristallin, ont été évalué.

La validité de toutes les formulations BBB, a été analysé dans les co-cultures BBB des cellules. L'activité des liposomes et niosomes encapsulés du traitement de pramipexole neutre, a été analysé par des recherches 6-OHDA in vivo et en faisant des lésions sur les rongeurs formant des modèles de PD par rotamètre et autoradiographie. Toutes les formulations ont montré environ 10% d'activité d'encapsulation et environ 100 nm la dispersion de la taille des particules et la diffusion cinétique au 1er degrès. Dans tous les travaux de culture de cellule, nous avons déterminé que la formulation BBB est possible par pénétration. Dans le traitement de la maladie de Parkinson chez les rats avec la lésion 6-OHDA, les niosomes neutres nanocristallins pramixole encapsulées a montré des résultats similaires voire légèrement meilleurs avec une solution de la même dose de pramipexole. Cette dose, 9 fois moins que les tablettes conventionnelles de pramixole, est couramment utilisée dans les cliniques neurologiques de Parkinson. Par conséquent avec l'application de nanocrystalin , niozome neutre et des faibles doses de paramipexole, a été obtenu chez les rongeurs à lésion 6- OHDA, un antiparkinson efficace. Chez les modèles de rongeur à PD , dû aux caracteristiques des niozomes non-ioniques, des niozomes neutres, de paramixoles encapsulés et nanocristalin, un effet potantiel thérapeutique antiparkinson a été décelé.

Mots-clés: liposomes emprisonnés de pramixole, niosomes emprisonnés de pramipexole, le ciblage du cerveau, traitement de la maladie de Parkinson, Dopamine Transporter autoradiographie.

Avec le soutien de : TUBITAK – SBAG (Projet no: 112S244).

L'ambassade de France-Service de coopération et d'action culturelle, Campus France.

CONTENTS

ABBREVIATIONS

AD	Alzheimer's disease
AET	Active efflux transport
AP	Anteroposterior
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BMVECs	Brain microvascular endothelial cells
Chol	Cholesterol
CMT	Carrier mediated transport
CNS	Central nervous system
COMT	Catechol-o-methyl transferase
CT	Computed tomography
DA	Dopamine
DAT	Dopamine transporter
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DSC	Differential scanning calorimetry
DTPA-PE	Diethylene triamine penta acetate-Phosphatidyl ethanolamine
EPR	Enhanced permeability and retention
FTIR	Fourier transform infrared
hCMEC/D3	Human brain endothelial cell line
HLB	Hydrophilic lipophilic balance
HPLC	High-performance liquid chromatography
L-DOPA	Levodopa
MAO-B	Monoamine oxidase B
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance ımaging
MRPs	Multidrug resistance proteins
MUVs	Multilamellar vesicles
NE	Norepinephrine

NMDA	N-methyl-D-aspartate
PD	Parkinson's disease
PEG	Polyethylene glycole
PET	Positron emission tomography
Pgp	P-glycoprotein
PMAT	Plasma membrane monoamine transporter
PPX	Pramipexole
R^2	Coefficient of determination
RES	Reticuloendothelial system
Rh	Rhodamine
RMT	Receptor mediated transport
ROI	Region of interest
SA	Stearylamine
SNc	Substantia nigra pars compacta
SPECT	Single-photon emission computed tomography
SURII	Non-ionic surfactant II (Alcool cetylique polyglycerole)
SUVs	Small unilamellar vesicles
TH	Tyrosine hydroxylase
TLC	Thin layer chromatography
TRIS	(Tris(hydroxymethyl)-aminomethan
UV	Ultraviolet
VTA	Ventral tegmental area
[^{125}I]PE2I	N-(3-iodopro-2E-enyl)-2beta-carbomethoxy-3beta-(4'-methylphenyl) nortropane
6-OHDA	6-hydroxydopamine

FIGURES

TABLES

1. INTRODUCTION

Depending on the increase in the lifetime of mankind according to the advance in the technology, a rise is observed in the incidence of geriatric diseases and by this way an increase is also observed in the reseach for this field in all around the world. One of the most recently observed neurodegenerative disease among aged-related diseases is PD. It is formed due to the degeneration of dopamine producing cells in the substantia nigra in the midbrain (1). Generally, PD is diagnosed over age 50, however it is not only seen in middle to old people but also in young people (at the age below 20 years) as Juvenile PD 5% of all cases. While the disease incidence is 0.2-0.3 % among the community, it is 1% among the people who are over the age of 55. While about 10 million people suffer from PD worldwide, about 100-130 thousand people are to date diganosed with PD in Turkey. It is expected that this number will be duplicated in 2030 in Turkey in which about 10 thousand people is diagnosed with PD every year. In contrast to this accelerated increase in number, only half of the patients can achieve a successful therapy which is mostly related with the late diagnosis of the disease (2).

Brain is protected by tight, protective barriers composed of tight endothelial cells and tight junctions such as blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) limiting delivery of drugs and other molecules for both diagnosis and treatment of brain diseases. These endothelial and epithelial structures comprise specialized and differentiated neurovascular system providing proper functioning of neuronal circuits, synaptic transmission and remodelling, neurogenesis and angiogenesis in the brain by separating and protecting neurons from blood circulation (3-5). Due to that, more than 98% of drugs can not penetrate into the brain. Although there is a variety of approaches for BBB penetration, the utilization of drug delivery systems is one of the most frequently investigated one.

Levodopa (L-DOPA) administration as a gold standart in the treatment of PD is very valuable, however, its long-term use may cause some motor complications such as abnormal involuntary movements (dyskinesia) and shortening response to each dose (wearing off phenomenon). To reduce the duration of

immobile off periods and dependence to L-DOPA therapy for maintaining or improving motor impairment, dopamine agonists were developed (6). Pramipexole is one of these effective dopamine agonists with high relative in vitro specificity and full intrinsic activity at D2 subfamily of dopamine receptors and with a higher binding affinity to D3 than to D4 or D2 receptor subtypes (7). To obtain therapeutical effect in brain it is required to increase the dose which results in the increase of side effects. Therefore, solutions are searching for increasing drug amount delivery without increasing drug dose. Nanosized drug delivery systems have important advantages in this issue. The term of Nano is called as manikin (dwarf, small man) in Greek and it is one in a million od a milimeter. Nanotechnology is first stated by a physicist, Richard Feynman, in 1959 about mesuring nanomaterials by miniaturized devices and the use for identifying new scopes. According to USA National Health Institute, one of the most important area in nanotechnology is nanomedicine and it comprises specific medical research in molecular level for the diagnosis and therapy of the diseases. Nanomedicine can be defined as the application of nanotechnology to health and can be described as a new field of science (8,9). Nanosystems that are used in nanomedicine comprise liposomes, niosomes, micelles, nanospheres, polymeric delivery systems, dendrimers, emulsions, nanoparticles and nanocapsules etc. Apart from other conventional drugs, the superiority of these drug delivery systems is their ability to accumulate in the desired tissue, increase bioavailibility of drugs, increase effectiveness even in lower concentrations and decrease side effects (8-11).

Liposomes and niosomes are one of the most commonly investigated drug delivery systems used for delivering both drugs and diagnostic agents to the targeted area. Liposomes are formed by self-sustainable bilayered structure comprising phospholipids (12,13). Niosomes are non-ionic surfactant vesicles (14). The composition of niosomes is very similar to that of liposomes, however, the substances used for the preparation of niosomes, non-ionic surfactants, attain them a more stable structure and more ability to penetrate through BBB. Both liposomes and niosomes have proper characteristics such as being non-toxic, biocompatible and biodegradable. They can also carry a variety of drugs with different physicochemical propeties such as hydrophilic, lipophilic and amphoteric drug

molecules by either entrapping inside hydrophilic core or anchoring on the lipid bilayer (15,16).

Passive targeting of liposomes and niosomes can be performed with the help of formation of a steric hindrance created by altering surface charge, surface properties, particle size, site membrane lipid packing, extent of steric hindrance and reducing particle size into nano-scale. These sterically stabilized long circulating delivery systems are distinguished from other conventional ones due to the altered pharmacokinetics and pharmacodinamics which enhances accumulation at diseased area (17). Coating was initially made by monosialogangliosides (18) to incease distribution half-life. The existence of steric hindrance achieved by coating with a hydrophilic biocompatible polymer such as PEG, propylene glycol, polypropylene oxide, polyethylene oxide or mannose coating for brain targeting prevents them from interaction between serum opsonins by lower contact angle between particles and phagocytic cells (15,16) and prevents them from opsonisation by tissue macrophages. Brain targeting can be obtained by this way rather than targeting to RES organs like liver, spleen, etc.

Although a number of studies have performed and CNS disorders are the first to endorse their research in the diagnosis and therapy with several framework projects in Europe and all around the world, there is still a huge gap in CNS drug delivery and the success of PD therapy.

Although a variety of studies were performed about therapeutic efficacy of Pramipexole (6,7,19-22) and use of some delivey systems for the therapy of PD (23,24), the formulation of Pramipexole encapsulated liposomes and nisosomes have not been studied before as an alternative to oral Pramipexole dosage form using in neurology clinics for PD treatment. As preliminary studies, it was tried to formulate active targeted liposomal and niosomal formulations as theragnostic systems for both diagnosis by radiolabeling and therapy by D2 or DAT targeting by a specific ligand modification of PD at the same time. It was tried to perform a chemical synthesis with computer aid coordinational chemistry. However, after discussions with different chemists and radiochemists from different countries, it was observed that the modification of a D2 or DAT specific ligand with liposomes

and niosomes is vey hard to perform and may most probably result in deactivation of specific part of ligand after modification.

Therefore, the aim of thesis was changed and modified as to formulate novel, nanosized for passive targeted, PEGylated for “stealth effect”, antiparkinsonian drug (Pramipexole Dihydrochloride Monohydrate) encapsulated, neutral and positively charged liposomes and niosomes for effective therapy of Parkinson’s Disease. Nanosized, pramipexole encapsulated, neutral and positively charged liposomes and niosomes were formulated. The characterization and release kinetics of formulations were performed. BBB penetration of nanosized, neutral, pramipexole encapsulated liposomes and niosomes was evaluated in BBB Cell Co-Culture model by using flurescent intensity and fluorescence microscopy images. In vivo therapeutic efficacy of neutral liposomes and niosomes was monitored and compared by complementary methods such as rotational behaviour and autoradiography in a rat model of PD obtained by a partial 6-hydroxydopamine (6-OHDA) unilateral striatal lesion. It is expected to obtain potential therapeutic results in 6-OHDA lesioned rats which leads further studies with large number of animals leading to develop commercial preparates for PD patients at clinics in the future with decreased side effects and decreased frequency of administration which is very significant for patient’s compliance to the therapy.

2. GENERAL INFORMATION

2.1. Parkinson's Disease

Parkinson's disease (PD) also known as idiopathic or primary parkinsonism) is a degenerative disorder of the central nervous system. Although its mechanisms and reasons are not clearly defined, it is thought that its motor symptoms result from the death of dopamine-generating cells in the substantia nigra, a region of the midbrain. It is commonly seen in people in the middle or older ages. PD takes its name from James Parkinson, English physician, in 1817 as trembling stroke and now PD is in the beginning orders among geriatric neurodegenerative disorders. The main abnormality is generally seen in parts regulating coordinate and basic movements in brain. Formation of alpha-synuclein aggregation and Lewy bodies are hallmarks of PD and other related diseases (25).

According to the litrature, environmental toxins, genetic factors and oxidative stress may cause PD onset. According to a study performed through the Europe, the minimal amount of pesticide exposure can increase the risk of PD in a large amount (26). Another factor is the genetic mutations. According to Kurosinski et al. (27), mutations in gene SYN (A30P and A53T) can cause PD in animal models by causing internal cellular α-synuclein agregation and deposition to form Lewy bodies which are significant signs of PD. Dopaminergic loss in the basal ganglia and substantia nigra of several animal species such as mice and flies can be seen after these mutations (28). Another cause of PD is related to the formation of unstable free radicals which are by-products of oxidative stress contributing to nerve cell death. It was seen that MTH1 suppresses cell death depending on oxidative stress in human PD patients (28,29).

- ***Dopaminergic Neurotransmission in PD***

Dopamine (3-hydroxytyramine; DA) is a catecholamine neurotransmitter which is a precursor for synthesis of the neurotransmitter norepinephrine (NE). DA in synthesized from tyrosine by a two step process, where tyrosine hydroxylase (TH) is the rate-limiting enzyme in the reaction (30). Tyrosine (L-Tyrosine) is a naturally occurring amino acid involved in the synthesis of neurotransmitters dopamine,

adrenaline and noradrenaline. Dopamine synthesis from tyrosine is given in Figure 2.1 (31).

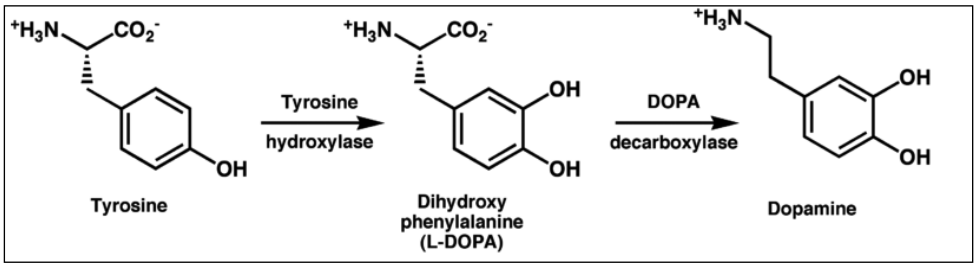


Figure 2.1. Dopamine synthesis from Tyrosine (31)

DA is a neurotransmitter that transmits messages from one nerve cell to another. Dopamine signals travel from the substantia nigra to brain regions including the corpus striatum, the globus pallidus and the thalamus in order to control movement and balance (32)

- ***Dopaminergic Pathways and Nigrostriatal Dopamine Pathway Affected in PD***

There are four main dopaminergic pathways; the tuberoinfundibular pathway, the nigrostriatal pathway, the mesocortical pathway and the mesolimbic pathway (Fig. 2.2)

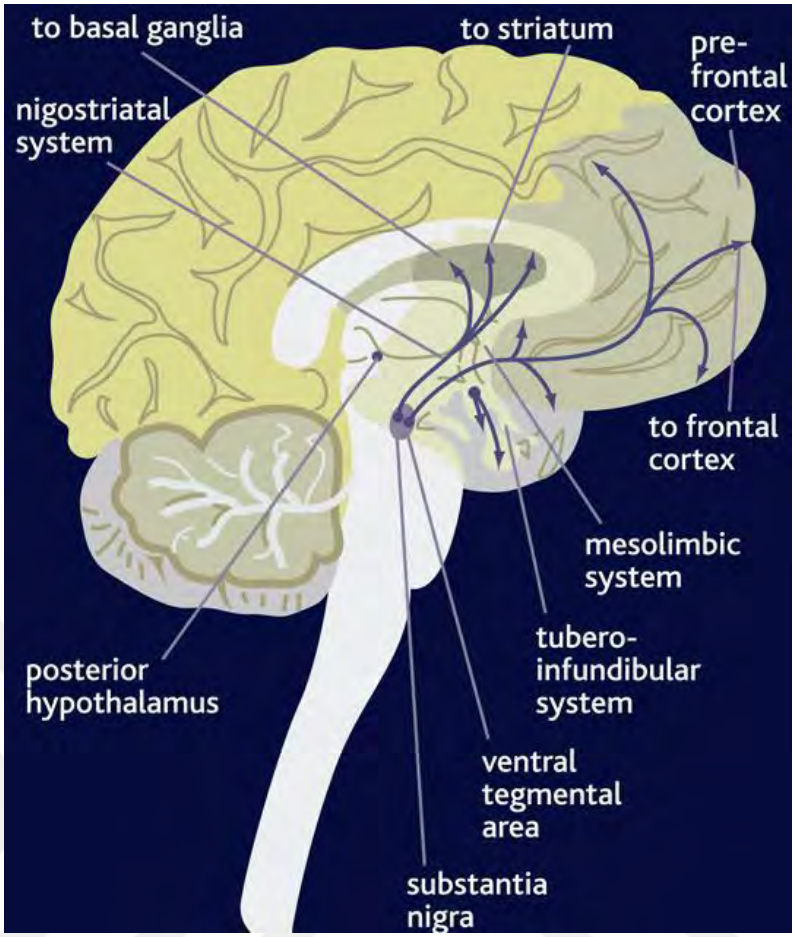


Figure 2.2. Pathways of dopamine signaling in the brain. (Illustration of major DA projections in the central nervous system. The nigrostriatal pathway originates in the substantia nigra and projects to the dorsal striatum. The mesolimbic and mesocortical projections originate in the ventral tegmental area and project both to ventral striatum and areas in the prefrontal cortex, respectively. The final system is the tuberoinfundibular system which projects from the hypothalamus to the pituitary). (30,33)

1. The tuberoinfundibular pathway, which refers to a group of DA neurons in the arcuate nucleus of the hypothalamus that project to the median eminence, controls prolactin secretion from the anterior pituitary gland (34) Hyperprolactinaemia is associated with a failure of this pathway (30).
2. Dopaminergic neurons in the mesocortical pathway project from the ventral tegmental area (VTA) to the frontal lobes of the cerebrum, particularly the prefrontal cortex, and are involved in cognition and

emotion. Attention deficit hyperactivity disorder, addicton and schizophrenia can be seen in any failure of this pathway (30).

3. Neurons of the mesolimbic pathway also originate in the VTA but instead innervate the ventral striatum, also known as the nucleus accumbens. This pathway is implicated in reward and pleasure. Attention deficit hyperactivity disorder, addicton and schizophrenia can be seen in any failure of this pathway (30).
4. Nigrostriatal pathway is related with PD. The nigrostriatal pathway in the midbrain consists neurons whose cell bodies originate in the substantia nigra and terminate in the dorsal striatum. This area is implicated in movement since degeneration of these projections has been shown to cause Parkinson's Disease; characterized by tremors, rigidity, and overall improper movement (35). This region is also important in feeding behavior (36). Addiction and chorea can also be seen in any failure of this pathway (30).

In PD, most of the dopamine signals from the substantia nigra are lost. Nigrostriatal pathway is the efferent connection between the susbtantia nigra and corpus striatum. Nigrostriatal pathway is particularly involved in the production of movement, as part of a system called the basal ganglia motor loop. Loss of dopamine neurons in the substantia nigra is one of the main pathological features of PD, leading to a marked reduction in dopamine function in nigrostriatal pathway. The symptoms of the disease typically do not show themselves until 80-90% of dopamine function has been lost.

The secretion of dopamine is actualized from membrane storage vesicles in the presynaptic neurons and binds to postsynaptic neurons and activates dopamine receptors to perform its physiologic effects (32). Afterwards, following dopamine signalization from one neuron to another at the synapse, it is removed via re-uptake back into the presynaptic cell by either the high-affinity DAT or the low-affinity Plasma membrane monoamine transporter (PMAT). Once it is taken back inside the cytosol, it is repackaged into vesicles at the end for new signaling. As an alternative way, dopamine is directly broken down into inactive metabolites by two enzymes

called monoamine oxidase B (MAO) and catechol-o-methyl transferase (COMT). but enzymatic degradation does not account for inactivation of DA in the synapse. Instead, termination of DA neurotransmission is regulated by DAT. DAT allows DA to be cleared out of the synapse and taken up into the presynaptic bouton (30,37,38). Insufficient dopamine biosynthesis due to loss of the substantia nigra dopaminergic neurons causes PD. Figure 2.3 shows dopamine levels in a normal and Parkinson's affected neurons. Figure 2.4 designates a horizontal section of subtantia nigra in normal and PD patients.

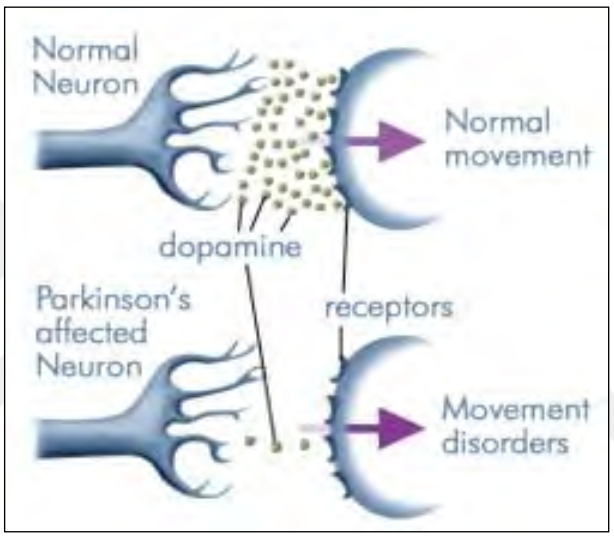


Figure 2.3. Dopamine levels in a normal and Parkinson's affected neuron (39).

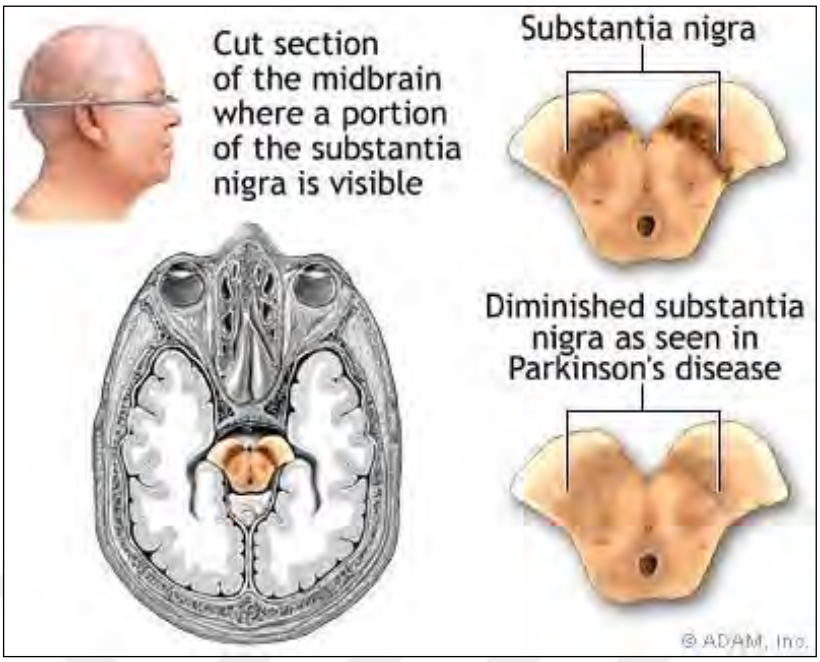


Figure 2.4. A horizontal section of subtantia nigra in normal and PD patients (37).

- ***Consequences of Dopamine Reduction in PD***

At the early phases of the disease, the most obvious symptoms are movement-related such as tremor (hands and head develop involuntary movements), muscle rigidity/rigour, slowness of movement/bradykinesia and postural instability (difficulty with walking and gait). At progressive stages, thinking and behavioral problems may arise and dementia can be occurred in the advanced stages of the disease generally (40). Additionally depression is the most common psychiatric symptom that can be observed in PD patients. The mechanism of depression in PD is not clear however, it may be due to the deficiency of multiple transmitters in mesocortical monoaminergic systems containing dopaminergic projections, noradrenergic and serotonergic projections (41-43). Other symptoms may comprise sensory, sleep and emotional problems such as hallucinations and sleep disturbance. Subtle cognitive deficits especially frontal lobe executive dysfunction may be seen in patients with early PD which can be detected with sensitive neuropsychological testing (44,45). Postural hypotension can also be seen in some PD patients. It is more frequently seen in PD patients with dementia when compared with PD patients without dementia (46).

2.1.1. Therapeutic Approaches of Parkinson's Disease

PD, which has a high incidence in neurodegenerative disorders, can be clinically diagnosed generally only when the symptoms are initiated. Early therapy can be managed by early diagnosis of PD such as the alterations are in molecular level before syptomatic changes are started. There is a balance in brain between acethylcholine and dopamine which increases and regulates the stimulation property. In PD, the balance is spoiled in favor of acethylcholine and dopamine should be substituted. Today, although the main therapy of PD comprises therapy with drugs, there are also other therapy approaches.

The patient should be protected to be withdrawn and seperate from the public and stay physically active. Surgery can be used in some conditions which comprising the damage of diseased area. One method is the deep brain stimulation for the treatment (47). Deep brain stimulation involves the implantation of a medical device called a brain pacemaker sending electrical impulses composed of implanted electrodes, to specific parts of the brain (brain nucleus) for the treatment of movement and effective disorders. The use of deep brain stimulation as the approved procedure by US Food and Drug Administration in the threatment of PD initiated in 2002 and since then almost 80.00 applications were administered by physicians in all around the world (48). However, the most commonly, easily and practically applied therapy approach is the drug therapy for PD.

Treatment approaches may be grouped as dopamine prodrugs, dopamine agonists, COMT inhibitors, MAO-B inhibitors, anticholinergics and NMDA inhibitors. They are defined below very briefly.

- ***Dopamine Prodrugs***

L-DOPA is a prodrug and a dopamine precursor. Dopamine can not be used for PD therapy due to its absence of penetration into CNS. This problem was solved by the use of L-DOPA in 1960s which is thought as gold standart in PD

therapy. It desigates its effects in CNS by turning into dopamine by the enzyme of Dopa-decarboxylase. A very low amount (such as 1-2%) of the given dose is only effective. Therefore, L-DOPA should be administered in high doses which may increase the side effects. The use of L-DOPA in some patients is effective, problem-free and successful for 2-4 years, however, after that the symptoms of PD can reappeared. The level of L-DOPA can be decreased at certain times which causes patients to designate PD symptoms (off period, or wearing off phenomenon). Dyskinesia is a common side effect of the long term use of L-DOPA and it is generaly observed in 40% of the patients suffering from PD after 5 years which can be assumed as the honeymoon period and in 80% of PD patients after 10 years of L-DOPA utilization (49,50). In the case of any contraindication or no therapy effect or resistance to L-DOPA, some other drugs such as dopamine receptor agonists, anticholinergics and other drugs can be chosen as single therapeutics or in conjugation with L-DOPA. Sometimes, L-DOPA can be given with a peripheral decarboxylase inhibitor like benserazide or carbidopa. Apart from this, amantadine or bromocriptine can be added to the therapy with L-DOPA to obtain synergistic effect (37,51).

- ***Dopamine Agonists***

Dopamine agonists mimic dopamine for stimulating dopamine system in the brain and they induce dopamine receptors and currently being used as a treatment for improving many of the symptoms characterizing PD (28). bromocriptine, pergolide, cabergoline and lisuride are specific dopamine agonists. Bromocriptine is the only ergot dopamine agonist approved for treatment of Parkinson in the U.S.A. Bromocriptine stimulates dopamine D2 receptors. Pramipexole is a non-ergot D2 and D3 receptor agonist. Pramipexole has similar selectivity with ropinirole. Apomorphine is used as a very efficient drug in people having very severe on-off effects which may require going off L-DOPA for a few days. It is FDA-approved drug for the treatment off-time episodes of PD. Apomorphine has high affinity on D4 receptor. Adverse effects of these dopamine agonists are nausea, headache, vomiting, dizziness, constipation (37,51).

- ***Catechol O-MethylTransferase (COMT) Inhibitors***

COMT enzyme is a peripheral metabolizer of L-DOPA. COMT Inhibition prevents L-DOPA metabolism into 3-O-methyldopa and prolongs L-DOPA's effect. Entacapone (Comtan, Stalevo) is COMT inhibitor. Wearing-off effect can be prevented and it can be given with dopamine precursor in PD's pharmacotherapy.

Tolcapone (Tasmar) may cause some side effects related with liver functions so it has been taken off the market in many countries. Entacapone is more safer. Headache, diarrhea, and abdominal pain are some other adverse effects (37,51).

- ***MonoAmine Oxidase-B (MAO-B) Inhibitors***

MAO-B inhibitors are used as adjunctive therapy for PD because they inhibit the breakdown of dopamine by MAO-B and increase dopamine amount in the brain. Selegiline (deprenyl) blocks MAO-B which is a dopamine degrading enzyme and it may have some mild benefit as an initial therapy. It is generally used in early-onset disease. Rasagiline is used for PD treatment and for both early-onset or moderate to advanced disease with combination with L-DOPA. MAO-B inhibitors can cause some severe side effects. Orthostatic hypotension is one of the crutial one. Hypertension can be observed if combined with drugs inducing serotonin level enhancements like antidepressants and feding with foods rich in the amino acid tyramine (37,51).

- ***Anticholinergic***

These were the first drugs for PD in 1949 however they were replaced by dopamine drugs. Trihexyphenidyl is still used in several PD cases and especially for controlling tremor in early stages. It may cause dryness of the mouth. Nausea,

dizziness, glaucoma, constipation, urinary retention, and some mental problems such as memory loss, confusion, and even hallucinations can also be observed (37,51).

- ***N-Methyl-D-Aspartate (NMDA) Inhibitors***

Amantadine (Symadine, Symmetrel) is an antiviral compound. It stimulates dopamine release and designates its effect against dyskinesia in PD patients. Memantine is an uncompetitive antagonist of NMDA receptor however it is not an option for PD therapy. NMDA inhibitors may cause swollen ankles and visual hallucinations. Rarely, it may cause acute delirium or neuroleptic malignant syndrome (37,51).

The mechanism of action of antiparkinsonian drugs in dopaminergic synapse is given in Figure 2.5. (51). For the general therapy of PD; it is generally recommended to use dopamine precursors and dopamine agonists at early phase (37,51-55).

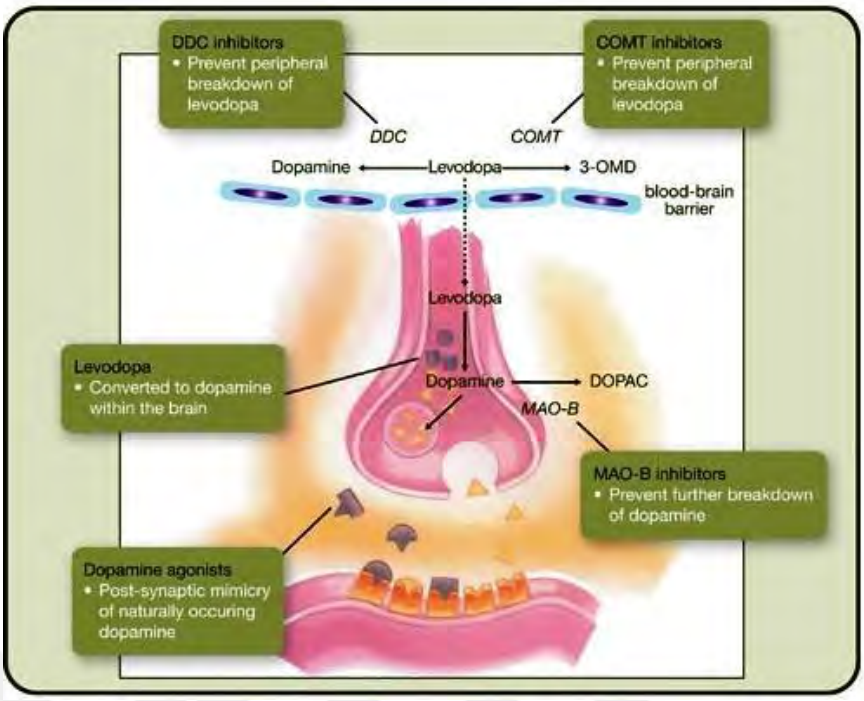


Figure 2.5. The mechanism of action of antiparkinsonian drugs in dopaminergic synapse (51).

*COMT: Catechol-C-methyltransferase, DDC: Dopa Decarboxylase, DOPAC: Dihydroxyphenylacetic Acid, MOA-B: Monoamine Oxidase-B, 3-OMD: 3-O-Methyldopa.

2.1.1.1. Pramipexole

Pramipexole dihydrochloride monohydrate ((S)-2-amino-4,5,6,7-tetrahydro-6-propylamine-benzothiazole dihydrochloride) is a white to off-white crystalline powder and stable under ordinary conditions. Its solubility is more than 20% in water, about 8% in methanol and 0.5% in ethanol. It is practically insoluble in dichloromethane (56). Due to proper solubility properties, Pramipexole Dihydrochloride Monohydrate is chosen instead of Pramipexole for PD treatment for this study (57-59).

Although its therapeutical mechanism is unclear, it is thought to stimulate dopamine receptors in striatum. Pramipexole is a non-ergot dopamine agonist. It binds more to D3 than D2 and D4 receptors. Its effect on D1 receptor is lesser. It can be used as individual in PD. The observation of side effects is very rare or none. The probability to see orthostatic hypotension is low when compared with

other dopamine agonists. Immeadiate release Pramipexole approved by FDA in 1997 which can be used individually or together with L-DOPA in idiopathic PD. FDA was approved the extended release fomulation (MirapexER®) for the therapy of early phase PD in February 2010. It is approved in March 2010 for the therapy of symptoms in idiopathic PD (60).

2.2. The Structure of Brain and Blood Brain Barrier

The brain is one of the most complex organs within the human body. It is made up of more than 100 billion nerves communicating lots of connections with synapses. It is thought that the cerebral cortex (the largest part) contains 15–33 billion neurons in a typical human (61). These neurons communicate with one another by axons carrying signal pulses called action potentials to every part of the body. The brain controls the rest of the body by generating patterns of muscle activity and driving the secretion of hormones. The lobes and general parts of the brain is given in Figure 2.6.

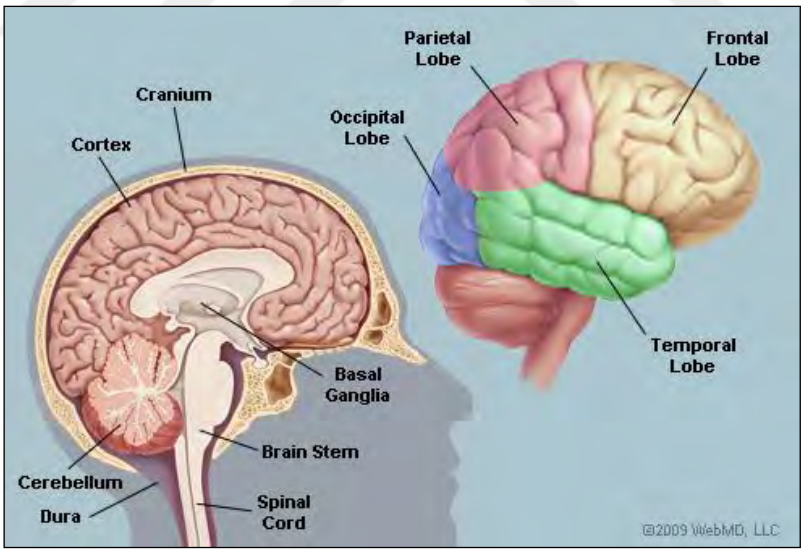


Figure 2.6. The lobes and general parts of the brain (62).

There are 2 physical barriers seperating brain extrasellular fluid from the blood called BBB and BCSFB.

1. BBB is characterized by tight junctions between endothelial cells by the absence of fenestration (63). Brain microvascular endothelial cells (BMVEC) and neuroparenchymal cells such as pericytes, microglia, astrocytes and neurons surrounding the microvessels form BBB. This barrier regulates trafficking of ions, molecules and leukocytes into and out of the brain. It limits and prevents the penetration and entry of compounds from blood to the brain.

2. Blood-cerebrospinal fluid barrier (BCSFB) exists at the choroid plexus and seperates the blood from the CSF (64). The epithelial cells exist on the choroid plexus forming BCSFB have complex tight junctions on CSF (apical) side of the cells. However, the tight junctions of the epithelial cells in the choroid plexus seems to be more permeable than the ones in the endothelial cells of BBB (65,66).

The schmeatic representation of two main barriers in CNS is given in Figure 2.7.

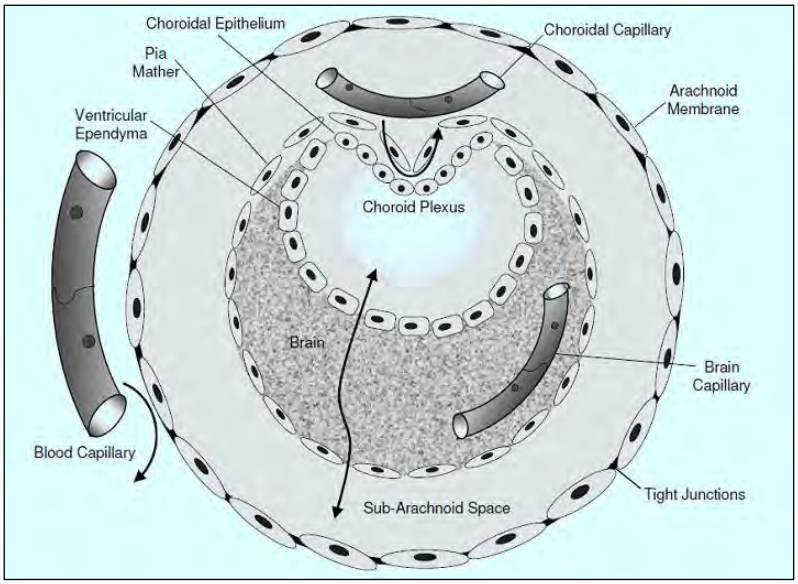


Figure 2.7. Schematic representation of two main barriers in the CNS (66).

2.2.1. Blood Brain Barrier Penetration Approaches

Drug and imaging agent delivery to the brain is still a kind of challenge for both the diagnosis and therapy of CNS disorders. Brain barriers can be spoiled and getting leaky in some conditions such as brain tumors, inflammation, etc. (67). This is because tumors develop their own vasculature to grow. The vessels within tumors have increased permeability depending on the presence of larger endothelial cell gaps when compared with the normal vessels and retantion effect which is called EPR effect (67).

Formulation of new and effective drug delivery systems is crutial for brain delivery depending on the existance of barriers. BBB restricts the entry of compounds to the brain from the periphery (68,69). By this way brain entry of many low molecular weight drugs, compounds, biomacromolecules such as DNA and proteins are restricted. Some substances can penetrate to brain depending on the passive diffusion across BMVECs due to the lipophilicity and proper molecular weight of these substances. One of the drawback is the rapid efflux from the brain into the blood by extremely effective efflux pumps such as P-glycoprotein (Pgp) and Multidrug Resistance Proteins (MRPs) in BBB (70-73).

The molecules penetrating BBB by passive diffusion should have some properties because as mentioned above almost every large molecules and drugs can not penetrate BBB in which about 98% of the small molecules and drugs can not cross BBB (74,75). Small particle size, low molecular mass (< 400 Da), a log octanol/water partition coefficient between -0.5 and 6.0, lipid-solubility, being either neutral or significantly uncharged at physiological pH:7.4, and forming <8 H-bonds with water (76) are some important properties for formulating BBB penetrating drugs. BBB only permits the passage of some small lipid-soluble drugs through this barrier (77). It was observed that almost 98% of small molecules and nearly all large molecules such as recombinant proteins or gene-based medicines can not cross the BBB (78). Due to the presence of tight junctions of endothelial cells present in BBB, penetration pathways comprise paracellular aqueous pathway, transcellular lypophilic pathway, transcellular lipophilic pathway, transport proteins, receptor-mediated transcytosis or adsorbtive-mediated transcytosis

(79,80). These nanocarrier systems generaly taken up by carrier-mediated transport, receptor-mediated endocytosis and adsorptive-mediated endocytosis and by this way reaching to the cerebral parenchyma or degraded within the lysosomes leading drug release (66).

In this section, different brain penetration mechanisms of drugs will be mentioned very briefly. A general table comprising drug delivery strategies to brain are summarized in Table 2.1.

There are 3 approaches to enhance brain penetration of drugs (66);

1. Invasive route is important to leave out the penetration through BBB and BCSFB by direct administration of the drug into the brain. Direct administration of drug into the brain can be applied via intracerebral, intracerebroventricular and intrathecal administration. By this way, a variety of compounds containing large and small molecules can be administered. However this method is inpractical depending on being invasive and requires surgery and craniotomy. Another option for transporting through BBB is the intranasal administration to use the connection between nose and brain which is called the olfactory bulb (81). A variety of materials such as small molecules, proteins, viruses, pathogens and toxic materials can be administered by this way (82-85).

2. Pharmacological strategy depends on the increase of lypophilic solubility of drugs. By this way, drug molecules can be chemically modified or encapsulated in liposomal formulations. Generation of a transient disruption of BBB which allows the entrance of therapeutic agents into the brain from blood. The disruption of brain can be managed by pharmacological means. Many different endogeneous proinflammatory vasoactive agents like bradykinin, histamine, nitric oxide can induce an increament in the BBB permeability. Bradykinin was designated to increase the ionic permeability of the BBB reversibly (86,87). The transient disruption of BBB can also be observed by systemic administration of different molecules especially alkyl

glycerols. BBB disruption depends on the length of the alkyl group and the number of glycerols exists in the structure (85,88).

3. Physical strategy depends on the penetration of nutrients, peptide and non-peptide hormons and transport proteins with the help of carrier mechanisms exist on BBB (85).

Table 2.1. General drug delivery strategies to brain (89).

Invasive Techniques	**Non-invasive Techniques**	
	Chemical Way	**Biological Way**
-BBB disruption	-Prodrug design and lipidization	-Receptor Mediated Transport (RMT)
-Direct drug injection		-Carrier Mediated Transport (CMT)
		-Inhibition of Active Efflux Transport (AET)

There are both chemistry-based and biology-based approaches for developing BBB drug-targeting strategies (Figure 2.8) (90,91). The chemistry-based strategies are the conventional approaches that rely on lipid-mediated drug transport across the BBB. The biology based strategies depends on numerous endogenous transport systems within the BBB. These transporters are conduits to the brain. The endogenous BBB transport systems may be broadly classified as carrier-mediated transport (CMT), active efflux transport (AET), and receptor-mediated transport

(RMT). These BBB transport systems are situated on the luminal and abluminal membranes of the brain capillary endothelium.

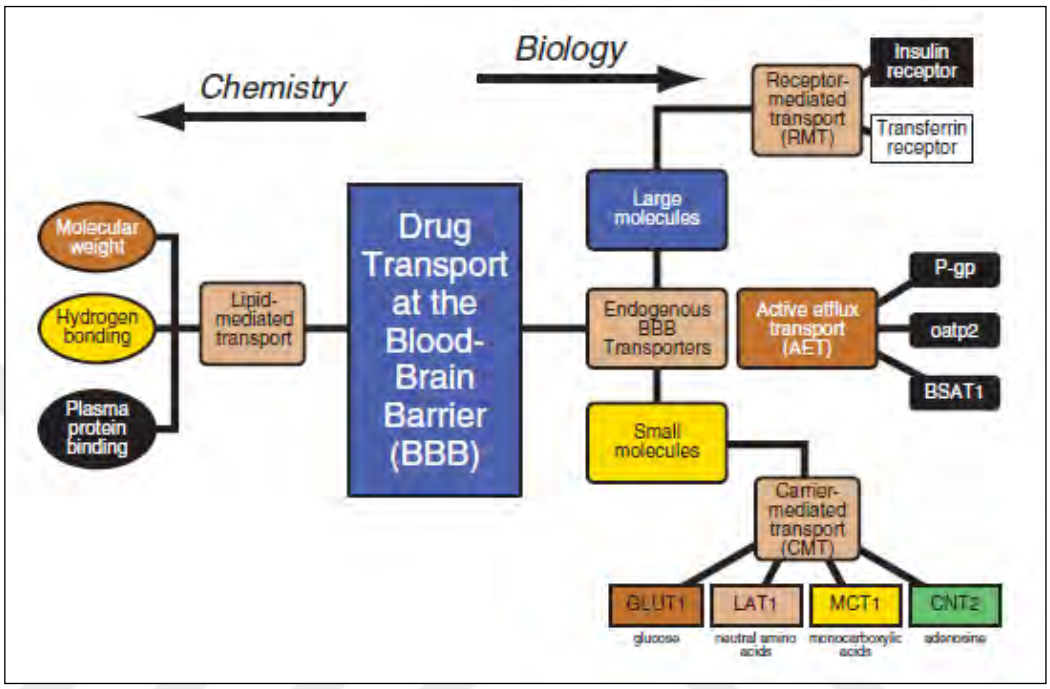


Figure 2.8. Brain drug targeting strategies comprising biological and chemical bases (Chemistry-based strategies emphasize lipid solubility, hydrogen bonding, and molecular weight of the drug. Biology-based strategies emphasize endogenous BBB transporters. Small molecules can be transported across the BBB by either accessing certain carrier-mediated transport (CMT) systems or by inhibiting certain active efflux transporters (AET). Large-molecule drugs such as recombinant proteins or gene medicines can be delivered across the BBB via the receptor-mediated transport (RMT) systems. Reprinted with permission (90,91).

*(Pgp: P-glycoprotein, oatp2: organic anion transporter polypeptide 2, BSAT1: BBB specific anion transporter type-1, GLUT 1: glucose transporter isophorm-1, LAT 1: Large amino acid transporter-1, MCT 1: Monocarboxylate transporter 1, CNT 2: Concentrative nucleoside transporter 2).

Drug delivery to the brain through the many endogenous transport systems within the BBB requires reformulation of the drug so that the drug can access the BBB transport system and enter the brain. Researchers within brain drug-discovery and brain drug-targeting should work together to formulate more effective formulations to enable BBB transport (90).

One of the most commonly researched approach is the formulation of effective drug delivery systems which can deliver to the brain which exists in pharmacological approach. Researchers work on improving drug delivery strategies to the brain to obtain an effective clinical outcome. This method is non-invasive for delivering drugs to the brain by the use of nanoparticular and nanovesicular delivery systems which will be explained in detail at the sections given below (81).

2.3. Nanotechnology and Nanomedicine

The major problem with drugs used for therapy of CNS diseases is the low amount of drug that penetrates from BBB and reaches the brain targeting. Dose increment to obtain the desired effect in brain may cause some side effects. Therefore, researchers are searching solutions for increasing drug amount without increasing drug dosage. Nanosized drug delivery systems have important advantages in this issue. The term nanotechnology is first stated by a physicist named Richard Feynman in 1959 about measuring nanomaterials by miniaturized devices and their use for identifying new scopes. Nanotechnology is generally related with materials and devices that are smaller than 100 nm. According to USA National Health Institute, one of the most important area in nanotechnology is nanomedicine and it comprises specific medical research in molecular level for the diagnosis and therapy of the diseases. Nanomedicine can be defined as the application of nanotechnology to the field of health and it can be described as a new field of science. Nano is one in a million of a milimeter (8,9).

Nanotechnology and nanomedicine are closely related research fields comprising a huge industry and projects with high budgets. Nature Materials Journal indicated that there was about 130 nanotechnology based drugs and drug delivery systems world-wide in 2006. Nanomedicine is a great field which

comprising intense researchs every year with nanosized drug sales about 6.8 billion dollars, factory over 200, products over 38 and R&D budget higher than 3.8 billion dollars world-wide. While nanotechnology R&D investments reached about 1 billion dolars in USA in 2005, it reached about 1.3 billion euros in European Union in 2003-2006. As in general, there was a total nanotechnology investment about 3 billion dollars world-wide in 2003. The market related with nanosized devices and molecular modelling designated 28% increase in one year and the income obtained from biomedical nanosized devices reached to 1.37 billion dollars in 2007. In the light of these datas, it is tought that in course of the development and improvement in this field, nanomedicine industry will bring significant benefits onto the economy of countries world-wide (92).

The potential applications of nanotechnology to drug field comprises subjects related with formulation, improvement and potential applications of drug delivery systems and diagnostic devices and gene therapy. So many benefits are obtained by the application of nanotechnology to pharmaceutical field also for brain delivery of drugs depending on very small particle size which can help passive targeting. Nanosystems that are used in nanomedicine as drug delivery systems comprise liposomes, niosomes, micelles, nanospheres, polymeric delivery systems, dendrimers, emulsions, nanoparticles and nanocapsules etc. and these systems are composed of an important part of nanomedicine.

2.4. Drug Delivery Systems

Apart from other conventional drugs, the superiority of drug delivery systems that use nanotechnology basically depends on small particle size. Nanosized drugs can be used in lower concentrations efficiently and faster effect can be obtained (8,10,11).

The significant properties that should be considered for a drug delivery system to become effective are high drug loading capacity, physical and chemical stability, low toxicity incidence of the carrier used, proper in vivo behavior of the carrier, the ability to scale up the producing process and the overall cost (93). Among drug delivery systems; polymeric or lipidic nanoparticles, liposomes, niosomes and polymeric micelles are mostly investigated for neuroprotection and

facilitating the delivery of drugs and small molecules into the brain (94,95).

The essential reasons that directing formulation of drug delivery systems are their controlled drug delivery and release properties, improved pharmacokinetic and pharmacodinamic properties, enhanced blood circulation, effective and specific targeting potential, biocompatibility, biodegradability and nontoxicity. They can be both passively and actively targeted to the target tissue by altering surface properties, surface charge and by specific ligand modification, respectively (75).

Nanoparticular and nanovesicular drug delivery systems are a reason of choice because of their sustained-release and controlled-release properties. These nanosized drug delivery systems are effectively used for diagnosis and therapy purposes in many diseases and although it is not very common, their use in neurodegenerative diseases such as PD, alzheimer's disease and dementia are also investigated by researchers. For the therapy of PD, the observed frequency of dyskinesia depending on the use of L-DOPA can be diminished by the utilization of these nanoparticular drug delivery systems (50).

According to a study performed by Kura et al (96) layered organic-inorganic nanocomposite material containing L-DOPA which is intercalated into the inorganic interlayers of a Zn/Al-layered double hydroxide was synthesized using a direct coprecipitation method. Obtained L-DOPA containing nanocomposites designated sustained release properties. Depending on the Fourier Transform InfraRed study, nanocomposite containing L-DOPA was found thermally more stable than free L-DOPA. It was also observed that a decrease in the cytotoxicity was seen in the toxicity potential of L-DOPA when exposed to normal cell lines after intercalated into Zn/Al-layered double hydroxide (96,97).

One of the most reliable data on the safety of nanoparticles towards CNS has been announced for liposomes. Liposomes are known from the 60s and they are biocompatible, biodegradable and non-toxic due to naturally occurring lipid composition (98).

2.4.1. Liposomes and Niosomes

Liposomes and niosomes are one of the mostly studied formulations among drug delivery systems depending on being in phospholipid structure, biocompatible and biodisintegrable and ability to target drugs. Niosomes are very similar systems to liposomes except comprising nonionic surfactants instead of phospholipids which may attain them a superiority for passive targeting through very tight endothelial junctions such as BBB.

Liposomes were first studied in 1960s by Bangham et al (99). They are synthetic analogous of natural membranes. These are generally composed of one or more consentric vesicles containing lipid bilayers that are seperated by aqueous buffer compartments. Their size distribution changes between 80nm-100 µm and they contain phospholipids, cholesterol and sometimes charge inducer substances in their structure. The encapsulation behaviour of drug molecules depends on the physicochemical behaviour of drug molecules and lipid composition. While hydrophilic drug molecules are generally encapsulated in the aqueous compartment, hydrophobic drugs can be carried within lipid vesicles (100-102). The schematic representation of liposome and its formulation is given in Figure 2.9.

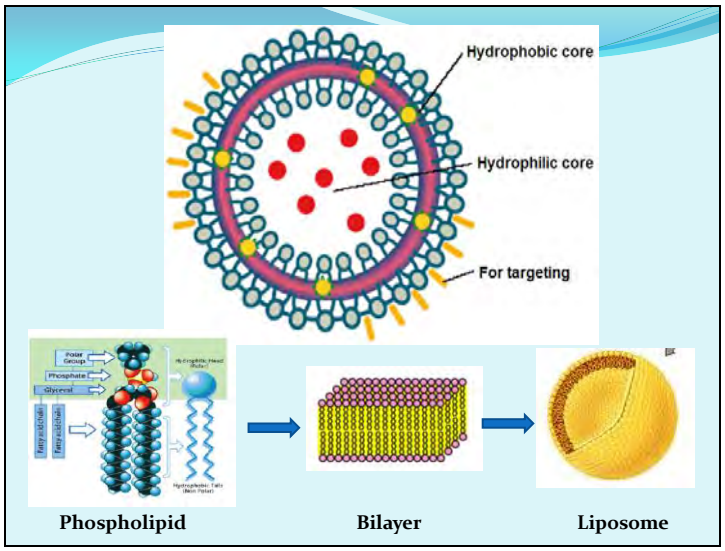


Figure 2.9. Structure of a liposome and its formation (103).

Liposomes are one of the most commonly investigated drug delivery systems used for delivering both drugs and diagnostic agents to the targeted area. Another crutial point is their proper characteristics such as being non-toxic, biocompatible and biodegradable due to the phospholipid structure. They can also carry a variety of drugs with different physicochemical propeties such as hydrophilic, lipophilic and amphoteric molecules by either entrapping inside hydrophilic core or anchoring on the lipid bilayer (104). Nanosized drug delivery systems can also be used for static or dynamic imaging purposes related with diagnosis and for in vitro function tests. There are a number of studies about the use of liposomes for imaging purposes for many years (105,106).

The use of liposomes in the therapy and diagnosis of different diseases is being searched intensively. There are a number of different liposomal based drugs such as anticancerogenic, antiinflammatory, peptid-protein based drugs, DNA, imaging agents, vaccines. By this way fine, different results were obtained depending on increased bioavailibility and decreased adverse effects (107). Some of them are at clinical trials of Phase III and Phase IV (Table 2.2). (104-106,108-110).

Table 2.2. Liposomal preparations in different phase studies for diagnosis or therapy of a variety of diseases (104,108,109,111-113).

Drug or Diagnostic Marker	**Application**	**Company**	**Position**
^{111}In radiolabel	Imaging of tumors such as melanoma, sarcoma and lymphoma	Vestar Inc.	Vescan® Clinical trial phase II/III
Daunorubicin	Treatment of AIDS, ovarian, breast cancer and Kaposi's sarcoma	Sequus Sequus NeXstar	Doxil® (USA) Caelyx® (Europe) Phase III Clinical trial phase

		Liposome Co.	II
Doxorubicin	Cancer treatment	Sun Pharmaceutical Industries Ltd.	LipoDox®
Daunorubicin, Daunorubicin citrate	Treatment of AIDS, cancer	NeXstar NeXstar	DaunoXome® Clinical trial
Doxorubicin	Cancer treatment	Celsion Corporation	Thermodox® Clinical trial phase III
Doxorubicin	Treatment of recurrent breast cancer	The Liposome Company, Inc.	Myocet®
Paclitaxel	Treatment of ovarian, breast and lung cancer	NeoPharm	LEP-ETU® Completing of clinical trial phase II
Vincristine	Treatment of metastatic malignant uveal melanoma	Talon Therapeutics	Marqibo®
Cisplatin	Cancer treatment	Regulon, Inc.	Lipoplatin®
Cisplatin	Treatment of head and neck cancer	-	SPI-077™ Clinical trial phase I-II
Amphotericin B	Treatment of fungal infection	NeXstar	AmBisome®
Amphotericin B	Treatment of fungal infection	Liposome Co.	Abelcet®
Amphotericin B	Treatment of fungal infection	Sequus	Amphocil®

Amikacin	Bacterial infection	NeXstar	Mikasome®
Trans-retinoic acid	Cancer treatment	Aronex Pharma.	Antagen®
Anamycin	Cancer treatment	Aronex Pharma.	Phase I/ II
Nystatin	Treatment of fungal infection	Aronex Pharma.	Nyotran®
Vincristine	Cancer treatment	Sequus	Clinical trial
Cisplatin	Cancer treatment	Sequus	Phase I
Muramyl tripeptide	Tumor macrophage activation	Ciba- Geigy	Clinical trial
Prostaglandin E1	Respiration hardness, Myocardial infarction	Liposome Co. Liposome Co.	Phase III
Verteporfin	Treatment of age-related macular degeneration, pathologic myopia and ocular histoplasmosis	Novartis Pharmaceutical Corporation	Visudyne®
Morphine sulfate	Treatment of postoperative pain following major surgery	EKR Therapeutics	DepoDur®
Cytarabine	Treatment of neoplastic meningitis and lymphomatous meningitis	Skye Pharma.	DepoCyt®
Amikacin	Treatment of lung infections	Transave Inc.	Arikace™ Phase III
Hepatitis B	Vaccine	Swiss Serum and Vaccine Institute	Hexapel®
Hepatitis A	Vaccine	Crucell company Berna Biotech Ltd.	Epaxal®

Influenza	Vaccine	Crucell company Berna Biotech Ltd.	Inflexal V®
Sulphur Hexafluoide	US imaging	Bracco Diagnostic Inc.	Sonovue®
Perfluorocarbon	US imaging	ImaRx Therapeutics Inc.	Aerosomes®

Niosomes are nonionic surfactant vesicles in which hydrophobic and hydrophilic active pharmaceutical ingredients can be encapsulated (14). The composition of niosomes are very similar to that of liposomes however the substances used for the preparation of niosomes, non-ionic surfactants, give them a more stable structure and more ability to penetrate through BBB (Figure 2.10.).

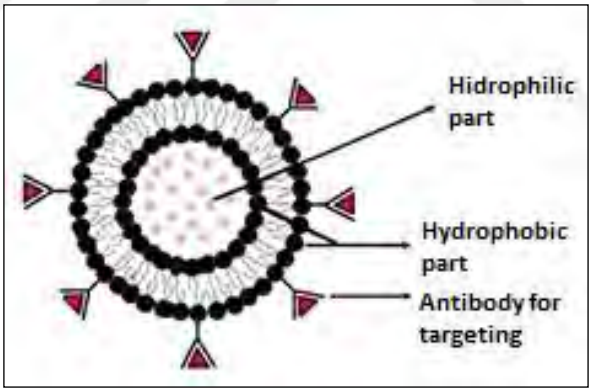


Figure 2.10. The structure of niosomes (114).

Niosomes have some advantages as follows;

-They can be prepared in high amounts (115).

- They can encapsulate a variety of drug molecules,

- Their storage doesn’t require any special conditions,

- The half-life of encapsulated drug can be increased and improved organ distribution and metabolic stability (116).

- They can deliver antiinfective agents, anticancer agents, antiinflammatuar agents and vaccine adjuvants, anticancer drugs and antiinflammatuar agents (117).

They have penetration enhancer properties depending on consisting of surfactant especially for skin administration (115).

Liposomes and niosomes are one of the mostly conspicuous sytems for brain delivery. Liposomes can be formed by self sustainable bilayered structure in various sizes like small unilamellar vesicles (SUVs) to multilamellar vesicles (MUVs) (12,13). Depending on the proper features of liposomal drug delivery systems, a single liposome vesicle can carry more than 10000 drug molecules (118). Liposomal and niosomal drug delivery systems have a variety of advantages making them desirable systems.

General Advantages of Liposomes and Niosomes:

Some advantages of liposomal and niosomal drug delivery systems are given below (119-123):

1. These systems are biocompatible systems which can deliver both lipophilic and hydrophylic drugs.
2. Liposomes and niosomes are biodegredable depending on phosphospholipid and nonionic surfactant composition, respectively and depending on that they do not designate immunogenic and toxic effect.
3. They can be formulated in variable size and physicochemical composition.
4. These systems can designate therapeutic effect in lesser doses and they have the ability to sustain the plasma active ingredient dose in therapeutical dose window in desired time interval.
5. While these delivery systems can increase dosage interval and half life, they decrease or prevent adverse or toxic effects.

6. They prevent active ingredients from enzymatic effects.
7. These systems can be used for targeting purposes to the desired tissues, organs or even cells.
8. They can deliver a wide range of drugs such as wide spectrum chemotherapeutics, imaging agents, antigens, immunomodulators, chelating agents, hemoglobin and cofactors, lipids, genetic materials.
9. These systems can behave as drug depots and the encapsulated drugs can be released very slowly within time. Due to their extended release profile, efficient but non-toxic therapeutic effect can be obtained and sustained within the blood circulation and local application area.

2.4.1.1. Targeting of Liposomes and Niosomes

One of the mostly observed problems with drugs applied systemically is their rapid removal and large distribution volume within the body. For this reason, it is needed to apply the diagnostic or therapeutic drug in higher doses or in repetitive doses to the patient to achive the desired concentration within the target tissue. This may cause higher toxicity and risk of low benefit/harm ratio. To prevent this effect drug delivery systems such as liposomes and niosomes are used succesfully as a solution.

The most commonly seen restricting properties for targeting of conventional (unmodified) liposomes are their recognition by RES cells located within liver and spleen and very limited blood circulation time due to rapid removal. Opsonins which are plasma proteins percieve liposomes as foreign materials within the body and bind onto the liposome bilayer. This binding process activates retantion of liposomes by RES cells and their removal from the blood circulation by this way (124,125). This process is the protection of immune system by removal of all foreign materials including liposomes by nonspecific elimination mechanism (fagositosis).

Especially in the first years of the development and use of liposomes, the property of fast retantion of liposomes by RES was used for the therapy of infections composed by patogens localized within RES organs and it was used as an advantage to deliver antimicrobial drugs to these organs (125). However, this property is also a disadvantage for liposomes because the fast removal of liposomes by RES causes a

significant decrease in therapeutical effect within the organs except RES and additionally sometimes a decrease in the toxicity risk of dose within RES (126,127).

A milestone for liposomal research is their lokalization in the pathological region depending on small particle size and altered surface propertis after i.v. administration which is known as ***passive targeting*** (128,129). With this development, the clinical studies of liposomes are getting a velocity and additionally it is possible to develop commercially available liposomal drugs.

Additionally, in some conditions like cancer therapy, gene therapy, it is desired to bind the drugs directly onto the target cell and take it within the cell. For this reason, to obtain cell binding of receptor-mediated liposome and target cell binding cell surface specific ligands are attached on liposome surface. This approach is named as ***active targeting*** (128,130). Briefly, active targeting is defined as redirecting of drug or drug delivery system to a specific tissue, organ or cell by the change of natural distribution of drug or drug delivery system. Active targeting can be performed by the conjugation of a target specific ligand onto liposomes (131-133) by magnetic targeting (134) or ultrasonic targeting (135,136). These specific vectors can be mAb specific to a receptor such as anti-transferrin receptor or human insulin receptor (HIR), trojan horses, cationized proteins such as cationized human serum albumin, endogenous peptides or plasma proteins. These active targeting vectors facilitate BBB penetration by coupling with brain drug transport vector through absorptive-mediated transcytosis or receptor-mediated transcytosis (75,137-139). However, active targeting is frequently hard to achieve due to high costs, time consuming and difficulty in manuplation depending on the necessity to the combination of many fields such as chemistry, pharmacy, biology, and in many cases the result in pratical applications is unsuccessful and does not meet with the theoretical expectations.

2.4.1.1.2. Passive Targeted Liposomes, Niosomes and Brain Penetration

The solution to increase the retention and localization of liposomes within the target tissue is to increase the blood circulation time of liposomes. Passive targeting can be performed with the help of a steric hindrance created by nano-vectors. These drug delivery systems are genereally administered through parenterally. Surface

charge, surface properties, particle size, site membrane lipid packing and extent of steric hindrance are some significant properties for passive targeting of sterically stabilized long circulating liposomes. These liposomes are distinguished from other conventional liposomes depending on non-linear kinetics of sterically stabilized liposomes (17). These liposomes are called ***stealth liposomes*** depending on the existence of steric hindrance achieved by coating with a hydrophilic biocompatible polymer prevents them from opsonisation by tissue macrophages. Due to this reason, they stay longer time within the blood circulation which helps them to localize more in the target disease area. This is also named as ***long-circulating liposomes***. (122,140,141).

These long-circulating liposomes or stealth liposomes are one of the mostly researching fields among drug delivery systems today which enriches the value of liposomes. These liposomes localize in the diseased area such as tumor, infection or inflammation in which the higher vessel permeability is observed depending on longer circulation time, small particle size and proper surface properties (122,142-144).

Brain targeting can be obtained by this way rather than targeting to other RES organs like liver, spleen, etc. Sterically stabilized long circulating liposomes can penetrate into the brain in the presence of a brain tumor with the help of enhanced permeability and retention (EPR) effect in the tumor tissue. Their accumulation in the desired tissue depends on their longer pharmacokinetic profile. It was reported that liposomes with a particle size smaller than 100 nm, non-charged and rigid bilayer structured due to saturated lipid and high cholesterol structure stay longer in blood circulation (145). Another approch to increase blood circulation time is the addition of specific glicolipids such as monosialoganglioside (GM1) phosphatidylinositol to the structure of bilayer of liposomes (146). A rigid bilayer structure in liposome is necessary to achieve these approaches successfully. However, this approach is not used in some in vivo applications in order to obtain more leaky bilayer composition for desired drug release profile.

The mechanism of preventing opsonisation by surface coating with a hydrophilic polimer depends on the increase of hydrophilicity and surface charge by surface coating and increased repulsive interactions between polymer coated

nanocarrier and blood ingredients and the formation of impermeable polymeric structure formation on particle surface even in low polymeric concentrations (123,133,142,147).

Coating was initially made by monosialogangliosides (18) to incease the distribution half-life. Recently synthetic polymeric matrials such as polyethylene oxide, propylene glycol, PEG, polyacrylamide, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), hydroxypropyl methylcellulose (HPMC) are intensly investigated for surface coating. Polymers' hydrophilic chains prevent them from interaction between serum opsonins by surface modification of liposomes with lower contact angle between particles and phagocytic cells (16). PEG is one of the mostly desired hydrophilic polymer due to its many advantages such as higher water solubility, having a higher elasticity within the polymer chain, very low toxicity, immunogenicity and antigenicity, very low accumulation within RES organs, biodisintegrability and minimum effect on specific biological properties of modified pharmaceuticals (133,142,148,149).

In order to add the polymer to the lipid bialyer, PEG-lipid conjugates are used. While the lipid part of the conjugate is attached to bilayer, hydrophilic part is used to perform steric hindrance to prevent plasma protein binding onto liposomes. There are a variety of commercially available PEGs having different molecular weight. PEGs having a molecular weight smaller than 40 kDa are eliminated from the body via kidneys instantly. For coating of delivery systems it is prefered to use PEGs with a molecular weight smaller than 1000-20.000 Da and 1-5 % concentration is frequently used as concentration (133,144).

PEGylating provides not only long circulation, but also the change from dose dependent (saturable) pharmacokinetic to dose independent (log-linear) pharmacokinetic within the clinical dose range. A drug delivery system with dose independent (log-linear) pharmacokinetic provides prediction of plasma drug level easily as a function of dose. The circulation half life of small, PEGylated liposomes is 45h generally and it is generally dose independent (122).

A study for observing the effect of phagocytic cells of the innate immune system, mainly neutrophils and monocytes in brain delivery of liposomes was performed by Afergan et al (150). This hypothesis mainly depends on the knowledge

of the brain's being under immunological surveillance by neutrophils and monocytes. For this purpose, serotonin encapsulated, negatively-charged, nanosized liposomes were formulated. The brain uptake of seratonin encapsulated intact liposomes found significantly higher at 4th h and 24th h after i.v. administration in rats when compared with seratonin solution. It was also observed that monocytes are the main transporters of liposomes to the brain.

With the use of liposomal delivery systems, anticancer drugs can accumulate more in cancer tissue. Research about this issue designated that liposomes can overcome the problems related with brain tumor delivery and higher amounts of drug can accumulate and deliver brain in intracranial models (151,152). Antitumor agent 5-FU loaded liposomes were prepared by Soni et al in 2008 (153) via film method either with a brain targeting mechanism of transferrin coupling or nontargeted liposomes. ^{99m}Tc was used for labeling and in vivo labeling was found efficient. While almost 10-fold increase in brain tumor tissue uptake was observed in the uptake of 5-FU by the use of its liposomal delivery, almost 17 fold increase was seen in the uptake of 5-FU by the use of transferrin coupled liposomes. Both passively targeted and actively targeted 5-FU encapsulated liposomes showed higher brain tumor uptake when compared with free 5-FU (153).

For the treatment of PD, a study was performed with the use of dopamine encapsulated liposomes comprising surfactants Span 20 (S20), Span 40 (S40), Span 80 (S80) and Span 80 - Tween 80 combination (ST 80) by Pichandy et al (154) . It was observed that ST 80 formulation is the most effective one in the treatment of parkinsonism than the other formulation and L-DOPA solution (Syndopa) as control by i.p. injection due to the reduction of parkinsonism's extrapyramidal side effects using actophotometer and rotorod after intrapritoneal injection to rats with haloperidol induced parkinsonism. Another study about preparation of L-DOPA derivatives as potential prodrugs was performed by Di Stefano et al (155) for PD treatment in order to decrease its side effects. The drug was encapsulated in unilamellar liposomes containing DMPC and cholesterol also to increase its bioavailibility. Striatal levels of L-DOPA and dopamine were designated 2.5-fold elevation after i.p. administration of liposomal formulation of prodrug (+)-1b ([(O,O-diacetyl)-l-dopa-methylester]-succinyldiamide) when compared with L-DOPA itself

or free prodrug (+)-1b. In vivo results designated a great difference between the effect of liposomal drug and drug solution (155).

Another similar research was performed by During et al (156) about formulation of dopamine-containing liposomes and they were implanted stereotactically into the partially denervated corpus striatum of PD model rats subjected to unilateral lesions of the substantia nigra. Extracellular dopamine levels were found higher with dopamine liposomes than control group. Additionally, partial behavioral recovery also observed higher in rats received dopamine liposomes.

In another study performed by Yurasov et al (157) the dose of L-DOPA was managed to reduce 10 folds by encapsulated in nanosized, unilamellar liposomes composed of PC and cholesterol when compared with free L-DOPA which also reduced the side effects.

Jain et al (23) developed dopamine HCl containing, positively charged liposomes to penetrate BBB for the therapy of PD. It was observed that dopamine effectively delivered to the brain by passive targeting and protected from degredation by incorporating it into liposomes when compared with plain dopamine HCl, L-DOPA preparations and marketed formulation of L-DOPA containing carbidopa (Syndopa®) (23). It was also observed that cationic liposomes can easily cross BBB by the mechanism of absorptive mediated transcytosis (137).

Another research performed by Amicarelli et al in 1999 (24) about tyrosinase encapsulated liposomes. These systems were administered by stereotactic injection and a significant increase in dopamin level was observed in rat brain. By this way they managed to provide successfully the lack in tyrosine hydroxylase with tyrosinase to produce L-DOPA from L-tyrosine (24).

A liposomal formulation of rivastigmine was prepared by Ismail et al (158) to obtain effective therapy in Alzheimer's therapy. Its efficacy was assessed in an Aluminium Chloride ($AlCl_3$)-induced Alzheimer's model rats. It was observed that both rivastigmine liposomes improved the deterioration of spatial memory induced by $AlCl_3$ more than rivastigmine solution (control). Additionally with the use of rivastigmine liposomes nearly preventing amyloid plaque formation was observed in rat brain which was not observed with the use of rivastigmine solution.

Kizelsztein et al (159) prepared PEGylated nanoliposomes loaded with antioxidant tempamine ameliorate to perform EAE in mice. It was seen that 3-5 fold enhancement was observed in diseased mice brain with EAE by liposomes depends on the partial disruption of blood-brain barrier (159). Another similar research was performed for therapy of EAE and relapses in multiple sclerosis by Schmidt et al (160) by formulation of prednisolone encapsulated PEGylated liposomes. ^{3}H-labeled designated higher accumulation in CNS of rats with EAE than in healthy control animals. Macrophage infiltration was clearly decreased only by prednisolone encapsulated liposomes. Prednisolone encapsulated liposomes found highly effective in EAE treatment and is superior to a 5-fold higher dose of free methylprednisolone.

A study was performed for brain PET imaging of rats with hypovolemic shock by Awasthi et al (161) by the formulation of ^{15}O-labeled, PEGylated hemoglobin encapsulating liposomes. It was observed that hemoglobin encapsulating liposomes efficiently picks up ^{15}O-labeled oxygen gas in a rat model of 40% hypovolemic shock due to ^{15}O-PET evaluation. Cerebral PET images of anesthetized rats inhaling ^{15}O-labeled O_2 gas showed efficient oxygen carrying and delivery capacity of hemoglobin encapsulating liposomes. Compared with control fluids, hemoglobin encapsulating liposomes significantly improved cerebral metabolic rate of oxygen when compared with control groups. An efficient MR imaging and neuroprotective agent was formulated with the use of nanosized, PEGylating long circulating liposomes by Shazeeb et al (162) for neuroprotective treatment of ischemic in brain. Liposomes encapsulated M40401, which is a synthetic enzyme mimetic that has a catalytic activity rate exceeding that of the native superoxide dismutase enzymes and it comprises paramagnetic Mn(II) cation, to prevent the cardiotoxicity of Mn(II) in free form. The superoxide anion (O_2^-) is an essential component of the immune system's defense against invasion of microorganisms however, its toxicity should be counteracted by superoxide dismutase enzyme. Succesful results were obtained and liposomal formulation of M40401 caused differential and region-specific enhancement of mouse brain after systemic administration.

A study was performed by Caraglia et al (163) about the therapy of neuropathic pain by formulation of nanosized, zoledronic acid encapsulated,

PEGylated liposomes. It was observed that zoledronic acid encapsulated liposomes can cross BBB when compared with free zoledronic acid after i.v. administration to mice with nerve injury.

Therefore, passive and active targeted liposomal and niosomal delivery systems can be successfully used for the purpose of BBB penetration and either diagnosis or therapy of different central nervous system disorders.

2.5. Release Kinetics of Drug Delivery Systems

Drug release is a significant issue for the regulation of drug delivery. Due to the development in pharmacy, chemistry, medicine, engineering and computer science engineering, novel drug delivery systems have been developed for many years. A variety of naturally derived and synthetic macromolecules are formulated or synthetized to improve and facilitate drug release. The definition of drug release refers to the process in which drug solutes migrate from the initial position in the polymeric system to the polymer's outer surface and then to the release medium (164). Drug release can be affected by different factors such as the physicochemical properties of the solutes, structural characteristics of the material system and release environment (165) .

The fundamental principle for evaluation of the kinetics of drug release was assessed by Noyes and Whitney in 1897 as the equation given below (166):

$$dM/dt = KS (C_s - C_t) \quad \text{.....Equation 1.}$$

M is the mass transferred with respect to time (t), by dissolution from the solid particle of surface (S), under the effect of the force of concentration (C_s-C_t). Ct is the concentration at t and Cs is the equilibrium solubility of the solute at the experimental temperature. Dissolution rate (dM/dt) is the amount dissolved per unit area per unit time (167).

A number of kinetic models are used to explain the drug release from dosage forms or drug delivery systems. The qualitative and quantitative changes in a

formulation may affect and change the drug release and in vivo performance of drugs. Evaluation of in vitro drug dissolution data to predict in vivo performance is very essential for the development of controlled release formulations (167-170)

The kinetics of drug release from controlled release formulation can be evaluated in 3 different sections (167):

1. Statistical methods: exploratory data analysis method, repeated measures design, multivariate approach [MANOVA: multivariate analysis of variance] (169,170).
2. Model dependent methods: zero order, first order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas, Baker-Lonsdale, Weibull, Hopfenberg, Gompertz and regression models (171-173)
3. Model independent methods: difference factor (f1), similarity factor (f2) (174-176).

Among these methods, model dependent methods are used more frequently chosen to evaluate the release kinetics of drugs. Only some of the model dependent methods will be mentioned in this section. These model dependent methods are based on different mathematical functions describing the dissolution profile (167,173,177)

- ***Zero Order Model***

This approch comprises drug dissolution from dosage forms that do not disaggregate and release the drug slowly and by the equation given below:

$Q_0-Q_t = K_0t$ …….Equation 2.

Rearrangement of this equation yields:

$Q_t = Q_0 + K_0t$ …….Equation 3.

where Qt is the amount of drug dissolved in time (t), Q_0 is the initial amount of drug in the solution (generally $Q_0 = 0$) and K_0 is the zero order release constant.

To study the release kinetics, data obtained from in vitro drug release studies were plotted as cumulative amount of drug released versus time (178,179). This approach can be used to describe the drug dissolution of several types of modified release pharmaceutical dosage forms such as some transdermal systems, matrix tablets with low soluble drugs in coated forms, osmotic systems, etc (167,180,181).

- ***First Order Model***

This model can be used to describe absorption and/or elimination of some drugs. The release of the drug which followed first order kinetics can be expressed by the equation:

$$dC/dt=-Kc \quad \text{.....Equation 4.}$$

where K is first order rate constant. This equation can be expressed as:

$$\log C = \log C_0 - Kt / 2.303 \quad \text{.....Equation 5.}$$

where C_0 is the initial concentration of drug, k is the first order rate constant, and t is the time (182). The data obtained are plotted as log cumulative percentage of drug remaining vs. time which would yield a straight line with a slope of -K/2.303.

This relationship can be used to describe the drug dissolution in pharmaceutical dosage forms such as those containing water-soluble drugs in porous matrices and some lipid containing drug delivery systems (167,178).

- ***Higuchi Model***

Its mathematical model was first investigated by Huguchi in 1961 (183) with the purpose of describing drug release from a matrix system. This approach based on some rules such as a) initial drug concentration in the matrix is much higher than drug solubility; b) drug diffusion takes place only in one dimension; c) drug particles are much smaller than system thickness; d) matrix swelling and dissolution are negligible; e)drug diffusivity is constant; and f) perfect sink conditions are always

attained in the release environment (167). The mathematical model is given by the equation:

$$f_t = Q = A \sqrt{D(2C-C_s)\, C_s\, t} \quad \text{.....Equation 6.}$$

where Q is the amount of drug released in time (t) per unit area (A), C is the drug initial concentration, Cs is the drug solubility in the matrix media and D is the diffusivity of the drug molecules (diffusion coefficient) in the matrix substance.

In a general way, it is possible to simplify the Higuchi model (183) as (generally known as the simplified Higuchi model):

$$f_t = Q = KH \times t^{1/2} \quad \text{.....Equation 7.}$$

where, KH is the Higuchi dissolution constant (177).

This relationship can be used to describe the drug dissolution from several types of modified release pharmaceutical dosage forms such as transdermal systems and matrix tablets with water soluble drugs (167,183).

2.6. In Vitro BBB Cell Co-Culture Model

Cell culture is the process in which cells are grown under controlled conditions, generally outside of their natural environment. These cells may be removed from the tissue directly and disaggregated by an enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain (184). The historical development and methods of cell culture depends on tissue culture and organ culture. English physiologist Sydney Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for maintaining the beating of an isolated animal heart outside of the body in 19th-century (185). In 1885, Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days and established the principle of tissue culture (186). In 1907, Ross Harrison succeeded to grow the frog neurons in a lymph liquid medium (187,188).

Primary culture is the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate called as rich confluence. Cells have to be subcultured by transferring them into a new vessel with fresh growth medium to provide enough space to continue growing (184).

Cell line or subclone term is called after the first subculture. This subclones have a limited life span and highest growth capacity due to the passaging process (184).

The use of cell culture studies became popular nowadays due to its significant role in enlighting molecular genetic, immunology, surgery, bioengineering and monoclonal antibody, drug development and vaccine formulation. Recently use of in vitro cell culture techniques are taking place of animal experiments due to its being cost effective and improved ethic rules in drug therapy, toxicity and permeability studies.

The conditions such as special medium and substrate carry a significant role in performing a proper culture medium. Cell confluent, cell-matrix interactions and nutritional factors are also essential issues for cell differantiation and cell proliferation. It is essential for supporting the cellular functions and production of new cells. However, it is obvious that different cells have different neccessities.

Cell/tissue neccessities and selection depends on the structure of the planned study. Embrionic tissues or cells proliferate faster than mature tissues or cells. Mature cells comprise less proliferating and non-splitting cells but more specialized cells. The proliferation speed is limited in normal cells when compared with neoplastic cells. There also some special cell cultures that can be used as in vitro models of brain uptake. To compose a human BBB cell culture model, cerebral microvessel endothelial cells were first isolated in 1970s (189). In order to perform quantitative permeability studies and drug discovery, brain microvessel endothelial cell (BMEC) culture can be used (190,191).

Transwell containing immortalized human brain endothelial cell line (hCMEC)/D3 BBB model is the most commonly used BBB model to evaluate the transport and penetration studies of drugs and drug delivery systems. A schematic in vitro BBB model in a Transwell comprising hCMEC/D3-NHA-BBB system

including hCMEC/D3 as endothelium and NHA as astrocytes in co-culture is illusterated in Figure 2.11.

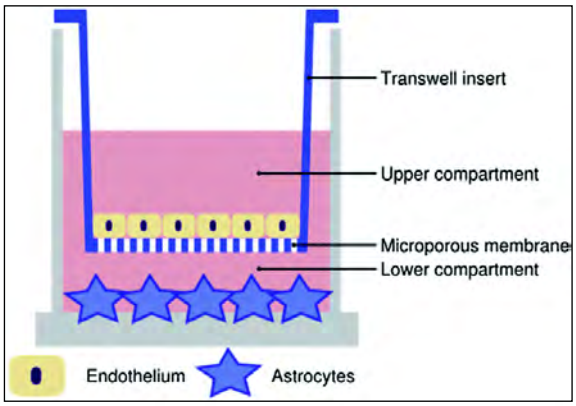


Figure 2.11. In vitro model of BBB (192).

The hCMEC/D3 cell line was derived from human temporal lobe microvessels isolated from tissue excised during surgery for control of epilepsy. The primary isolate was enriched in CECs. The hCMEC/D3 cell line as human BBB model which is the closest human BBB model for evaluating nanoparticle interaction with a cellular barrier (189,193). To perform a better a BBB model, it then supported by direct/indirect co-culture with human astrocytes (194) which provides evaluating of cell–cell signalling and other physiological effects.

T dos Santos et al designated the interactions of this model, uptake into and transport across the BBB (195). In the concept of continuing studies, with the imaging modalities, very small numbers of nanoparticles can cross in vitro BBB. According to another study performed by Raghnailll et al that the effect of nanoparticle accumulation in the BBB on lysosome health and paracrine signalling affecting BBB cells. This effect can be essential for the protection of vulnerable tissues by the biological barriers (192). Raghnaill et al investigated the behaviour of carboxylated polystyrene nanoparticles in vitro BBB model. It was observed by TEM imaging that nanosized nanoparticles accumulate but not degradated within the lysosomes over time. It was observed that in vitro BBB does not restrict the uptake of NPs completely. However, the uptake of these nanoparticles was observed to slow

down in a large amount in BBB monolayer compared to that of in single hCMEC/D3 cells (192).

Among this thesis, we chose and used a non-contact BBB co-culture model. Endothelial cell layer divides this system as apical (blood side) and basolateral side (brain side). This BBB co-culture model cmprise Human brain microvascular endothelial cell and astrocytes.

2.7. Parkinson's Model in Small Animals

To investigate the pathogenesis and patophysiology of PD, PD animal models have been widely used. To perform PD animal models, neurotoxins can be applied either by systemic or local (intracerebral) administration in mammals like rodents or primates (196). Toxic and transgenic animal PD models have their own properties and limitations which should be kept in mind when choosing the appropriate model specific to the purpose of the research. In case of obtaining a substantial and reproducible nigrostriatal lesion to test therapeutic interventions aimed at counteracting PD-related cell death, a classical toxin application model such as MPTP for mice or 6-OHDA for rats can be used. If the purpose is to investigate the selected molecular mechanisms of PD pathogenesis, transgenic models can be used however, this is not the issue of this thesis. Therefore, these models are valuable till obtaining the 'perfect' model in the future (196).

2.7.1. 6-OHDA

6-OHDA is a hydroxylated analog of dopamine (Fig. 2.12.) (197,198). The mechanism of 6-OHDA induced neurotoxicity was given in Figure 2.13. Briefly, 6-OHDA is taken up from the extracellular space by DAT or noradrenaline membrane transporter (NAT). Then, 6-OHDA is stored in catecholaminergic neurons. 6-OHDA undergoes both enzymatic degradation by monoamine oxidase (MAO-A) and autoxidation inside neurons, generating several cytotoxic species which, by damaging endocellular proteins and nucleus, produce neuronal damage. Moreover, 6-OHDA might induce neurotoxicity by impairing the activity of mitochondrial complex I. In experimental animals, 6-OHDA is usually administered in association

with NAT blockers, such as desipramine (DMI), to prevent its uptake by noradrenergic terminals and to selectively target dopaminergic neurons (199).

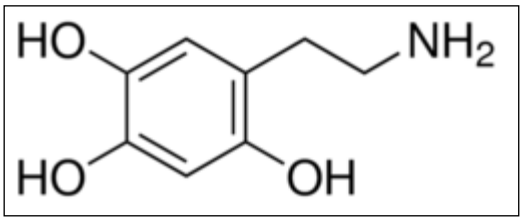


Figure 2.12. The molecular structure of 6-OHDA (200).

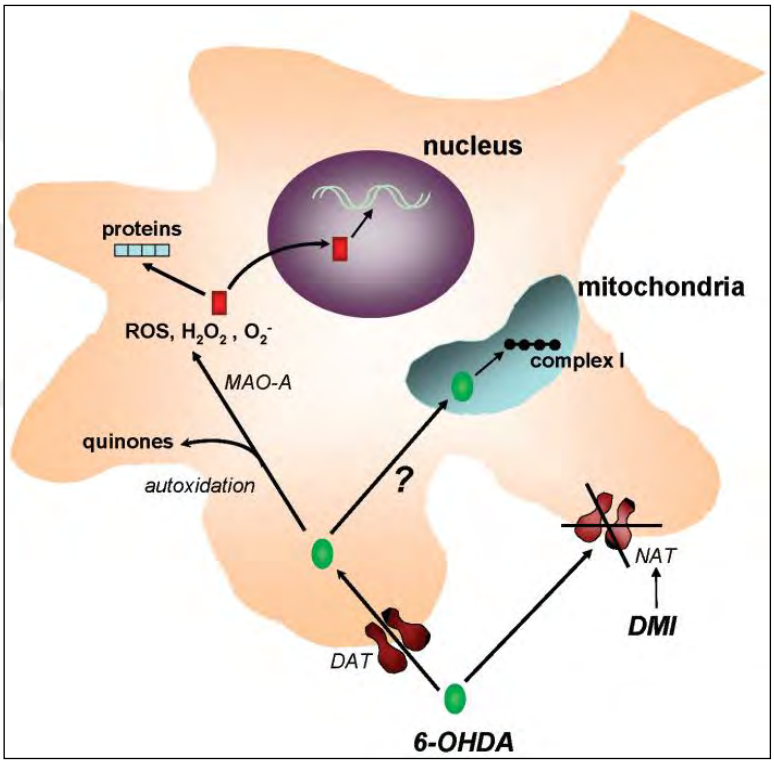


Figure 2.13. Mechanisms of 6-OHDA-induced neurotoxicity (199).
*Malondialdehyde (MDA) level, the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

It was reported at the begining that 6-OHDA caused depletion of noradrenaline in the mouse heart (201,202). 6-OHDA was also used for inducing selective degeneration in sympathetic adrenergic nerve terminals (203) a specific cell population (204). The neurotoxin 6-OHDA has been widely used as a model of PD

through the lesion of nigrostriatal dopaminergic system. 6-OHDA is responsible from the degeneration of both dopaminergic and noradrenargic neurons (205). 6-OHDA accumulates in the cytosol of neurons and there to generate reactive oxygen species it is oxidized and afterwards oxidative stress-related cytotoxicity is formed (206,207). To degenerate specific neurons after located stereotactically, 6-OHDA is carefully applied in specific regions in brain to target specific neurons and region of interest. It is pereferred to administer 6-OHDA to rats when compared with mice to perform PD model due to larger body size when compared with mice (208). 6-OHDA can be also used in cats, guinea pigs, dogs, and monkeys (200,209,210).

The importance of the site of injection and the exact coordinate within the brain depends on magnitude and characteristics formed by neurotoxin 6-OHDA (211-213). The degree of neurodegeneration affects the therapy effectiveness of drugs or drug delivery systems. It is commonly pereferred to administer 6-OHDA unilaterally to the substantia nigra, medial forebrain bundle or striatum (214,215). When delivered to the striatum, 6-OHDA induces slow, progressive, and partial damage to the nigrostriatal structure in a retrograde level over a period of up to 3 weeks (212,216). The administration to the striatum has three advantages: First, the progressive and less extensive lesion model is more proper to early PD. Second, this model generally produces PD nonmotor symptoms with cognitive, psychiatric, and gastrointestinal dysfunction (217-219). Third, the simplicity in administration to stereotactically located small animals with large structure such as the striatum increases the succeess of the model (200).

The main benefit of 6-OHDA model in animals is its specific effect on quantifiable circling motor abnormality (220). It is generally administered to one hemisphere (hemiparkinsonian model) unilaterally which gives the chance to have a contact unlesioned side as control. To evaluate the success and performance of the lesion, some drugs such as dopamine receptor agonists (apomorphine), L-DOPA (the dopamine precursor), or dopamine releasing compounds (amphetamine) can be systemically administered inducing asymmetrical rotation (220,221). This circling motor behaviour of animals depends and correlates with the magnitude of nigrostriatal lesions (205,212,220,221). Briefly, unilateral 6-OHDA rat model is generally used for evaluating antiparkinsonian effects of drugs and neuroprotection

effects of PD (222-224). Unilateral 6-OHDA rat model can also be used to evaluate the clinical improvement of cell transplantation (225-227). This model has also some defficiencies such as lacking of progressive and age-dependent effects of PD (200).

2.7.2. MPTP

There is also another neurotoxin model to produce PD model in mice. However, this model is given very briefly because it was not used within the concept of our thesis. We used Wistar rats among our thesis depending on that we had chosed 6-OHDA lesion for PD formation.

The first study about 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model was performed in 1980s (228,229). The use of dopaminergic neurotoxin, MPTP, induces replication of pathological signs and motoric signs related with PD in primates and rodents by selective destruction of dopamine (DA) neurons of substantia nigra (25). It was reported that MPTP induces loss of nigrostriatal structures in postmortem parts of patients (228,229). The use of MPTP significantly inceased the number of PD research to provide information about pathogenesis and mechanisms for cell death in PD. Additionally, work with this model provides development of some current treatments for PD (230). MPTP toxicity was studied and characterized extensively (231,232). Because, MPTP can easily cross BBB due to its lipophilic property. In astrocytes, MPTP is metabolized by monoamine oxidase-B, and the active toxic cation form 1-methyl-4-phenylpyridinium (MPP+) is formed (Fig. 2.14.).

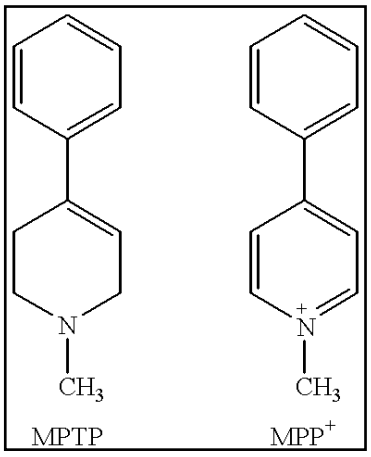


Figure 2.14. The molecular structures of MPTP and MPP^+ (233).

MPP+ is released into the extracellular space from the nigral and striatal astrocytes through organic cation transporter 3 (232,234). Afterwards, it is taken up by neighboring dopaminergic neurons and terminals through the DAT. MPP+ induces neurotoxicity mainly by inhibiting complex I of the mitochondrial electrontransport chain after accumulated in dopaminergic neurons. This causes ATP depletion and oxidative stress increament (235,236). MPTP is widely used for preclinical testing of therapeutic strategies for PD especially in mice however, it can also be used for various mammalian species, including sheeps, dogs, guinea pigs, cats, mice, rats and monkeys (209,237,238). The reason of broad usage of MPTP in mice rather than in rats depends on lesser sensitive of rats to MPTP toxicity (239). MPTP primarily causes damage to nigrostriatal dopaminergic pathways in both mice and monkeys (230,231,240). The advantage of MPTP model is its specific and reproducible neurotoxic effect to the nigrostriatal system (240). Another advantage is that the motor deficits induced by MPTP can be characterized in both monkeys and mice. These abnormal phenotypes can be reversible by L-DOPA or a dopamine agonist, confirming a connection between these symptoms and damage in the nigrostriatal system (241,242). MPTP neurotoxin can be used in PD research based on its ability to produce PD-like effects in humans and nonhuman primates, its reproducible L-DOPA responsive lesion on nigrostriatal system and the simplicity of administration (typical intraperitoneal injection) as a commonly used PD model (200).

2.8. Monitoring Therapeutic Efficacy in PD by Rotational Behaviour and Imaging

Therapeutic effiacy can be observed by some tests. In case of neurodegenerative diseases such as PD, AD, dementia and epilepsy some tests comprising rotational behavior for unilateral lesions and movement tests can be used to evaluate therapeutic efficacy. Imaging can be used for different purposes to improve various different aspects of drug delivery and drug therapy of novel drug molecules or drug delivery systems. This can be used for vizualizing and quantifying the site of drug and drug delivery system accumulation and to asses their efficacy by

this way non-invasively. Furthermore, image-guidance is highly useful for monitoring the success of targeting and release of drug delivery systems such as liposomes, niosomes, micelles, nanoparticles, microbubles, nanocapsules etc. This can be performed by two ways. One is the radiolabeling of the outer shell or encapsulating the desired radioligand within the core of drug delivery systems and then imaging targetability of drug encapsulated drug delivery system. Second way is the imaging of therapy efficacy of drug encapsulated drug delivery system by administering target specific radioligand or radionuclide seperately, afterwards. With the combination of drug targeting and imaging protocol, biodistribution and drug release kinetics can be visualized, patients can be pre-selected, proper and minimum effective dose can be selected and personalized therapy protocol can be administered (243). Medical imaging has an enlarging role in new drug development.

It is anticipated that greater use of imaging during pre-clinical stages will facilitate better translation from animal models to human subjects. The main emphasis is on the application of medical imaging in therapeutic drug trials is very similar to the development of novel imaging contrast agents and radiopharmaceuticals (244). The use of pre-clinical imaging with a proper imaging instrument such as micro-PET, SPECT, CT, MRI, optical imaging and autoradiography in neurodegenerative diseases in PD animal models can contribute to the identification of new imaging biomarkers and also evaluation the therapeutic efficacy of active drug encapsulated drug delivery systems. It is important to use a specific molecular imaging ligand.

2.8.1. Rotational Movements

This is another method than imaging for evaluation of PD lesion and potential efficacy of therapeutic efficacy of new drug delivery systems. Rotational behaviour is one of the generally used measurement tests to evaluate the efficacy of drugs for the treatment of neurodegenerative diseases such as PD, AD, dementia and epilepsy in unilateral lesined animal models. Rotational movement test is used in 6-OHDA rats evaluating lesion-induced motor impairment. Among other rotational behaviour and movement tests, rotameter test can be used for the evaluation of measuring skilled motor performance. 6-OHDA neurotoxin is generally administered

to one hemisphere (hemiparkinsonian model) unilaterally which gives the chance to have a contact unlesioned side as control. Systemic administration of some drugs such as apomorphine and amphetamine induces asymmetrical rotation to evaluate lesion performance (220,221). This circling motor behavior of animals increases with the enhanced magnitude of nigrostriatal lesions (205,212,220,221). This behavior can be used for the evaluation of the efficacy of some new antiparkinsonian drug molecules and drug delivery systems. Antiparkinsonian therapeutic efficacy of drug molecules and drug delivery systems is inversly proportional with the number of ipsilateral turns in rotameter test of lesioned PD model animals after dopamine agonist apomorphine or amphetamine injection (245). Apomorphine induced rotation test is a useful tool for primary screening of 6-OHDA lesion (246). Although 6-OHDA induced rotameter test has some drawbacks with the use of apomorphine test such as its use in the prediction of rotational performance of 6-OHDA rats to L-DOPA, (245) it is very frequently used in rats performed PD for assessing both the success of neurodegeneration and the efficacy of drug therapy.

2.8.2. Imaging Modalities

The use of imaging and drug monitoring or following up with a proper imaging modality such as There are many methods to monitor PD as other CNS neurodegenerative diseases like AD, multiple sclerosis (MS), dementia and epilepsy. CT, MRI, PET, SPECT, PET/CT, SPECT/CT, PET/MRI, optical imaging and autoradiography are some of the commonly used and desired imaging techniques and instruments. All of these techniques have some pros and cons however they can all be used for evaluating different aspects of brain structures or functions (247-249). These modalities can also contribute to the identification and formulation of new drug delivery systems, drugs and imaging biomarkers.

Another mostly and simply used imaging technique is the autoradiography. Autoradiography with Beta imager is an imaging technique providing a fast, accurate and efficient detection of many diseases and gives the opportunity to work on small sections and slides. However, to perform research on sections obtained from specific slices of desired organs or tissues in small animals, some rapid and practical imaging modalities can be performed such as autoradiography in frozen brain sections after

incubating with the specific radioligands (249). In this research, autoradiography was used to evaluate and monitor the therapeutic efficacy of nanosized, PEGylated, neutral, Pramipexole encapsulated liposomes and niosomes in brain sections of 6-OHDA lesioned rats after sacrification at 21 dpl.

2.8.2.1. Autoradiography

Autoradiography is the process to obtain the autoradiograph which is an image on an X-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., β-particles or α-rays) from a distribution of a radioactive substance. It is also available as a digital image (digital autoradiography), with the use of scintillation gas detectors (250) or rare earth phosphorous imaging systems. Autoradiography was first used in 1867 accidentally after observing a blackening on emulsions of silver chloride and iodide by uranium salts. These studies were contributed to the discovery of radioactivity. Although, the discovery of autoradiography was very early, its use as a biological technique was begun after World War II after the development of photographic emulsions and silver halide comprising stripping films (251-253). Lacassagne and collaborators developed the first autoradiographic method as film emulsion to localise radioactive Polonium in biological specimens in 1924 (254). Film has some special characteristics that make it suitable for autoradiography. The sensitivity of the film and the background noise are controlled by the proportion of the crystals per unit area. The number of crystals should not exceed 10 crystals per 10,000 square micron, higher counts of crystals may cause low signals and effect the evaluation of experimental data (255,256). These grains are ionised by beta-particles emitted from the tissue sample and cause image formation. The extremely small size of these grains causes an excellent intrinsic spatial resolution (256). Figure 2.15. designates the schematic presentation of autoradiography from a radioactive ligand incubated tissue section (257).

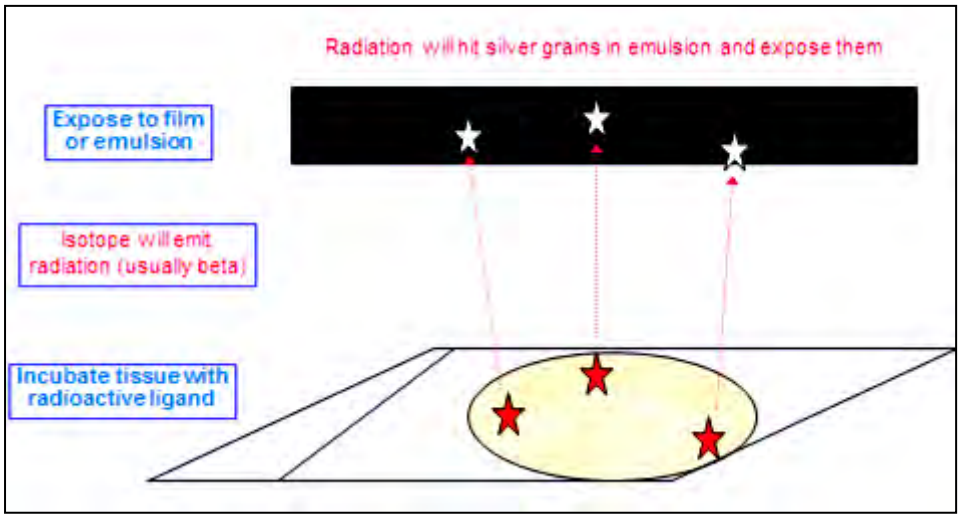


Figure 2.15. The schematic representation of autoradiography from a radioactive ligand incubated tissue section (257).

Typically, biomolecules are labeled with ^{32}P or ^{35}S and detected by overnight film exposure. Other from these radionuclides, ^{125}I and ^{3}H can also be used for the detection in autoradiography. These radioisotopes emit beta-particles which are fast electrons. Table 2.3. gives the amount of commonly used isotopes which can be detected by overnight autoradiography (258,259).

Table 2.3. Detection limits of some isotopes (258).

Isotope	CPM Necessary for Detection	Energy Per Emission (MEV)
^{3}H	$>10^{7}$	0.0055
^{14}C	2000	0.050
^{35}S	1000	0.167
^{32}P	100	0.70
^{125}I	10	(gamma)

The film or emulsion is apposed to the labeled tissue section to obtain the autoradiograph or autoradiogram. It was indicated by the auto-prefix that the radioactive substance is within the sample in which the sample is X-rayed using an external source. In the case of micro-autoradiography, the localization of silver

grains on the interiors or exteriors of cells or organelles can be detected microscopically (259,260).

Autoradiography has many applications in the laboratory including measuring the pathways of many different biomolecules**.** These applications are generally based on the identification, localisation and quantification of neurotransmitter receptors in brain tissue sections (256). For instance, autoradiography can be used to analyze the length and number of DNA fragments after they are separated by gel electrophoresis and to determine tissue or cell localization of a radioactive substance, either introduced into a metabolic pathway, bound to a receptor (261,262) or enzyme, or hybridized to a nucleic acid (259,263). Autoradiography is a method used to map the distribution of radiolabeled biomolecules, tracers, deposited in thin tissue specimens (256) and the location of radiolabeled ligands to visualize and quantify receptors in tissue. Autoradiography can also be used to determine hormonal uptake and indicate receptor location in the field of endocrinology (259). It is used to trace neurons by axonal transport of radioactively labeled amino acids, certain sugars or transmitter substances. It measures DNA production (e.g., ^{3}H-thymidine). Autoradiography produces a permanent record of the positions and relative intensities of radiolabeled bands in a gel or blot.

Autoradiography has 2 types. One is in vivo autoradiography and the other one is in vitro autoradiography. Receptors are labeled in intact living tissue by systemic administration of the radioligand for in vivo autoradiograhy. Slide-mounted tissue sections are incubated with radioligand so that receptors are labelled under very controlled conditions which is called in vitro autoradiography (257). In this research, in vitro autoradiography was used to observe the therapeutic efficacy of antiparkinsonian effect of formulations by the decrease in the loss of DAT binding (%) at the ipsilateral (lesioned) side in striatum of brain sections. DAT specific ligands are generally labeled with ^{3}H or ^{125}I.

Autoradiography can also be used to define the mechanism of drugs, ligands and their interaction with specific receptors after radiolabeling with a suitable radioisotope. Thus when the ligand under study binds with a specific receptor, the location of this receptor binding is identified by detection of the radioactivity emitted by the radioisotope (256).

The principle of autoradiography with Beta-imager was given as a scheme in Figure 2.16.

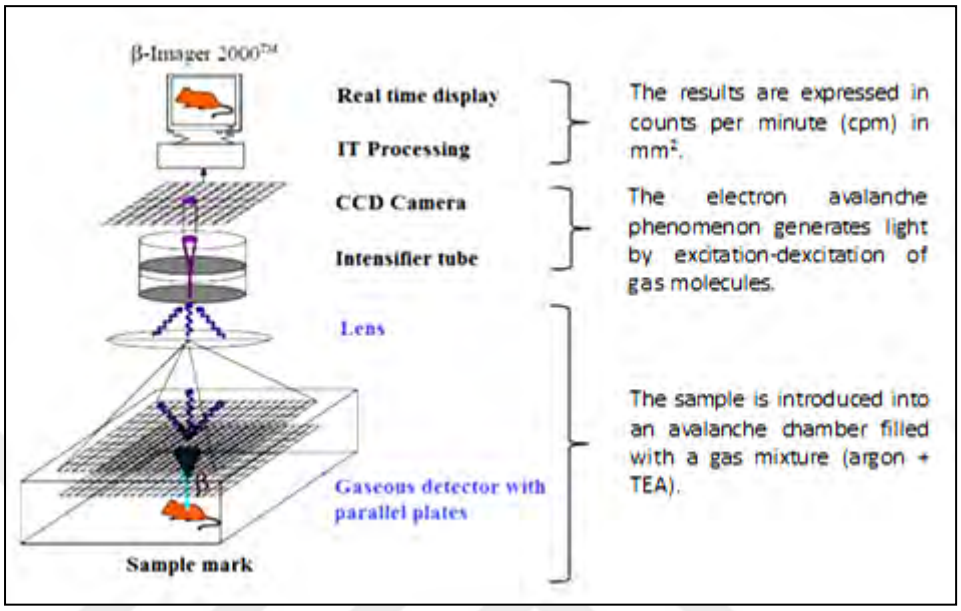


Figure 2.16. Autoradiography with a Beta-imager (264).

Autoradiography has some disadvantages. Binding to everything can cause some misinterpretations in the results. This process needs no biochemical or physiological criteria for assessing the binding specificity. The presence of a high-affinity radiolabeled receptor does not necessarily imply that the receptor has physiological significance and ligands are not always very specific.

Although autoradiography has some disadvantages, it also has many advantages such as; being a highly specific tool to pharmacologically characterize receptors in tissue, providing location of receptor or protein in tissue, enabling characterization of receptors in different tissues between different animals or brain regions and being a technically easy process (257). Autoradiography is a less costly and practical method to detect the small animals in preclinical research field.

3. MATERIALS and METHODS

3.1. Chemicals

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Avanti Polar Lipids, USA
1,2-Distearyl-sn-glycero-3-phosphoethanolamine-N-[Metoxy (Polyethylene glycole)-2000] (Ammonium salt) (PEG2000-DSPE)	Avanti Polar Lipids, USA
2,4,5-Trihydroxyphenethylamine hydrochloride, 2,5-Dihydroxytyramine hydrochloride, 2-(2,4,5-Trihydroxyphenyl)ethylamine hydrochloride (6-OHDA)	Sigma-Aldrich, USA
2-Methylbutane (Isopentane)	Sigma-Aldrich, USA
Absolute ethanol	Riedel-de-Haen, Germany
Ascorbic acid	Sigma-Aldrich, USA
Ascorbic asid (Vit C)	Atabay, Turkey
Bone Wax	Ethicon, France
Carboglace (Dry Ice)	Carboglace
Chloroform (%98)	Riedel-de-Haen, Germany
Cholesterol (5-Cholesten-3β-ol)	Sigma Chemicals, USA
Cocaine	Cooper, France
D-Amphetamine-d_3 sulfate salt	Sigma-Aldrich, USA
Diethyl ether anhydride	Merck, Germany
Diethylene Triamine Penta Acetic Acid (DTPA)	Sigma-Aldrich, USA
D-saccharose	Sigma-Aldrich, USA
Eye Protectan (Ocry-gel®)	TVM, France
Hydrochloric acid (%36-38)	J.T.Baker, Netherlands
Hydrogen bromide (solution in acetic acid (%32))	Merck, Germany
Instant freezing spray for anatomical pieces	Cryoral, RAL Diagnostics, France.

Isoflurane (Aerrane®)	Baxter, Dublin
N-(3-iodopro-2E-enyl)-2beta-carbomethoxy-3beta-(4'-methylphenyl) nortropane (PE2I)	Synthesized by Team 3, Molecular Imaging and Brain, U930, Tours Univ., France.
Pargyline hydrochloride	Sigma-Aldrich, USA
Perchloric acid (%70)	Riedel-de Haen, Germany
Potassium chloride (KCl)	Sigma-Aldrich, USA
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich, USA
Pramipexole dihydrochloride monohydrate	Abdi Ibrahim, Turkey
Rhodamine-PE (0,5% mmole)	Avanti Polar Lipids, USA
Sodium chloride (NaCl)	Sigma-Aldrich, USA
Sodium phosphate dibasic anhydrous (Na_2HPO_4)	Sigma-Aldrich, USA
Stearylamine (SA)	Sigma Chemicals, USA
SURII (Alcool cetylique polyglycerole)	L'Oreal, France
TRIS [(Tris(hydroxymethyl)-aminomethan]	Meck, Germany
Attachment factor (4Z0-210)	Cellsystems, Germany
Fetal bovine serum (5%)	Sigma Chemicals, USA
Penicilin/streptomycin (1%) solution	Sigma Chemicals, USA
ECGS (%1)	Sigma Chemicals, USA
Asyrocyte growth factor (1%)	Sigma Chemicals, USA
Rhodamine-PE (0,5% mmole)	Avanti Polar Lipids, USA

3.2. Equipments

Aspiration Unit	Minerve, France
Asyrocyte growth factor (1%)	ScienCell, USA
Attachment Factor	CSC Certified, Cell-Ststems.
Automatic Shaker	Lab-line, India
Balance	Mettler Toledo AB104-S, Swiss
β-imager	β-imager TM 2000, Biospace Lab, Paris, France
Cryotome	Leica, CM 3050 S, France
Dialysis Cellulose Membrane (13000 MW cut off)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Dynamic Light Scattering	Malvern Instruments, United Kingdom
Endothelial Cell Medium (ECM)	ScienCell, USA
Extrusion Unit	Lipex, Biomembranes, Canada
Fethal Bovine Serum (5%)	ScienCell, USA
Fluorescent microscope	Inverted microscope Olympus, U.K.
Fluorospectroscopy	Spectrofluorometry (RF-5301PC), Shimadzu, Japan
Gas-Tight Microliter Syringe	Stoelting Co., Sd Scientific, France
Isoflurane TEC3 Vaporiser	Minerve, France
Magnetic Stirrer	Heidolph MR 3001, Germany
Microdialysis Probe Holder	Stoelting Co., Sd Scientific, France
Micromotor High-Speed Drill	Stoelting Co., Sd Scientific, France
Oxygen Concentrator	Minerve, France
Penicilin/streptomycin (1%) solution	ScienCell, USA
pH-meter	Inolab pH meter, Turkey
Polycarbonate Membrane Filter	Whatman, USA
Pore Dialysis Membrane	Spectra, Spectrum Laboratories, Inc., USA
Rotavapor	Buchi 461, Swiss
Rotometer	Imetronic Behaviour Instruments, Bordeaux, France

Sonicator	Bronson 1210, OSI, USA
Stereotaxic Apparatus	Stoelting, Phymep, France
Temperature Controller	CMA/150, Fredom, USA
Transwell cell culture inserts	Sigma-Aldrich, Germany
UV Spectrophotometer	Agilent 8453, USA
Vacuumed Incubator	Shel Lab (SL), Spain
Veterinary Anesthesia Equipment	Minerve, France
Vortex	Heidolph Reax Top, Germany
Water Bath	GFL, Germany
Wistar rats	CERJ, Le Genest-St-Isle, France
Zeta Sizer	Malvern Instruments, Nano-ZS, United Kingdom

3.3. Physicochemical Controls on Active Substance

Pramipexole Dihydrochloride Monohydrate was encapsulated in the core of both neutral and positively charged liposomal and niosomal formulations as an antiparkinsonian agent.

3.3.1. Structure of Pramipexole Dihydrochloride Monohydrate

Pramipexole dihydrochloride monohydrate was used in this research as antiparkinsonian agent and it was obtained from drug company (Abdi Ibrahim®) as a gift with its analysis certificate. Pramipexole dihydrochloride monohydrate ((S)-2-amino-4,5,6,7-tetrahydro-6-propylamine-benzothiazole dihydrochloride) is a white to off-white crystalline powder (302,27 g mol^{-1}) and stable under ordinary conditions. Its solubility is more than 20 % in water, about 8 % in methanol and 0.5 % in ethanol. It is practically insoluble in dichloromethane (265). Pramipexole dihydrochloride monohydrate is classified as BCSC1 substance depending on high solubility and high permeability properties (58). Due to proper solubility properties, Pramipexole dihydrochloride monohydrate is chosen instead of Pramipexole for PD treatment for this study. 87.37 µg of Pramipexole is approximately equivalent to 125 µg of Pramipexole Dihydrochloride Monohydrate (58). The word ‘‘Pramipexole’’ is used in the meaning of ‘‘Pramipexole Dihydrochloride Monohydrate’’ within the thesis. The molecular structures of Pramipexole and Pramipexole Dihydrochloride Monohydrate are given in Figure 3.1. (57).

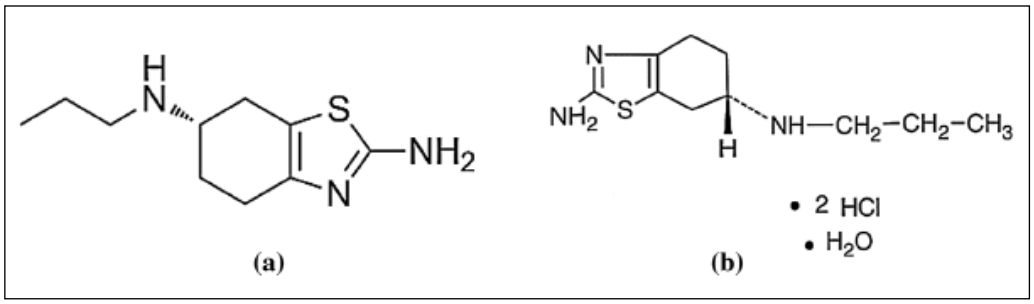


Figure 3.1. Structure of (a) Pramipexole (58) and (b) Pramipexole Dihydrochloride Monohydrate (57,59).

3.3.2. UV Spectrum and Calibration Curve of Pramipexole

3.3.2.1. Determination of UV Spectrum and Standart Curve of Pramipexole in Tris Buffer

To determine the released amount of Pramipexole from liposomal and niosomal formulations and obtaining release kinetics, UV spectra of Pramipexole was obtained in Tris buffer (20 mM, pH=7.4) at wavelength between 200-500 nm and maximum wavelenght was determined. Tris buffer was used to prepare liposomal and niosomal drug delivery systems. It was also used as control group. To prepare 500 mL of Tris buffer, 1,2114 g Tris was weighted and the volume was completed with distilled water to 500 mL by adjusting pH:7,4. Stock solution of Pramipexole in Tris buffer (0.6 $mg.mL^{-1}$) was prepared and diluted solutions in concentrations of 5, 10 15, 20, 25, 30, 40 ve 50 $\mu g.mL^{-1}$ were prepared. Standart line was drown and standart line equation and correlation coefficient ”r” and determination coefficient “r^2” were calculated by obtaining absorbance values for Pramipexole in these variety of concentrations at determined maximum wavelength.

3.3.2.2. Determination of UV Spectrum and Calibration Curve of Pramipexole in Ethanol

To determine the unencapsulated amount of Pramipexole in liposomal and nisomal formulations and to calculate encapsulation efficiency, UV spectra of Pramipexole was obtained in ethanol at wavelength between 200-500 nm and maximum wavelength was determined. Stock solution of Pramipexole in ethanol (0.6 $mg.mL^{-1}$) was prepared and diluted solutions in concentartions of 5, 10 15, 20, 25, 30, 40 ve 50 $\mu g.mL^{-1}$ were prepared. Standart line was plotted and the equation and correlation coefficient ”r” and determination coefficient “r^2” were calculated by obtaining absorbance values for Pramipexole depending on the varieties of concentrations at determined maximum wavelength.

3.3.2.3. Analytical Method Validation

Analytical method validation is a process to evaluate the reliability of the method of determination of the amount of the active ingredient within the way of

aimed usage (58,266) The related parameters to provide analytical validation was given below;

-Linearity

-Accuracy

-Precision

-Repeatability

-Reproducibility

-Specificity

-Stability (within the determination of the active ingredient).

- **Linearity**

Linearity of an analytical method is the linearity of the experimental results and as being in a definite concentration of the substance in the sample indirectly. Calibration lines were obtained as given in Sections 3.3.2.1 and 3.3.2.2. 7 pointed 6 series were studied by using stock solutions. Standart lines were drawn by using absorbance values and correspondance concentrations. The linearity of the equation was calculated by designating complementary coefficients.

- **Accuracy**

Accuracy expresses the degree of proximity within the consecutive measurements. It has no number equivalance and it expresses in standart deviation (SD), stanadart error (SE) or coefficient of variation (CV). The accurarcy of an analytical method is evaluated with the calculation of arithmetic mean ($\bar{x}$), SE and CV by the measurement of samples which is sufficient in number to perform a statistical analysis and in the same concentrations. Repeatibility and reproducibility provide expression of accuracy.

- **Precision**

Repeatibility

5 $\mu g.mL^{-1}$ and 20 $\mu g.mL^{-1}$ were selected as low and high concentrations, respectively and they were sampled from the stock solution and absorbance values were measured at 262 nm with UV spectroscopy 6 times. $\bar{x}$, SE and CV were

calculated from the concentrations corresponding to the related absorbance values. The value of CV lower than 2% designates the repeatibility of the method.

Reproducibility

One low (5 µg.mL^{-1}) and one high (20 µg.mL^{-1}) concentrations were selected and taken from the stock solution 6 times and UV absorbtion was measured at 262 nm. $\bar{x}$, SE and CV were calculated according to the concentrations corresponding to the absorbance values. The value of CV lower than 2% designates the reproducibility of the method.

- **Specificity**

To evaluate the state of belonging of absorbance value to Pramipexole, the spectra of empty, neutral and positively charged liposomes and niosomes were measured at 262 nm.

- **Stability**

The stability of Pramipexole was evaluated at definite concentrations within the measurement process. The absorbance values of the samples prepared at 5 µg.mL^{-1} and 20 µg.mL^{-1} concentrations were measured at the begining and at 1, 5, 10 and 24 h. and the alteration in the concentrations was evaluated for one day.

3.4. Physicochemical Controls of the Ingredients within Liposome and Niosome Dispersions

While 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) is used as phospholipids in liposomal dispersions, alcool cetylique polyglycerole (SUR II) is used as non-ionic surfactants in niosomal dispersions. Cholesterol is used as stability enhancer,1,2-Distearyl-sn-glycero-3-phosphoethanolamine-N-[Metoxy (Polyethylene glycole)-2000](Ammonium salt) (PEG2000-DSPE) is used as hydrophilic coating and stearylamine (SA) is used as positive charge inducer. Pramipexole dihydrochloride monohydrate dihydrate is used as antiparkinsonian agent.

3.4.1. Physicochemical Controls of Phospholipid

3.4.1.1. Standardization of Phospholipid

The analitical certificate of phospholipid was obtained from the company and phosphotidylcholine content of phospholipid was determined.

3.4.1.2. Identification of Phospholipid by Thin Layer Chromatography (TLC)

The solution of phospholipid DPPC (20 $\mu g.mL^{-1}$) was administered via a capillary tube on Silicagel 60 F_{254} plates with the size of 5x10 cm which was activated previously in an incubator for 60 min at 110ºC for the determination of DPPC by TLC. Plate was put in a container saturated with a solution system containing chloroform:methanol:water (80:20:2). After drying, the plate was put in a container saturated with iodine vapor and R_f value was calculated after observing spot under UV light at 254 nm (267).

3.4.1.3. Identification of Phospholipid by Differential Scanning Calorimetry (DSC)

Thermal analysis curves of DPPC were obtained and phase transition temperature was identified by DSC. For this procedure the flow rates of nitrogen gas was 50 $mL.min^{-1}$ and O_2 gas was 50 $mL.min^{-1}$ and temperature increase was $10ºC.min^{-1}$. The spectrum was obtained at -20 and 160ºC.

3.4.1.4. Identification of Phospholipid by Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Potassium bromide tablets were compressed for Fourier transform infrared (FTIR) spectrum of DPPC and with the use of these discs (tablets), spectra were obtained within 4000-650 cm^{-1} wave number intervals.

3.4.2. Physicochemical Controls of Non-Ionic Surfactant

SUR II was used in the composition of niosomal formulations as non-ionic surfactant. The analitical certificate of non-ionic surfactant was obtained from the company.

3.4.2.1. Identification of HLB Value of Surfactants

The Hydrophilic Lipophilic Balance (HLB) value of surfactant was evaluated according to the formulation given by Griffin et al (268,269) which is explained below;

HLB= (MH / M) x 20……Equation 8

MH: is the molecular mass of the hydrophilic portion or chain of the Molecule.

M: is the molecular mass of the whole surfactant molecule giving a result on an arbitrary scale of 0 to 20.

3.4.2.2. Identification of Surfactant by Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Potassium bromide (KBr) tablets were compressed for Fourier Transform Infrared (FTIR) spectrum of SUR II and with the use of these discs (tablets), spectra were obtained within 4000-400 cm^{-1} wave number intervals.

3.4.2.3. Identification of Surfactant by Differential Scanning Calorimetry

Thermal analysis curves of SUR II was obtained and phase transition temperature was identified by DSC. For this procedure the flow rates of nitrogen gas was 50 $mL.min^{-1}$ and O_2 gas was 50 $mL.min^{-1}$ and temperature increase was $10ºC.min^{-1}$. The spectrum was obtained at -20 and 160ºC.

3.4.2.4. Identification of Surfactant by Melting Point Determination

Melting point of SUR II was performed by melting point determination apparatus after placing of certain amount of SUR II in a capillary tube.

3.4.3. Physicochemical Controls of Stability Enhancer Ingredient

In this study, cholesterol was used as stability enhancer for liposomal and niosomal formulations.

3.4.3.1. Identification of Cholesterol by TLC

The identification of cholesterol by TLC was performed according to the procedure previously described in Section 3.4.1.2. However, as different from phospholipid, spot was observed under UV lamp at a wave-length of 254 nm after spraying sulfuric acid solution (50%) (50 mL H_2SO_4 : 50 mL Methanol) and warming in an incubator for 5-10 min (270,271).

3.4.3.2. Identification of Cholesterol by DSC

Thermal analysis curve of cholesterol was obtained by DSC according to the procedure previously described in Section 3.4.1.3.

3.4.3.3. Identification of Cholesterol by FTIR Spectroscopy

Potassium bromide tablets were compressed for FTIR spectrum of cholesterol and with the use of these discs, spectra were obtained within 4000-400 cm^{-1} wave number intervals.

3.4.3.4. Identification of Cholesterol by Melting Point Determination

Melting point of cholesterol was performed by melting point determination apparatus after placing of certain amount of cholesterol in a capillary tube.

3.4.4. Physicochemical Controls of Coating Agent

In this study, PEG2000-DSPE was used as PEGylating agent for liposomal and niosomal formulations to achieve stealth effect and longer blood circulation. PEG2000-DSPE is a hydrophilic polymer and to achive anchoring on drug delivery systems. Its DSPE conjugate was obtained as a commercially available product.

3.4.4.1. Identification of PEG2000-DSPE by TLC

The solution of PEG2000-DSPE (1 $mg.mL^{-1}$) was administered by a capillary tube on silicagel 60 F_{254} with 0.25 mm tickness which was activated in an incubator at 110ºC for 60 minutes to obtain TLC identification of PEG2000-DSPE. Plate was put in a container saturated with a solution system containing chloroform:methanol:water (65:25:4). After drying, ninhydrin reactive (1g.30 mL^{-1} ethanol) was sprayed onto the plate. The plate was put in an incubator at 170ºC for 5-10 minutes to observe primer amines as colorful. R_f value was calculated after observing spot under UV light at 254 nm (272).

3.4.4.2. Identification of PEG2000-DSPE by FTIR Spectroscopy

Potassium bromide tablets were compressed for FTIR spectrum of PEG2000-DSPE and with the use of these discs, spectra were obtained within 4000-400 cm^{-1} wave number intervals.

3.4.5. Physicochemical Controls of Charge Inducer

Stearylamine (SA) was used in some of the liposomal and niosomal formulations to obtain positive charged formulations.

3.4.5.1. Identification of SA by FTIR Spectroscopy

Potassium bromide tablets were compressed for FTIR spectrum of SA and with the use of these discs, spectra were obtained within 4000-400 cm^{-1} wave number intervals.

3.4.5.2. Identification of SA by DSC

Thermal analysis curve of SA was obtained by DSC according to the procedure previously described in Section 3.4.1.3.

3.5. Preliminary Studies on Liposomal and Niosomal Formulations

Before determination of the optimum formulation approach, several preliminary studies on formulations were performed following an intensive literature

survey about studies related with BBB delivery of liposomal and niosomal dispersions about one year.

- ***Metal Chelator for Radiolabeling of Liposomes and Niosomes***

DTPA-PE was synthesized and determined with TLC and FTIR spectroscopy analysis for the purpose of ^{99m}Tc-radiolabeling of liposomal and niosomal formulations.

- ***DTPA-PE Synthesis***

DTPA-PE was synthesized for the purpose of ^{99m}Tc-radiolabeling of liposomal and niosomal formulations due to its metal chelator properties. To prepare DTPA-PE for radiolabeling of liposomes, 4 mL of chloroform was added on 0,2 mmol Egg PC and stirred. 30µL triethylamine was added on the mixture. 1 mmol DTPA (Diethylene triamine penta acetate) anhydride was dissolved in 20 mL of DMSO (dimethysulfoxide) seperately and this mixture was added dropwise on the other mixture under argon gas and incubated for 3 h. Precipitation was obtained after adding distilled water on the mixture and filtered and lyophilized (273,274). The synthesis of DTPA-PE was observed by FTIR spectra with pressed potassium bromide tablets at 4000-400 cm^{-1} bands. TLC determination was also performed for the formation of the complex by observing the spot at UV lamp with 254 nm.

- ***Identification of DTPA-PE by TLC***

DTPA-PE was applied on activated silicagel 60 F_{254} plate with chloroform:methanol:water:formic acid (65:25:4:1) solvent system. The spot was observed after spraying of sulphiric acid:ethanol (1:2.75) solution and keeping for a few minutes in a drying oven at 170ºC. The R_f value was calculated by observing the spot on the chromatogram under the UV lamp at 254 nm (273,275).

- ***Identification of DTPA-PE by FTIR Spectroscopy***

Potassium bromide tablets were compressed for FTIR spectrum of DTPA-PE and with the use of these discs, spectra were obtained within 4000-400 cm^{-1} wave number intervals.

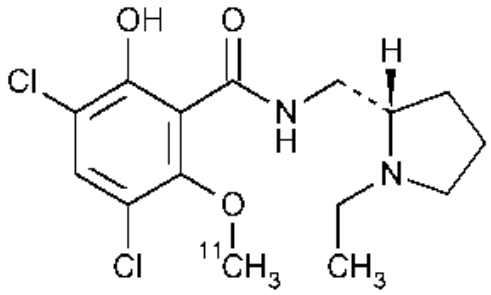
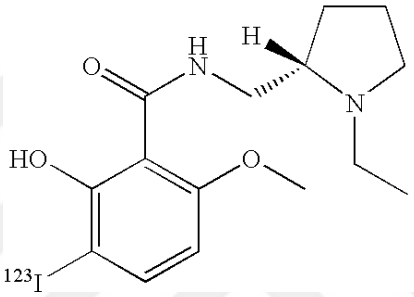
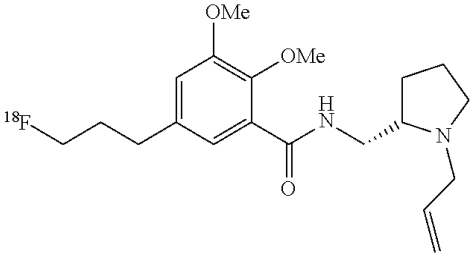
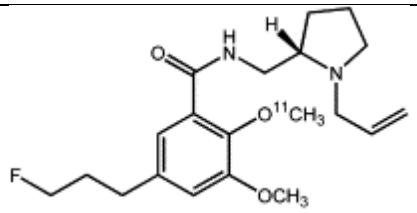
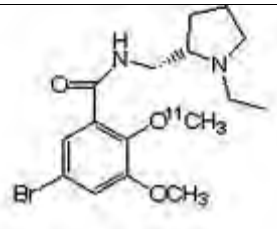
- ***Preliminary Studies for Specific Ligand Modification for Active Targeting of Liposomes and Niosomes***

The availability of ligand modification was assesed by evaluating the synthesis procedures, activity loss and anchoring of the ligand specific to dopamine D2/D3 receptor or DAT.

- ***Preliminary Studies for Synthesis and Anchoring of Dopamine D2/D3 Receptor or DAT Specific Ligand***

It was planned to synthesize a dopamine D2/D3 receptor or DAT specific radioligand and modify it with neutral and positively charged liposomal or niosomal drug delivery systems with a linker to be actively targeted as theragnostic delivery sytems for both imaging and therapy of PD. At the begining, this part was thought and planned to perform at François Rabelais de Tours University/France within the concept of co-directed doctorate cotutelle thesis. The name, molecular structure and synthesis process of some of D2/D3 receptor and DAT specific radioligands are given in Table 3.1. and Table 3.2., respectively.

Table 3.1. Dopamine D2/D3 receptor radioligands.

Radioligand	**Molecular Structure**	**Availability**
[^{11}C]Raclopride		Radiochemical synthesis
[^{123}I]IBZM ((S)-N-[(l-ethyl-2-pyrrolidinyl)methyl]-5-iodo-2-methoxybenzamide)		Commercially available
^{18}F-Fallypride		Commercially available
^{11}C-Fallypride		Commercially available
^{11}C-FLB457		Radiochemical synthesis

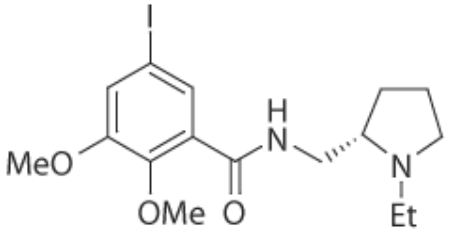
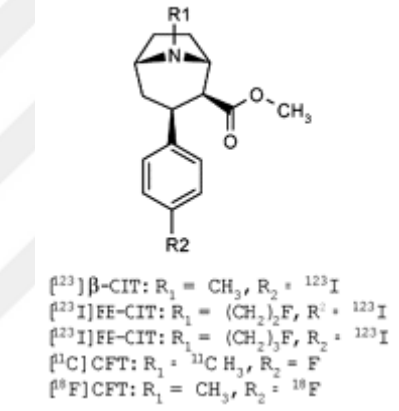
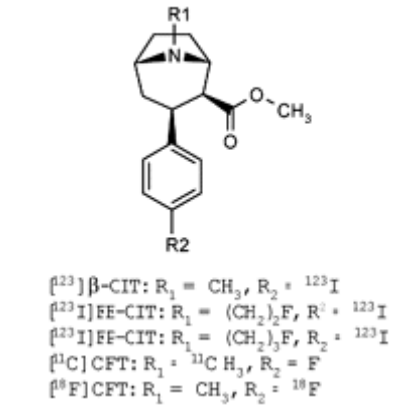
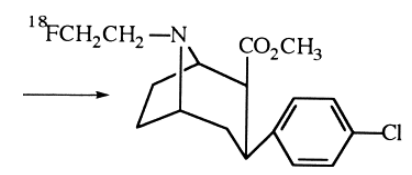
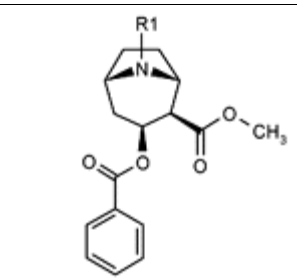
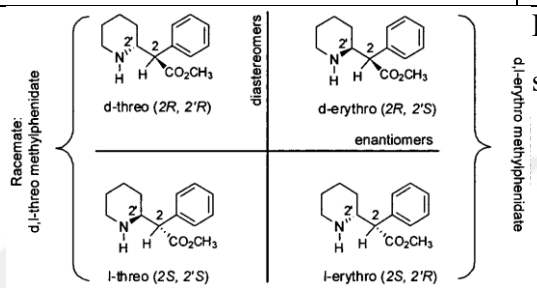
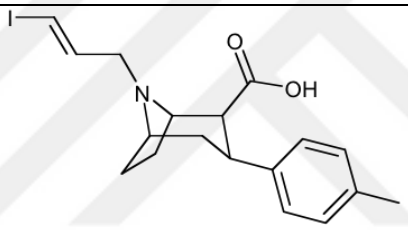
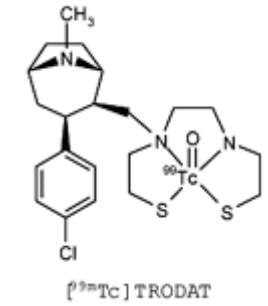
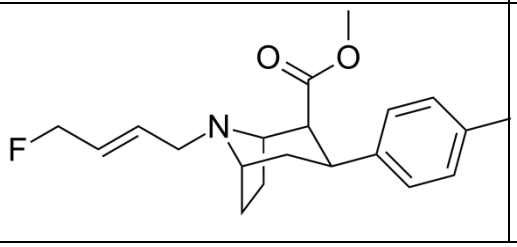
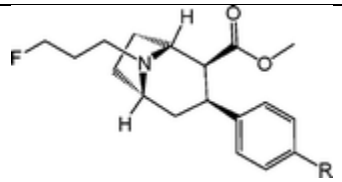
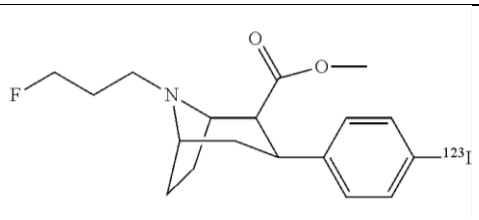
[$^{123/125}$I]Epidepride		Commercially available

Table 3.2. DAT radioligands.

Radioligand	**Molecular Structure**	**Availability**
^{123}I-β -CIT		Radiochemical synthesis
^{123}I-FPCIT ((123)I-2-β-carbomethoxy-3β-(4-iodophenyl)-N-(3-fluoropropyl) nortropane)		Radiochemical synthesis
^{18}F-FECNT (2beta-carbomethoxy-3beta-(4-chlorophenyl)-8-(2-(18)F-fluoroethyl)		Radiochemical synthesis

nortropane)		
^{11}C-cocaine		Radiochemical synthesis
[^{11}C]d-threo-methylphenidate		Radiochemical synthesis
^{123}I-PE2I ((123)I-N-(3-iodoprop-2E-enyl)-2-β-carbomethoxy-3β-(4-methylphenyl) nortropane)		PE2I stannous precursor is commercially available
^{99m}Tc-TRODAT		Raw material is commercially available and radiochemical synthesis can be performed in some labs.
LBT-999		Radiochemical synthesis

FBCFT (E)-fluorobutenyl substituted 4'-halophenyl		Radiochemical synthesis
^{123}I-FP-β-CIT (DATscan®) (^{123}I-Ioflupane)		Commercially available

3.6. Formulation Studies of Nanosized Liposomal and Niosomal Dispersions

3.6.1. Preparation and Characterization of Pramipexole Encapsulated Nanosized, PEGylated Liposomal Dispersions

The formulation, characterization, stability and release kinetics of neutral and positively charged, nanosized, Pramipexole encapsulated liposomal dispersions are described in this section.

3.6.1.1. Formulation of Nanosized, PEGylated, Pramipexole Encapsulated Neutral and Positively Charged Liposomes

The formulations of nanosized, PEGylated, Pramipexole encapsulated neutral and positively charged liposomes were prepared according to Bangham film method (99) and lipid ratios were chosen according to the study of Oku et al (276).

The molar compositions of nanosized, PEGylated, Pramipexole encapsulated neutral DPPC:PEG2000-PE:Chol and positively charged DPPC:PEG2000-PE:Chol liposome dispersions, phospholipids and other ingredients are given in Table 3.3. Phospholipid and other ingredients were solved in chloroform. Chloroform was evaporated in low pressure vacuumed rotavapor to obtain a thin film layer and afterwards the films were put in a vacuumed incubator to remove remaining chloroform for 12 h. The film was hydrated with Pramipexole (5 mg.mL^{-1}) solution in Tris buffer (20 mM, pH=7.4) at 65ºC for 30 min with the help of glass beads. This dispersion was then vortexed and hand-shaked for one min each. Liposomes were

extruded through polycarbonate membranes having 0.6 μm, 0.4 μm ve 0.2 μm pore sizes, respectively at a certain pressure (600 psi) by nitrogen efflux. To remove unencapsulated Pramipexole existing in liposomal dispersions, it was dialysed against Tris buffer (20mM, pH: 7.4) through dialysis membrane with a molecular weight cut off 13000 MW for 12 h. The schematic representation of this preparation procedure and size reduction of neutral and positively charged liposomal formulations is given in Figure 3.2.

Table 3.3. The molar compositions of neutral and positively charged, Pramipexole encapsulated, nanosized liposomal dispersions.

LIPOSOMAL FORMULATION	MOLAR COMPOSITION (%)
DPPC:Chol:PEG2000-PE	10:5:1
DPPC:Chol:PEG2000-PE:SA	10:4:1:1

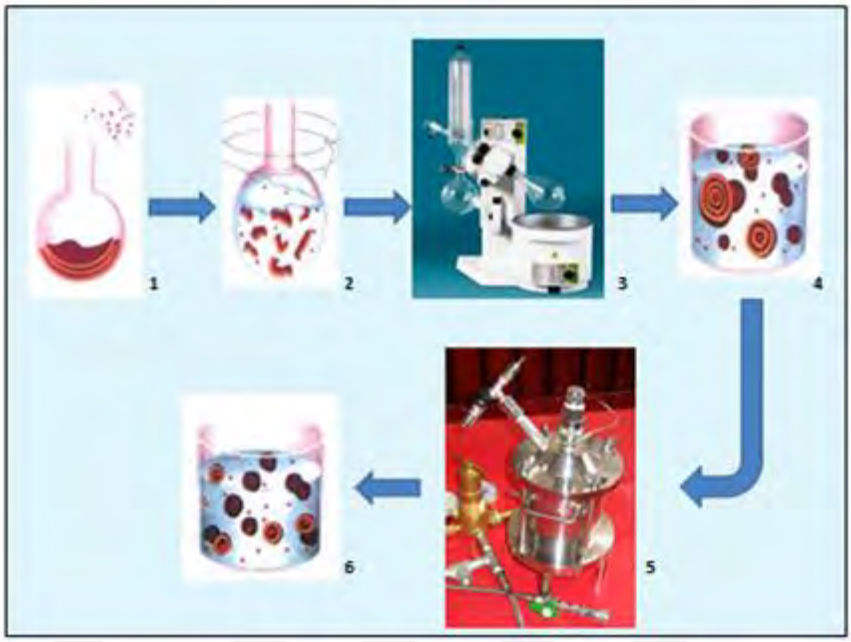


Figure 3.2. The preparation and size reduction of neutral and positively charged liposomal and niosomal formulations **(1.** Adding of phospholipids/non-ionic surfactants and cholesterol in chlorophorm, **2.** Dissolving of ingredients in chlorophorm by shaking, **3.** Removal of chlorophorm by evaporation with rotavapor, **4.** Dissolving of lipid film by the help of drug dissolved buffer solution at 10ºC above phase transition temperature of phospholipids/non-ionic surfactants, **5.** Particle size reduction by extrusion unit of liposomes/niosomes, **6.** Obtaining of nanosized liposomes and niosomes).

3.6.1.2. Characterization of Nanosized, PEGylated, Pramipexole Encapsulated Neutral and Positively Charged Liposomes

The characterization of nanosized, Pramipexole encapsulated, PEGylated neutral and positively charged liposomal formulations was performed by mean particle size and zeta potential mesurement, determination of encapsulation efficiency and phospholipid amount.

3.6.1.2.1. Mean Particle Size and Size Distribution and Zeta Potential

The avarage particle size and zeta potential of Pramipexole encapsulated, nanosized, neutral and positively charged liposomes were measured according to dynamic light scattering method at 25°C by using Nano-Zeta Sizer. This is a

technique which uses some functions of light beam striked onto the surface of dribblet like scattering and absorption. In this technique, light density digitalysis converted into the current and saved, particle size information is obtained with an evaluation by the use of a computer software.

3.6.1.2.2. Encapsulation Efficiency of Pramipexole

After removal of unencapsulated Pramipexole by dialysis, both neutral and positively charged liposome vesicles were lysed with ethanol and encapsulated Pramipexole amount was calculated by measuring its absorbance at 263 nm wavelength spectrofotometrically. Encapsulation efficiency was calculated with the use of standart curve and line equation which were prepared according to the procedure described in Section 3.3.2.2.

3.6.1.2.3. Determination of Liposomal Phospholipid Amount

The phosphorous amount of both neutral and positively charged, nanosized, Pramipexole encapsulated, PEGylated liposomes was determined according to Rouser method (277). The schematic representation of this method is given in Figure 3.3.

To obtain the standart line which is essential for calculation of phospholipid amount, standart line solutions were prepared freshly before every test and standart line was drawn and standart equation was caluculated by measuring absorbans at λ=797 nm, spectrophotometrically. The phospholipid amounts of liposomes were determined by the use of absorbance values of test solutions obtained at 797 nm wave-lenght and by the use of obtained standart line equation.

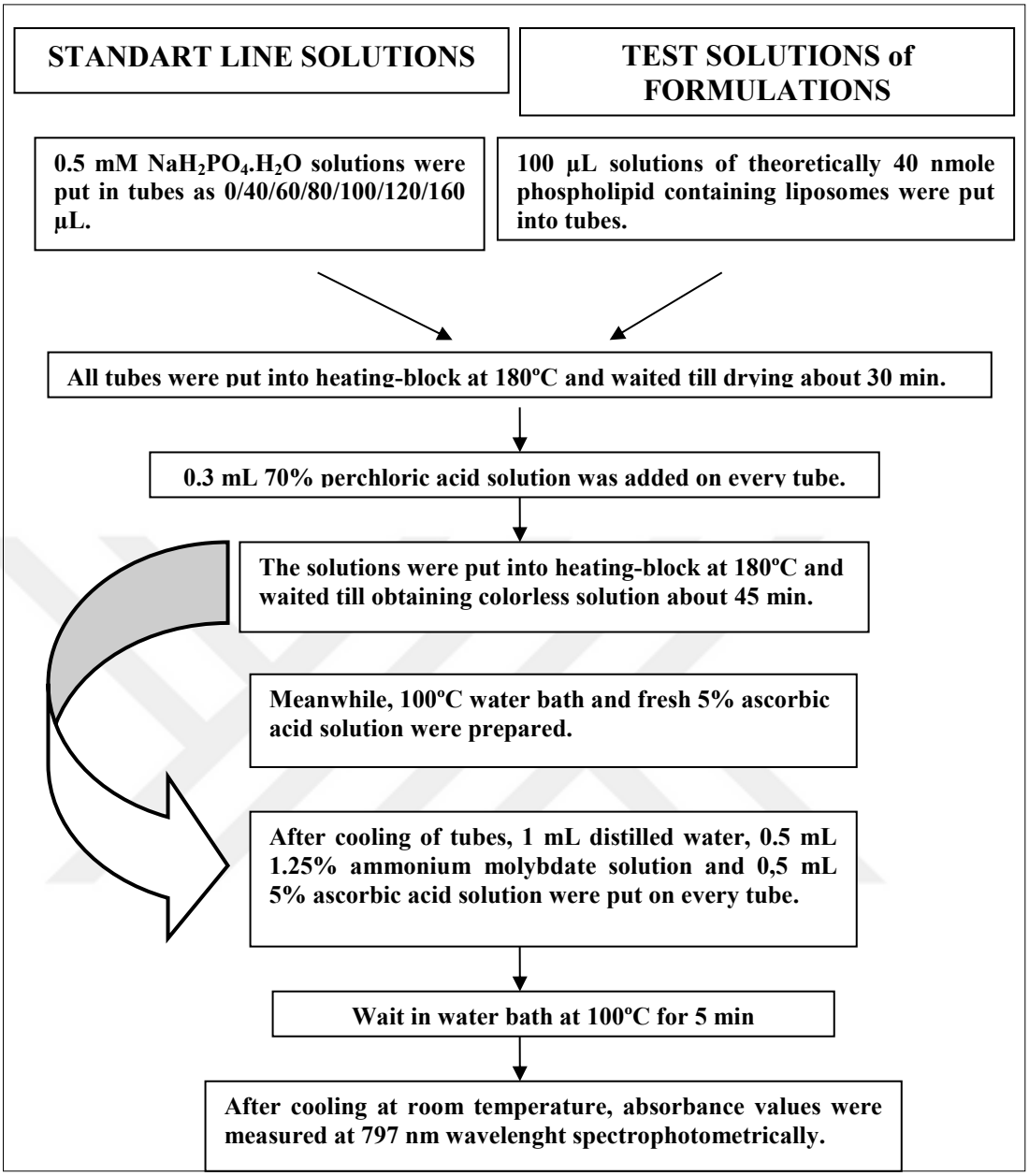


Figure 3.3. Determination of liposomal phospholipid amount (277).

3.6.2. Preparation and Characterization of Pramipexole Encapsulated Nanosized, PEGylated Niosomal Dispersions

The formulation, characterization, stability and release kinetics of neutral and positively charged, nanosized, Pramipexole encapsulated niosomal dispersions are described in this section.

3.6.2.1. Formulation of Nanosized, PEGylated, Pramipexole Encapsulated Neutral and Positively Charged Niosomes

The formulations of nanosized, PEGylated, Pramipexole encapsulated neutral and positively charged niosomes were prepared according to Bangham film method (99) and ratios non-ionic surfactant and other ingerients were chosen according to the method of Oku et al (276).

The molar compositions of nanosized, PEGylated, Pramipexole encapsulated neutral SURII:PEG2000-PE:Chol and positively charged SURII:PEG2000-PE:Chol niosome dispersions, non-ionic surfactants and other ingredients are given in Table 3.4. Non-ionic surfactant and other ingredients were solved in chlorophorm. The rest of the formulation studies of neutral and positively charged niosomes were performed same as the protocol described in Section 3.6.1.1. The only difference is the hydration process which was performed at 60ºC depending on the phase transition temperature of SURII. The schematic representation of preparation and size reduction of neutral and positively charged niosomal formulations is given in Figure 3.2.

Table 3.4. The molar compositions of neutral and positively charged, Pramipexole encapsulated, nanosized niosomal dispersions.

NIOSOMAL FORMULATION	MOLAR COMPOSITION (%)
SURII:Chol:PEG2000-PE	10:5:1
SURII:Chol:PEG2000-PE:SA	10:4:1:1

3.6.2.2. Characterization of Nanosized, PEGylated, Pramipexole Encapsulated Neutral and Positively Charged Niosomes

The characterization of nanosized, Pramipexole encapsulated, PEGylated neutral and positively charged niosomal formulations was performed by mean particle size and zeta potential mesurement, determination of encapsulation efficiency and phospholipid amount.

3.6.2.2.1. Mean Particle Size and Size Distribution and Zeta Potential

The avarage particle size and zeta potential of Pramipexole encapsulated, nanosized, neutral and positively charged niosomes were measured according to dynamic light scattering method at 25°C by using Nano-Zeta Sizer. The mechanism of this technique was described in Section 3.6.1.2.1.

3.6.2.2.2. Encapsulation Efficiency of Pramipexole

After removal of free Pramipexole by dialysis, both neutral and positively charged niosome vesicles were lysed with ethanol and encapsulated Pramipexole amount was calculated by measuring its absorbance at 263 nm wavelength spectrofotrometrically. Encapsulation efficiency was calculated with the use of standart curve and line equation which were prepared according to the procedure described in Section 3.6.1.2.2.

3.7. In Vitro Release Kinetics Studies of Liposomal and Niosomal Formulations

In vitro release studies of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged liposomes and niosomes are described in this section.

3.7.1. In Vitro Release Kinetıcs of Pramipexole Encapsulated, Nanosized, PEGylated, Neutral and Positively Charged Liposomal Dispersions

In vitro drug release studies of Pramipexole encapsulated, nanosized, PEGylated neutral and positively charged liposomal dispersions were performed by dialysis method with a dialysis membrane (MWCO:3.5 kDa) at 37 ± 1°C with 100 rpm against a release medium of Tris buffer (20 mM, pH=7.4) (278). A volume of 1 mL of Pramipexole encapsulated neutral and positively charged liposome dispersions in dialysis tubing were immersed in 10 mL of release medium, and agitated in a shaker at a rate of 100 oscillations per minute at 37°C. Then, 1 mL samples of release medium were taken at certain time intervals (15 min, 30 min, 1, 1,5, 2, 2,5, 3, 3,5, 4, 4,5, 5, 5,5, 6, 6,5, 7 h) and replaced with the same volume of fresh Tris buffer (20 mM, pH=7.4). Pramipexole amount in release medium was measured by spectrophotometrically at 262 nm wavelength. The release kinetics of Pramipexole

encapsulated neutral and positively charged liposomes were calculated by using absorbance values with *In Vitro-In Vivo Kinetics, Version 1.0.40* programme.

3.7.2. In Vitro Release Kinetics of Pramipexole Encapsulated, Nanosized, PEGylated, Neutral and Positively Charged Niosomal Dispersions

In vitro drug release studies of Pramipexole encapsulated, nanosized, PEGylated neural and positively charged niosomal dispersions were performed by dialysis method with a dialysis membrane (MWCO:3.5 kDa) at 37 ± 1°C with 100 rpm against a release medium of Tris buffer (20 mM, pH=7.4) (278). The applied procedure was identical with that of described in Section 3.7.1. The release kinetics of Pramipexole encapsulated neutral and positively charged niosomes were calculated by using absorbance values with *In Vitro-In Vivo Kinetics, Version 1.0.40* programme.

3.8. In-Vitro BBB Cell Co-Culture Experiments

3.8.1. Rhodamine Labeled Liposome and Niosome Formulations

Rhodamine-PE (0,5% mmole) was added to neutral and positively charged liposomes and niosomes in the preparation procedure (99) while phospholipid or non-ionic surfactant was added before hydration process. After preparation procedure Rhodamine labeled formulations were dialysed against Tris (20 mM, pH 7.4) buffer through a dialysis cellulose membrane (13000 MW cut off) for 12 hours with stirring to remove unlabeled free Rhodamine within the preparation process which was given in Section 3.6.1.1.

3.8.2. BBB Cell Co-Culture Studies

In vitro BBB penetration of nanosized, PEGylated, Pramipexole encapsulated neutral and positively charged liposomal and niosomal dispersions were performed in BBB Cell Co-Culture model in Transwell® plate composed of astrocyte at the bottom and endothelial cells at the top part. Figure 3.4 gives different in vitro cell-based BBB models.

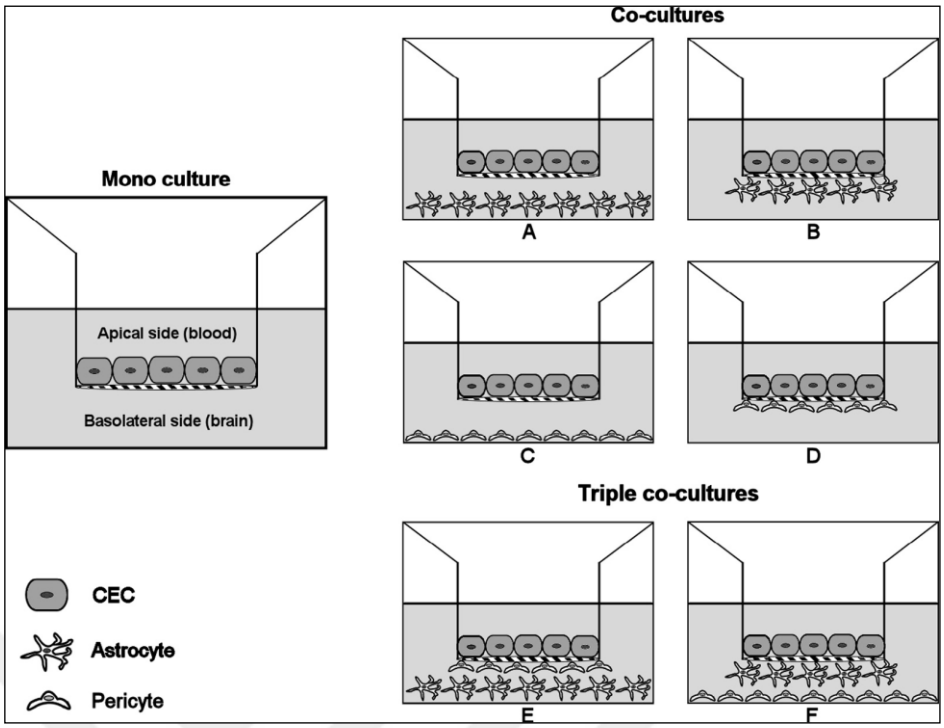


Figure 3.4. In vitro cell-based BBB models. Monocultures of cerebral endothelial cells (CECs) are being replaced by co-culture or triple co-culture systems, in which CECs are seeded with other elements of the neurovascular unit, such as astrocytes, pericytes or neurons, in a non-contact (A, C) or contact format (B, D). In triple co-culture systems, more than one cell type is seeded with CECs (E, F) (279).

We applied non-contact BBB co-culture model. To obtain a polarized BBB symbolizing co-culture model, 50,000 cell.cm^{-2} was planted on attachment factor coated 6 well plates with 0,4 µm pores in semi-transparent membrane indentations (0.3 cm^2/indentation). Endothelial cell layer divides this system as apical (blood side) and basolateral side (brain side). This setup provides application on both sides of BBB. Human brain microvascular endothelial cell membrane sustained with fetal bovine serum (5%), penicilin/streptomycin (1%) solution and ECGS (%1). In order to compose BBB co-culture model astrocytes were seeded on 6 well-plates (1.9 cm^2.well^{-1}) as 50,000 cell.cm^{-2}. Human astrocyte medium sustained with fethal bovine serum (5%), penicilin/streptomycin (1%) solution, asyrocyte growth factor (1%). All cells were cultured in humidified incubator at 37ºC in the presence of CO_2 (5%). Medium was changed for 5-7 days untill the cells are properly attached.

The neutral and positively charged liposomes and niosomes were added on endothelial cells (1.5 mL) (n=6 for each group). Formulations were incubated with human astrocyte and human brain microvascular endothelial cells at 37ºC in the existance of CO_2 (5%) and wait for penetration. To observe penetration of Pramipexole encapsulated different formulations (DPPC:Chol:PEG2000-PE, DPPC:Chol:PEG2000-PE:SA, SURII:Chol:PEG2000-PE, SURII:Chol:PEG2000-PE:SA), 100 µL samples were collected from the bottom of each Transwell® plates. Fluorescent microscope images were taken (40 times extension) at 0 and 120 min. after incubation and relative fluorescence intensity was measured with a fluorospectroscopy at 0, 30, 60, 90, 120, 150 and 180 min. To observe fluorescence intensity of penetration, excitation and emission spectra were scanned. Excitation was determined as 560 nm and emmission was determined as 583 nm.

3.9. Decision of Optimum Pramipexole Encapsulated, Nanosized, PEGylated Liposomal and Niosomal Formulations

The characterization and stability studies of neutral and positively charged liposomes and niosomes were compared and maximum BBB penetration was also regarded as a policy to select the optimum liposomal and niosomal formulations to administer to the PD rats model for therapy.

3.10. In Vivo Animal Studies

Rats were treated in accordance with the European Community Council Directive 2010/63/EU for laboratory animal care and the experimental protocol was validated by the Regional Ethical Committee (No: 00434.02). Experiments were performed on adult male Wistar rats weighing 250–300 g at the beginning of the experiments.

Animals were housed in groups of two per cage in a temperature and humidity controlled environment (temperature 22ºC ± 1ºC; relative humidity 40% ± 7%), throughout a 12 h light/dark cycle with standard feeding condition ad libitum.

3.10.1. Study Design of Animal Studies

In this part a brief summary of in vivo animal studies was given. Each application, test and method will be explained in detail at their own sections below. A total of 26 Wistar rats were used after lesioned with 6-OHDA. They were divided into four groups containing 6 rats in each for **i.p.** administration of nanosized, PEGylated, Pramipexole encapsulated, neutral liposomes (**liposomes**); nanosized, PEGylated, Pramipexole encapsulated, neutral niosomes (**niosomes**); Pramipexole solution (0,5 $mg.mL^{-1}$) or Tris buffer (20mM, pH=7,4) as control group aproximately on alternate days (evey Monday, Wednesday and Friday). All formulations contained the same amount of Pramipexole (0,5 $mg.mL^{-1}$) to compare the efficacy of different formulations. Two rats with a rotational behaviour of less than 8 ipsilateral turns/min were also divided into 2 groups and nanosized, PEGylated, Pramipexole encapsulated neutral liposomes (**liposomes**), nanosized, and PEGylated, Pramipexole encapsulated neutral niosomes (**niosomes**) were administered **i.v.** to observe if any lesion or occlusion formation due to the chronic administration by tail vein.

In the first set of experiments, to evaluate the occurence of formation of partial striatal unilateral lesion in rats administered with 6-OHDA, rotational behavior test was applied. The number of turns per min were measured during 90 min in animals in an automated rotometer bowl after 7 dpl (days post-lesion) which were previously injected with amphetamine solution.

In the second set of experiments, therapy effectiveness was evaluated by rotational behaviour at the same rats due to the number of turns per 90 min placed in automated rotometer bowl after 7, 14 and 21 dpl for every group.

In the third set of experiments, monitoring of therapeutic efficacy of all groups are evaluated by obtaining autoradiograms of both lesioned and intact side of the brain sections of striatum and substantia nigra. [^{125}I]PE2I radioligand was used to quantify DAT by in vitro DAT autoradiography after sacrifying of rats at 22 dpl. A schmatic representation of experimental protocol of in vivo animal studies was given in Figure 3.5.

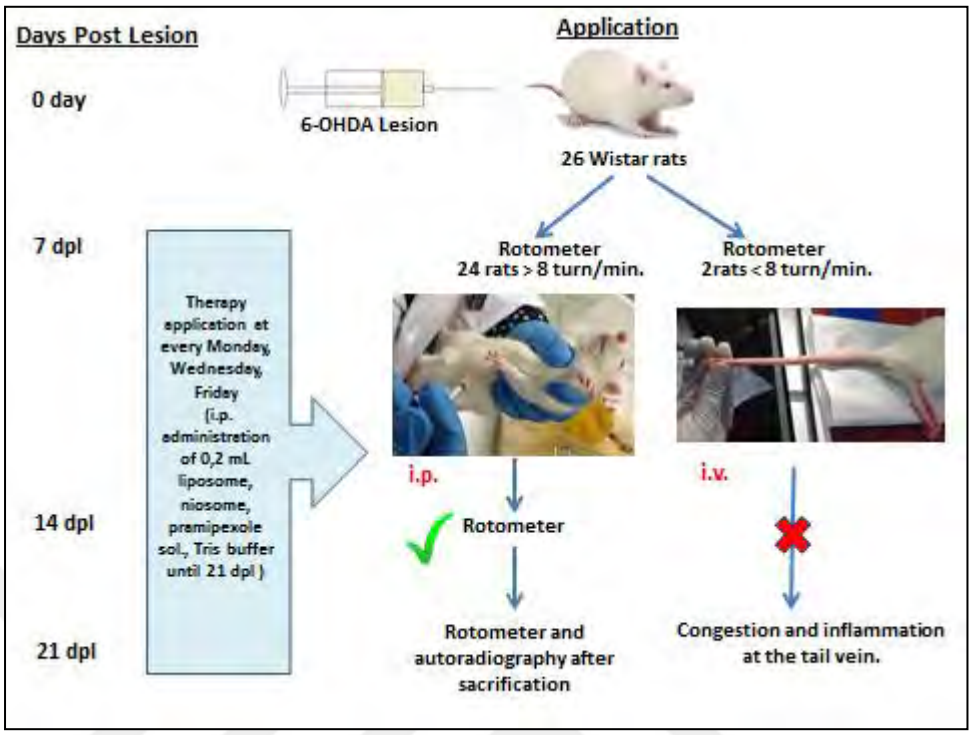


Figure 3.5. Schematic representation of experimental protocol.

3.10.2. 6-OHDA Induced Unilateral Partial Striatal Lesion

3.10.2.1. Preparation of 6-OHDA Injectable Solutions

- Preparation of Pargyline Solution

A premedication of Pargyline is usually administered to rodents prior to injection of 6-OHDA to increase the efficacy of 6-OHDA-induced lesions. The monoamine oxidase inhibitor, Pargyline, enhances the sensitivity of dopaminergic terminals to 6-OHDA, by reducing its extrasynaptic breakdown (280,281). To pepare 50 $mg.kg^{-1}$ Pargyline solution, 75 mg Pargyline was dissolved in 500 µL ethanol and 2 mL NaCl 0,9% 50 x weight/30000.

- ***Preparation of Phosphate Buffer***

182 mg KH_2PO_4 was dissolved in 20 mL of NaCl 0,9% (Solution A) and 238 mg Na_2HPO_4 was dissolved in 20 mL NaCl 0,9% (Solution B). Afterwards 20 mg

ascorbic acid was dissolved in a mixture of 2 mL of Solution A and 18 mL of Solution B and the pH was adjusted to 4,5 with H_3PO_4.

- ***Preparation of 6-OHDA Solution***

To prepare 6-OHDA solution, 10 mg 6-OHDA was dissolved in 10 mL phosphate buffer.

3.10.2.2. 6-OHDA Lesion by Brain Surgery

In this study, partial striatal 6-OHDA lesioned PD model was developed in rats and the therapeutic effect of nanosized, Pramipexole encapsulated, PEGylated liposomes and niosomes was evaluated in this model over a time period (from 7 to 21 dpl). The diversity of rat models using 6-OHDA makes it possible to choose the approximate stage of human PD which is expected to imitate, depending on the site, dose and number of injections of neurotoxin. It was used here a **partial lesion model** which induces progressive and retrograde degeneration of dopaminergic neurons corresponding to an early symptomatic stage of PD (282,283).

Twenty minutes before surgery, animals were injected intraperitoneally with Pargyline (50 $mg.kg^{-1}$). Rats were then anesthetized with Isoflurane (5% for induction) and placed on a stereotaxic apparatus. They were maintained under Isoflurane 3% during surgery. To protect eye and prevent drying of eye, a protector gel (Ocry-gel) was administered. The skull was exposed and small holes were made with a dental drill. Lesion was carried out by unilateral intrastriatal injection of 6-OHDA hydrochloride (1 $mg.mL^{-1}$). A total of 10 µg of 6-OHDA was administered in two points of the right striatum (1 $mg.mL^{-1}$ in 0.01% ascorbic acid, pH 4.5, i.e., 5µg in 5 µL for each point) with a Hamilton syringe (gauge 25) at a flow rate of 1 $\mu L.min^{-1}$ for 5 minutes. Coordinates from bregma were AP = +0.5 mm, L = -2.5 mm, P = -5 mm, and AP = -0.5 mm, L = -4 mm, P = -5 mm according to the atlas of Paxinos et al (284,285). A schematic representation of a rat bregma by dorsal and lateral views of a rat skull is given in Figure 3.6. The syringe was left in place for 4 min after injection and then removed slowly to optimize toxin diffusion. The photograph of 6-OHDA lesion administration and the view of brain bregma to one of the rats placed in a stereotaxic apparatus is given in Figure 3.7. After surgery, bone

wax was applied on 2 drills and the incision was sewed with suture (Figure 3.8.). To prevent surgery area from microorganisms, an antiseptic solution was applied and buprenorphin (0,3 $mg.mL^{-1}$) was administered subcutaneously as analgesic agent after the surgery.

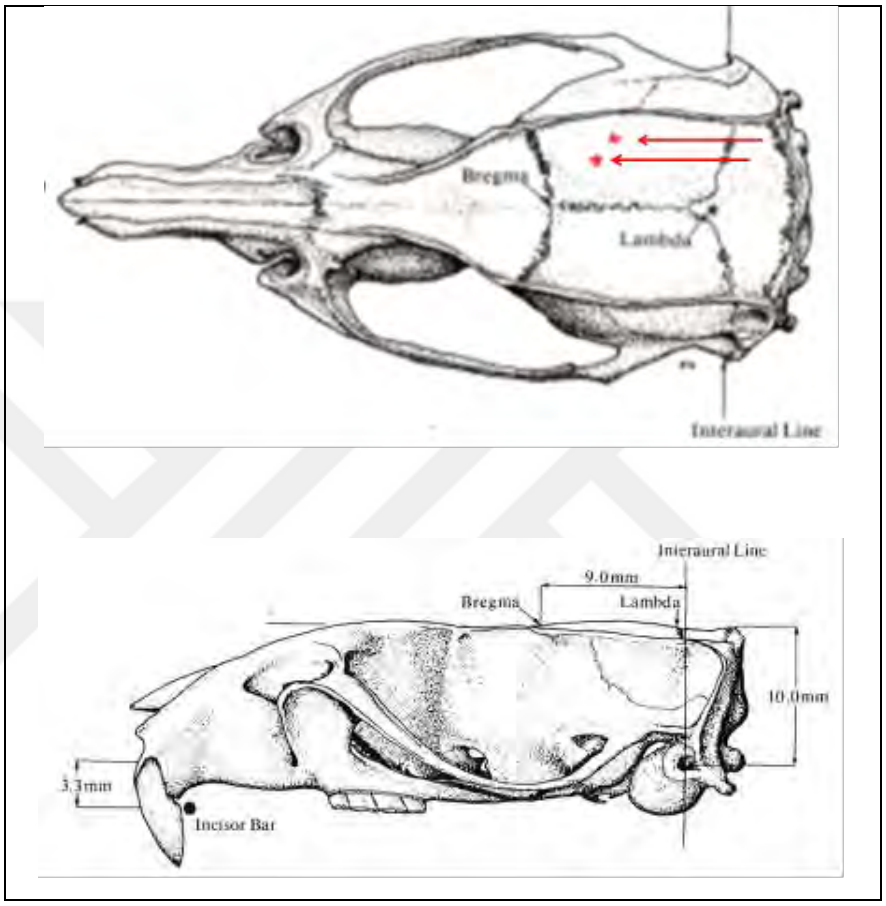


Figure 3.6. The schematic represantation of dorsal and lateral views of the skull of a Wistar rat and the approximate places of 6-OHDA administration. The positions of bregma, lambda and the plane of the interaural line are shown above the lateral view (The approximate 6-OHDA lesion administration places were shown in dark points in the figure above (284).

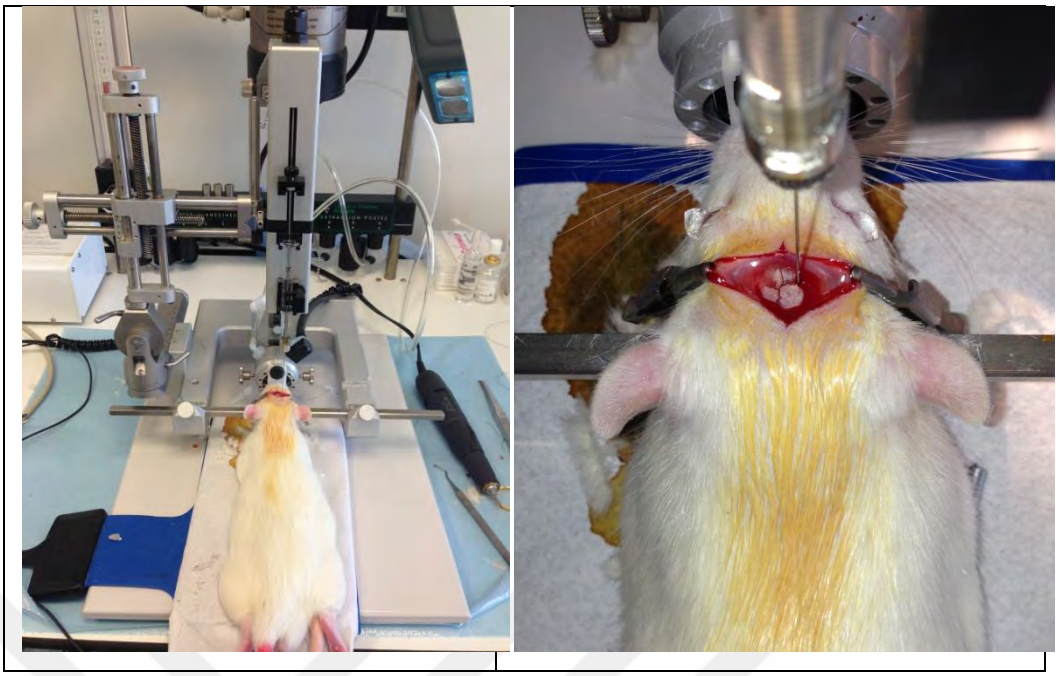


Figure 3.7. 6-OHDA lesion administration and the view of brain bregma to one of the rats placed in a stereotaxic apparatus.

Figure 3.8. Skull skin suturation of one of the rats after 6-OHDA lesion administration.

3.10.3. Rotational Behavior Studies

The rotational behavior test was used for observing formation of partial striatal unilateral lesion in rats and the theurapeutic efficacy of formulations The mechanism of this model depends on the rotation of animals towards the side with the 6-OHDA lesion (ipsiversive) when injected with amphetamine which is a dopamine releasing compound. This model is used unilaterally lesion of the nigrostriatal DA system with 6-OHDA neurotoxin (220,286,287).

3.10.3.1. Preparation of Amphetamine Solution

15 mg D-Amphetamine sulfate was put in 7 mL tubes and dissolved in 5 mL NaCl 0,9% taken from refrigerator and strongly shaked till obtaining a complete dissolution. It was waited at ambient temperature for a couple of minutes before injection.

3.10.3.2. Determination of Injected Amphetamine Solution Volume

Amphetamine solution volume should be calculated before each injection by weighing of rats previously. The weight of rats were registered and the volume of amphetamine solution was calculated as given below;

X/1000 mL …….Equation 9.

[X: The weight of rat (in mg)]

Afterwards this volume of amphetamine solution was injected into the rats i.p.

3.10.3.3. Placement of the Animal to Rotometer

After i.p. administration of amphetamine solution in appropriate volumes, animals were put in the bottom of sawdust containing blue plastic dustbins. Rats were bound to the rope of the rotometer linked to the software of the computer counting the number of turns. Rats were left to get acquainted with its new environment for approximately 15 min in a quiet and gloomy dark mood.

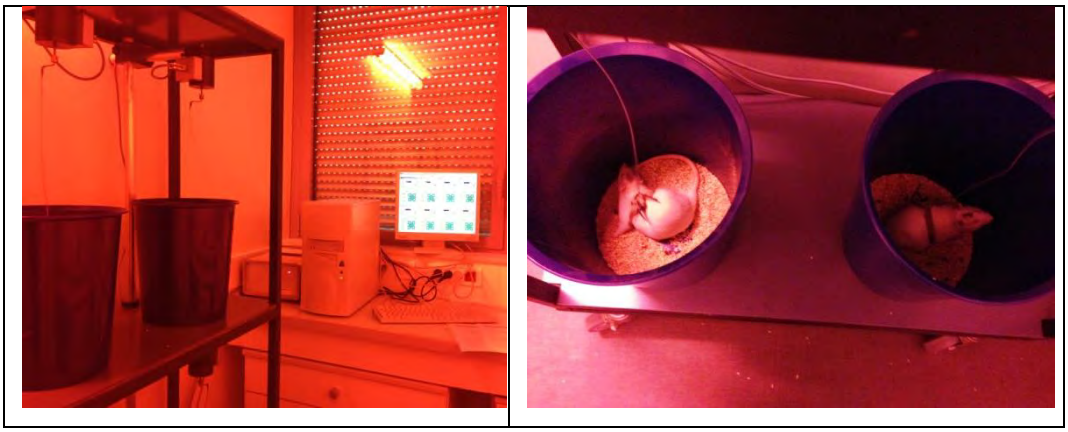
3.10.3.4. Evaluation of the Rotational Behavior by Rotometer

Rotometer is a device counting the number of tours which makes the animal on itself throughout 90 min clockwise (ipsilateral rotations) **(tours-)**, and it also counts the possible rotations anticlockwise (contralateral rotations) **(tours+)**. **N** general activity can be calculated by **(tours(-) - tours(+))** and some possible behaviors such as grooming, rearing or absence of behavior are out of this count.

Rotometer test was used for observing and evaluating both the validity of 6-OHDA lesion at 7 dpl and the therapeutic efficacy of nanosized, PEGylated, Pramipexole encapsulated, neutral liposomes and niosomes when compared with Pramipexole and control group at 14 dpl and 21 dpl.

3.11. Testing of 6-OHDA Lesion

For the purpose of evaluating the occurence of formation of partial striatal unilateral lesion and the success of lesion in rats administered with 6-OHDA, rotational behavior test was applied. For the test, animals were injected with D-amphetamine sulphate injection (3 $mg.kg^{-1}$ i.p.), placed in automated rotometer bowls, and 15 min later left and right full body turns were monitored by a computer for 90 min. The photographs of (a) Rotometer system and (b) ipsilaterally turning rats which were administered amphetamine placed in plastic dustbins were given in Figure 3.9.



(a) **(b)**

Figure 3.9. (a) Rotometer system and (b) Ipsilaterally turning rats which were administered amphetamine placed in plastic dustbins.

Only those rats showing on average more than eight ipsilateral turns per min at 7 dpl of 6-OHDA administration were selected for further experiments as assumed that they performed a good PD model. Results were expressed as the mean numbers of ipsilateral turns/min ± standart error of the mean (SEM) (245,285,288).

3.12. Administration of Nanosized, Pramipexole Encapsulated, PEGylated Neutral Liposome and Niosome Dispersions

Nanosized, PEGylated, Pramipexole encapsulated, neutral liposomes or niosomes, Pramipexole solution or Tris buffer (20 mM, pH:7,4) were applied i.p. or i.v. to 6-OHDA partial striatal lesion rat model of PD at alternate days for a period of 21 days (Figure 3.10).

Figure 3.10. Therapeutic application to PD model Wistar rats by i.p. and i.v. administration, respectively.

3.13. Monitoring of Therapeutic Efficacy with Rotometer Test

Therapeutic efficacy was evaluated by amphetamine induced rotometer test at 14 and 21 dpl after application of nanosized, PEGylated, Pramipexole encapsulated, neutral liposomes, niosomes, Pramipexole solution and Tris buffer (20 mM, pH:7,4) applied i.p. to 6-OHDA partial striatal lesion rat model of PD.

Rotometer test was performed as given in Section 3.10.3.3, Section 3.10.3.4 and 3.13 . Briefly, all rats were tested for their rotational behavior in response to D-amphetamine sulphate injection (3 $mg.kg^{-1}$ i.p.) both for determination of a successful PD model development and therapeutic efficacy of Pramipexole encapsulated, nanosized liposomal or niosomal drug delivery systems at 14 dpl, 21 dpl, respectively. For the test, animals were injected with amphetamine, placed in

automated rotometer bowls, and 15 min later left and right full body turns were monitored by a computer for 90 min as explained above. The photographs of (a) Rotometer system and (b) ipsilaterally turning rats which were administered amphetamine placed in plastic dustbins were given in Figure 2.9. Results were expressed as mean numbers of ipsilateral turns/min ± SEM (285).

3.14. Monitoring of Therapy Efficacy with Autoradiography

Therapeutic efficacy was also evaluated with in vitro autoradiography at 21 dpl after treatment to 6-OHDA partial striatal lesion rat model of PD.

After sacrifying, brains were removed and frozen in isopentane cooled to -35ºC (Fig. 3.11).

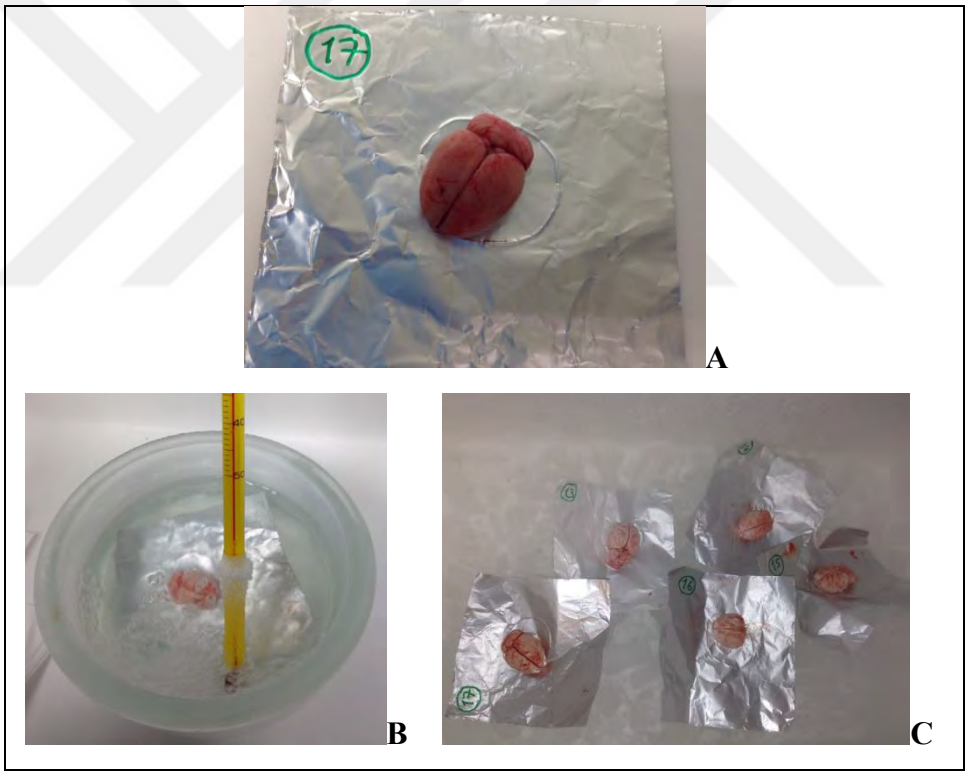


Figure 3.11. Brain removal and preparation for coronal sectioning in sequence (A-C).

Coronal sections (16 µm thickness) were then cut in a cryostat microtome and thaw-mounted on Super-FrostPlus® slides (Fig. 3.12.).

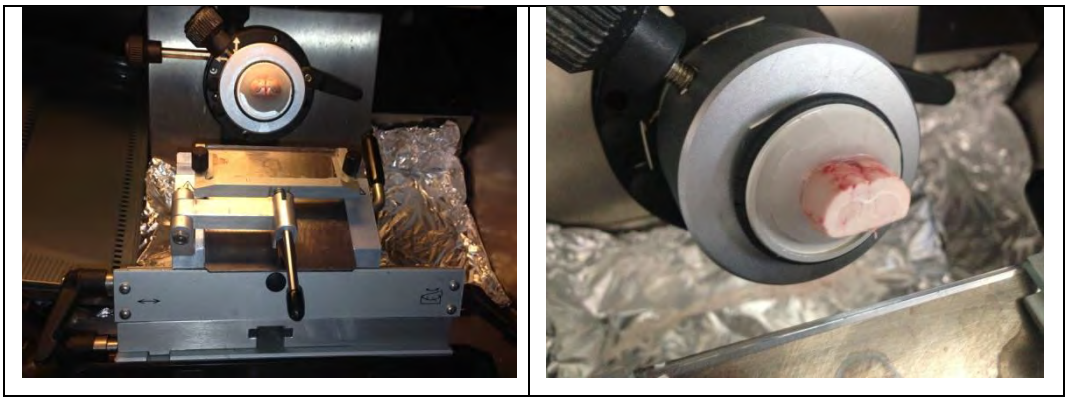


Figure 3.12. Coronal brain sectioning by cryotome after freezing.

Sections were kept at -80ºC until use. Autoradiographic experiments were performed on consecutive sections from each brain, either at the level of 6-OHDA injected striatum (AP coordinates between +0.5 and -0.5 from the bregma) or in substantia nigra pars compacta (SNc) (AP coordinates between -5 and -6 from the bregma) according to the atlas of Paxinos and Watson (284). For each animal, the ipsilateral lesioned side of the brain in the region of the striatum or SNc were compared with the intact controlateral side (285).

PE2I is a cocaine derivative and very potent radiopharmaceutical to image the DAT. PE2I and its radiolabeling precursors were prepared from cocaine as starting material, going through nortropane (289).

Several methods are available to obtain PE2I labeled with iodine-123 or -125, carbon-11 and tritium. The pharmacological properties of PE2I have demonstrated that it has good affinity for the DAT (4 nM) and is one of the most selective DAT ligands. Radioiodinated PE2I was obtained by iododestannylation of the corresponding tin precursor 2. Typically, the labeling procedure involves [$^{125/123}$I] NaI in the presence of an oxidizing agent to provide more than 50% yield of iodination. The half life of I-125 is 59.43 days. It decays by electron capture to an excited state of Tellurium-125 (289). The molecular structure of PE2I was given in Figure 3.13.

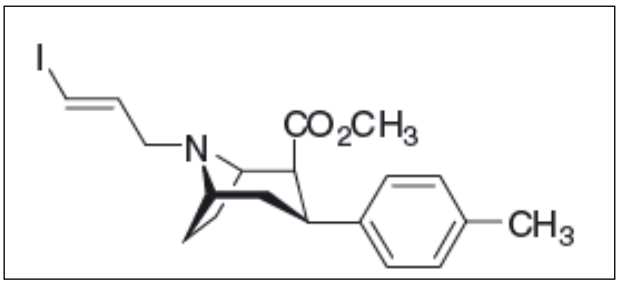


Figure 3.13. The molecular structure of PE2I.

N-(3-iodopro-2E-enyl)-2β-carbomethoxy-3β-(4-methylphenyl) nortropane ([^{125}I]PE2I) was prepared according to the conditions as previously described (290).

Although [^{125}I]PE2I was used as a finished radioligand as synthesized and radiolabeled at the lab of Tours Univ., Equipe 3 Imagerie Moléculaire du cerveau, Inserm U930 Imagerie et cerveau for DAT autoradiography, the chemistry and radiochemistry of PE2I was briefly touched.

- ***Chemistry and Radiochemistry of PE2I***

PE2I was prepared from the starting material, cocaine, going through nortropane 1, which is N-alkylated with 3-(tributyltin)-prop-(2 E)-enyl chloride to provide the tin derivative 2 (Fig 3.14). Then tin was used to produce either cold or radioiodinated PE2I (291).

The synthesis was performed as given above in Figure 2.14.(289).

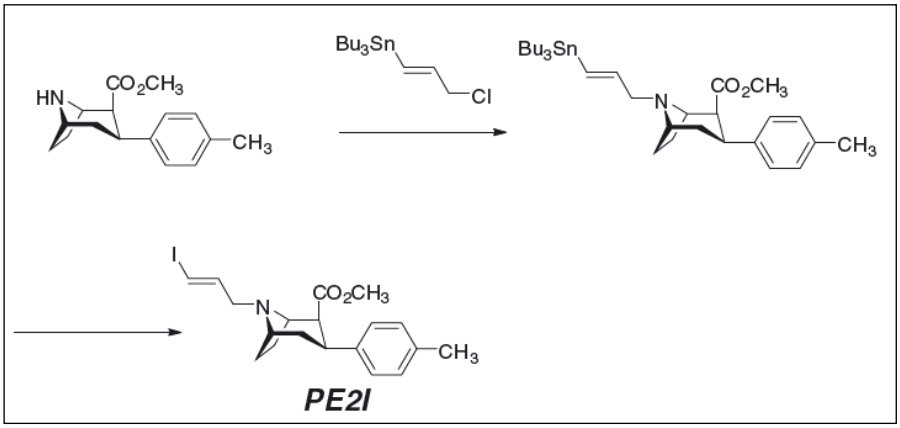


Figure 3.14. Synthesis of precursor for radiolabeling with iodine-125 (289).

- ***Iodine Radiolabeling***

PE2I was radiolabeled by iododestannylation of the corresponding tin precursor (Figure 3.15). Typically, the labeling procedure involves [^{125}I] NaI in the presence of an oxidizing agent to provide more than 50% yield of iodination.

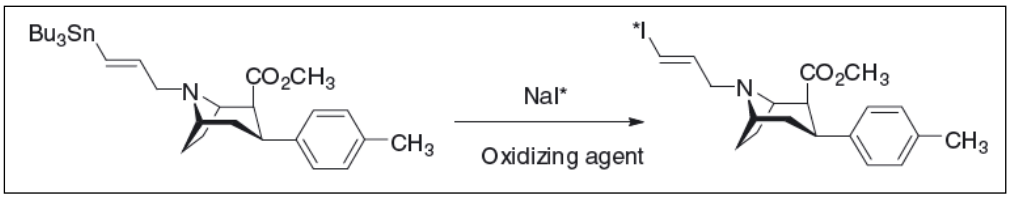


Figure 3.15. Radeolabeling of PE2I with iodine-125 (289).

Briefly, 50 µg of the tin precursor in 50 µg of EtOH and 3% w/v of hydrogen peroxide were used to oxidize NaI (125), and, after a purification step on reverse phase HPLC, pure radioiodinated PE2I was obtained with more than 50% radiochemical yield, more than 95% radiochemical purity and specific activity (no carrier-added) of 75 TBq/mmol for [^{125}I]PE2I, respectively (292). After a sterilization step, the radiochemical yield in these conditions was 70-85%, with radiochemical purity of more than 98% and an estimated specific activity of 8.7 TBq/µmol (289,293).

[^{125}I]PE2I tracer was used as a finished product in our studies for determining the loss of DAT binding (%) for evaluating therapeutic efficacy of different formulations at 6-OHDA lesioned rats by in vitro autoradiographic experiments.

- ***Autoradiography Studies***

The density of brain-specific DAT binding sites were measured by in vitro autoradiographic experiments using [^{125}I]PE2I. Brain sections were allowed to equilibrate at room temperature for 3 h, then they were incubated with 100pM [^{125}I]PE2I in 100µL of a pH 7.4 phosphate buffer (10,14 mM NaH_2PO_4, 137 mM NaCl, 2,7 mM KCl, 1,76 mM KH_2PO_4) at room temperature for 90 min. Nonspecific binding on the adjacent sections were assessed in the presence of 100 µM cocaine. Slides were then washed twice for 20 min in ice cooled phosphate buffer (4°C) for 4 min, and rinsed for 1 sec in distilled water and dried at room temperature. Dried

slides were made conductive by an application of metal electric tape on the free side and then they were placed in the gas chamber of the β-imager. Data from brain sections were collected during two or more hours. Striatum was selected manually and identified in the Franklin and Paxinos atlas (2008). Using the β-vision software, the level of bound radioactivity was directly determined by counting the number of β-particles emitted from the delineated area. The radioligand signal in the ROIs was measured on at least eight sections for each rat and expressed as counts per minute per square millimeter ($cpm.mm^{-2}$). Specific binding was determined by subtracting nonspecific binding from total binding (SB = TB-NSB). DAT expression in the striatum and SNc (identified by visual inspection of the radioactive signal) was observed to delineate ROIs. ROIs were defined manually on the ipsilateral side showing high radioligand uptake (ROI-1), and mirror ROIs (ROI-2) were drawn symmetrically in the homologous contralateral region. The results were expressed as the percentage of binding on the ipsilateral compared to contralateral side (285,290).

In vitro binding conditions were summarized in Table 3.5.

Table 3.5. Experimental in vitro binding conditions used for autoradiography (285).

Tracer	**[^{125}I]PE2I**
Concentration (nM)	0,1
Specific activity (GBq µmol^{-1})	80
Buffer	PBS buffer (0,1 M, pH 7,4) containing 137 mM NaCl, 2,7 mM KCl, 10,14 mM Na_2HPO_4, 1,76 mM KH_2PO_4 and 109 g.L^{-1} D-saccharose
Incubation duration at RT (min)	90
Competitor for nonspecific binding	Cocaine 100 µm (Cooper, France)

3.15. Statistical Analysis

All values were expressed as mean ± SEM. To evaluate the statistical significance of differences among the results, statistical analysis were performed. Depending on the number of data which is less than 30, nonparametric test methods were used for data evaluation. Depending on the group number, Mann-Whitney U test was used for comparison of two groups and Kruskal Wallis was used for comparison of three or more groups. To compare the rotational behavior between groups and in different days, one way Anove and Mann-Whitney U test were performed. To compare the autoradiography data, Mann-Whitney U test was used. All tests were performed two-sided. The significance level was set at $P < 0.05$. And the other one of the statistical analysis were performed using Prism software version (285).

4. RESULTS

In this section, results related with physicochemical properties of ingredients, formulation, characterization, release kinetics, BBB penetration and therapeutic efficacy of nanosized, Pramipexole encapsulated, PEGylated liposomal and niosomal formulations were given on 6-OHDA lesioned partial PD model rats by rotometer test and autoradiaography.

4.1. Physicochemical Control of Active Substance

The properties of Pramipexole (as the salt of dihydrochloride monohydrate) was admitted depending on its analytical sertificate obtained from the drug company (Abdi İbrahim).

4.1.1. UV Spectrum and Standart Line of Pramipexole

4.1.1.1. UV Spectrum and Calibration Curve of Pramipexole in Tris Buffer

UV spectra of Pramipexole were obtained in Tris buffer (20 mM, pH 7.4) in the mean of 6 series as explained in Section 3.3.2.1 and λ_{max} was observed at 262 nm and its spectrum was given in Figure 4.1.

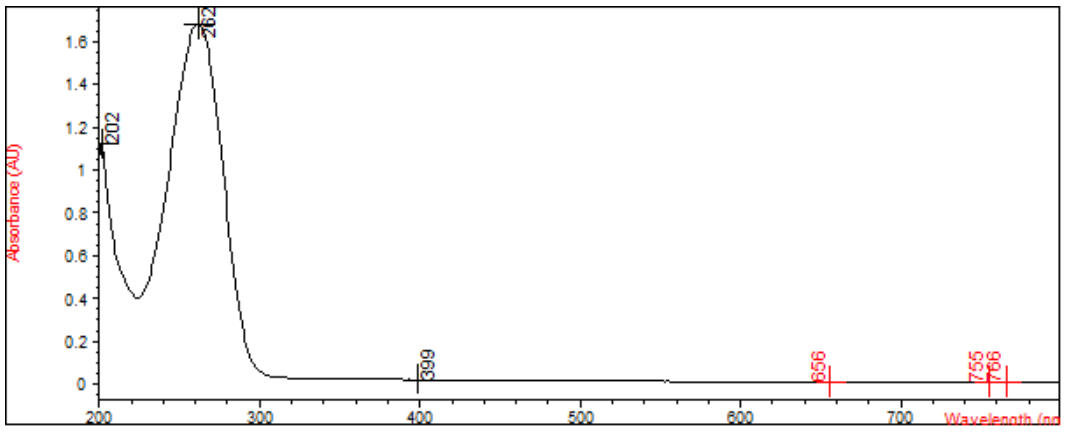


Figure 4.1. UV spectrum of Pramipexole in Tris buffer (20 mM, pH 7.4).

The standart line of Pramipexole was plotted after performing the process given in Section 3.3.2.1 at λ=262 nm (Figure 4.2.).

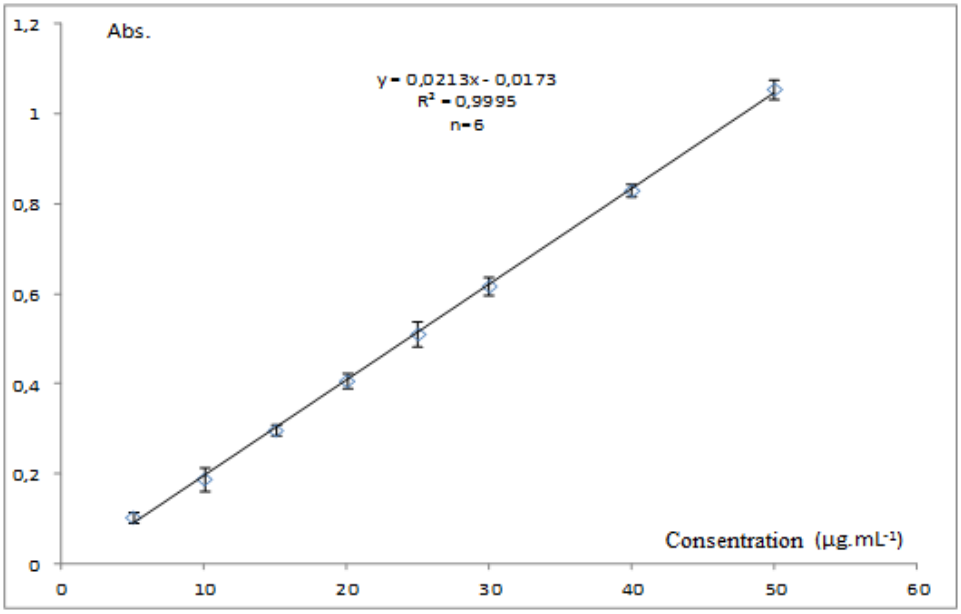


Figure 4.2. Standart line of Pramipexole in Tris buffer (20 mM, pH 7.4).

4.1.1.2. UV Spectrum and Calibration Curve of Pramipexole in Ethanol

UV spectra of Pramipexole were obtained in ethanol as the mean of 6 series as explained in Section 3.3.2.2 and λ_{max} was observed at 262 nm and its spectrum was given in Figure 4.3.

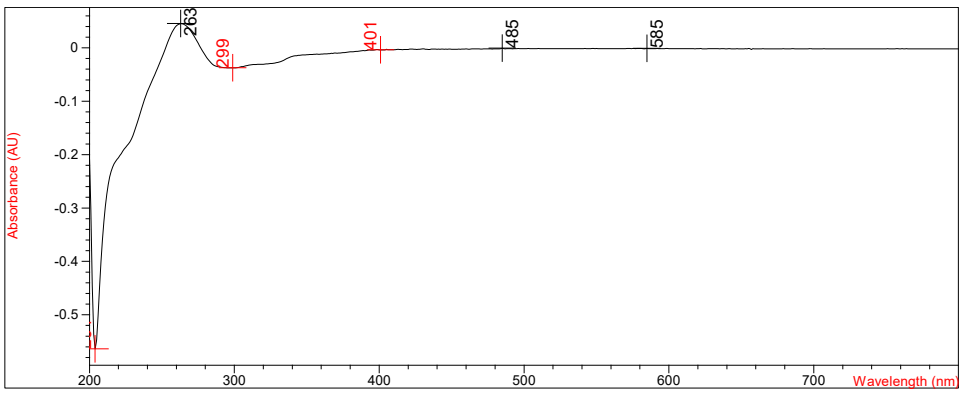


Figure 4.3. UV spectrum of Pramipexole within ethanol.

The standart line of Pramipexole was plotted after performing the process given in Section 3.3.2.2 at λ=262 nm (Figure 4.4.).

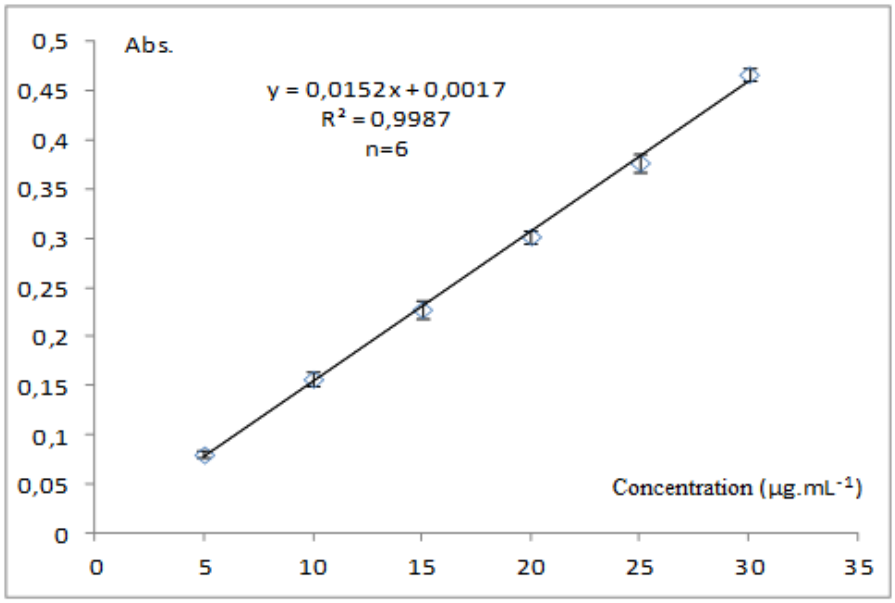


Figure 4.4. Standart line of Pramipexole in ethanol.

4.1.1.3. Analytical Method Validation

Analytical method validation was performed by using calibration lines given in Figure 4.2. and Figure 4.4. and the quantitation of Pramipexole was investigated by linearity, accuracy, precision, specificity and stability. The results related with analytical validation of calibration lines of Pramipexole in two systems.

- **Linearity**

UV spectra of Pramipexole were obtained in Tris buffer (20 mM, pH 7.4) and ethanol as explained in Section 3.3.2.1 and Section 3.3.2.2 at 262 nm. The absorbance values were obtained and following the obtaining of standart calibration line, the accuracy of line equations were calculated as R^2= 0,9995, R^2=0,9987, respectively (Fig. 4.2., Fig. 4.4.).

- **Accuracy**

Repeatibility

5 µg.mL^{-1} and 20 µg.mL^{-1} dose of Pramipexole were selected as low and high concentrations, respectively and they were sampled from the stock solution and

absorbance values were measured at 262 nm with UV spectroscopy 6 times. $\bar{x}$, SE and CV were calculated from the concentrations corresponding to the related absorbance values and the results were given in Table 4.1. and Table 4.2. The value of CV lower than 2% was assumed as the indicator of repeatibility.

Table 4.1. The repeatibity results of different concentrations of Pramipexole in Tris buffer (20 mM, pH 7.4).

Sample	Absorbance (λ=262 nm)	Concentration (µg.mL^{-1})	$\bar{x}$±SE	CV (%)
5 µg.mL^{-1}	0,10408	5,6985	5,6968±0,001	0,02
	0,10404	5,6967		
	0,10409	5,6990		
	0,10403	5,6962		
	0,10402	5,6957		
	0,104	5,6948		
20 µg.mL^{-1}	0,40757	19,9469	19,9227±0,015	0,07
	0,40724	19,9314		
	0,4071	19,9248		
	0,40672	19,9070		
	0,40685	19,9131		
	0,40685	19,9131		

Table 4.2. The repeatibity results of different concentrations of Pramipexole in ethanol.

Sample	Absorbance (λ=262 nm)	Concentration (µg.mL^{-1})	$\bar{x}$±SE	CV (%)
5 µg.mL^{-1}	0,08223	5,2980	5,299±0,007	0,31
	0,08218	5,295		
	0,08201	5,2838		
	0,08246	5,3131		
	0,08201	5,2838		
	0,08265	5,3259		
20 µg.mL^{-1}	0,30196	19,7539	19,711±0,009	0,11
	0,30133	19,7125		
	0,301	19,6907		
	0,3013	19,7105		
	0,30121	19,7046		
	0,30113	19,6993		

Reproducibility

One low (5 µg.mL^{-1}) and one high (20 µg.mL^{-1}) concentrations were selected and taken from the stock solution and UV absorption was measured 6 times at 262 nm. $\bar{x}$, SE and CV were calculated according to the concentrations correspondance to the absorbance values. The results were given in Table 4.3. and Table 4.4. The value of CV lower than 2% designates the reproducibility of the method.

Table 4.3. The reproducibility results of different concentrations of Pramipexole in Tris buffer (20 mM, pH 7.4).

Sample	Absorbance (λ=262 nm)	Concentration (µg.mL^{-1})	$\bar{x}$±SE	CV (%)
5 µg.mL^{-1}	0,10414	5,701	5,7086±0,004	0,15
	0,10427	5,707		
	0,10407	5,698		
	0,10452	5,719		
	0,10426	5,707		
	0,1045	5,718		
20 µg.mL^{-1}	0,40755	19,946	19,9431±0,006	0,07
	0,40726	19,932		
	0,407	19,920		
	0,40754	19,945		
	0,40783	19,959		
	0,40775	19,955		

Table 4.4. The reproducibility results of different concentrations of Pramipexole in ethanol.

Sample	Absorbance (λ=262 nm)	Concentration (µg.mL^{-1})	$\bar{x}$±SE	CV (%)
5 µg.mL^{-1}	0,08522	5,4951	5,434±0,012	0,53
	0,08417	5,4262		
	0,08420	5,4277		
	0,08446	5,4447		
	0,08401	5,4152		
	0,08465	5,4575		
20 µg.mL^{-1}	0,30294	19,8184	19,7368±0,019	0,23
	0,30132	19,7118		
	0,3012	19,7039		
	0,3014	19,7171		
	0,30123	19,7059		
	0,30211	19,7638		

- **Specificity**

As given in Section 3.3.2.1. and Section 3.3.2.2., empty neutral and positively charged liposomes and niosomes were investigated if they gave any peak at UV spectra at 262 nm wavelength in Tris buffer (0.6 $mg.mL^{-1}$) and ethanol. No peak or absorbance was identified at UV spectra at 262 nm.

- **Stability**

The stability of Pramipexole was evaluated at its solutions in diffrent concentrations within the measurement process. The absorbance of solutions prepared at 5 $\mu g.mL^{-1}$ and 20 $\mu g.mL^{-1}$ concentrations was measured in the beginning and at 1, 5, 10 and 24 h and the alteration in the concentrations was evaluated for one day. Concentration differences versus time was found statistically insignificant for both solution systems ($p>0,05$). This result designated that Pramipexole was stabile in both Tris buffer (0.6 $mg.mL^{-1}$) and ethanol (Table 4.5. and Table 4.6.).

Table 4.5. The stability results of Pramipexole in Tris buffer (20 mM, pH 7.4).

Time (h)	Concentration ($\mu g.mL^{-1}$)	$\bar{x}$±SE	CV (%)
0	5	5,6968±0,001	0,02
1	5	5,6086±0,004	0,05
5	5	5,511±0,002	0,13
10	5	5,533±0,009	0,06
24	5	5,618±0,010	0,08
0	20	19,9227±0,015	0,07
1	20	19,562±0,014	0,03
5	20	19,576±0,012	0,09
10	20	19,833±0,014	0,02
24	20	19,888±0,011	0,06

Table 4.6. The stability results of Pramipexole in ethanol.

Time (h)	Concentration (μg.mL^{-1})	$\bar{x}$±SE	CV (%)
0	5	5,299±0,007	0,31
1	5	5,297±0,009	0,40
5	5	5,289±0,008	0,35
10	5	5,276±0,009	0,33
24	5	5,246±0,003	0,39
0	20	19,711±0,009	0,11
1	20	19,613±0,002	0,06
5	20	19,410±0,003	0,04
10	20	19,562±0,005	0,12
24	20	19,615±0,004	0,09

4.2. Physicochemical Controls on the Ingredients of Liposome and Niosome Dispersions

4.2.1. Physicochemical Controls of Phospholipid

DPPC was employed as the main ingredient/phospholipid of liposome dispersions.

4.2.1.1. Standardization of Phospholipid

The phosphotidyl content of phospholipid DPPC is higher than 99% according to the analitical certificate obtained from the companies (Phospholipids GmbH, Almanya).

4.2.1.2. TLC Determination of Phospholipid

As given in Section 3.4.1.2, plates were observed in UV light at 254 nm wavelength. If the dragged substances were performed, purple spots seemed. R_f value was determined as 0,137 for DPPC in the chromatogram. The chromatogram for DPPC was given in Figure 4.5. The R_f value was found compatible and proper with the previous studies (271,294,295).

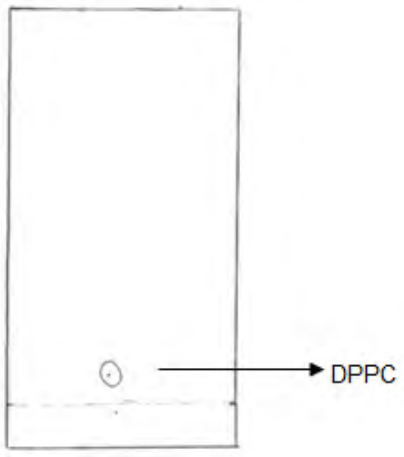


Figure 4.5. TLC chromatogram of DPPC.

4.2.1.3. DSC Determination of Phospholipid

The thermal analysis of the phospholipid used for liposome preparation was performed as explained in Section 3.4.1.3. Thermal analysis slope of DPPC was given in Figure 4.6.

When DSC thermogram of DPPC was evaluated, the phase transition temperature was observed at about 53°C. This value was found compatible with the literature (296).

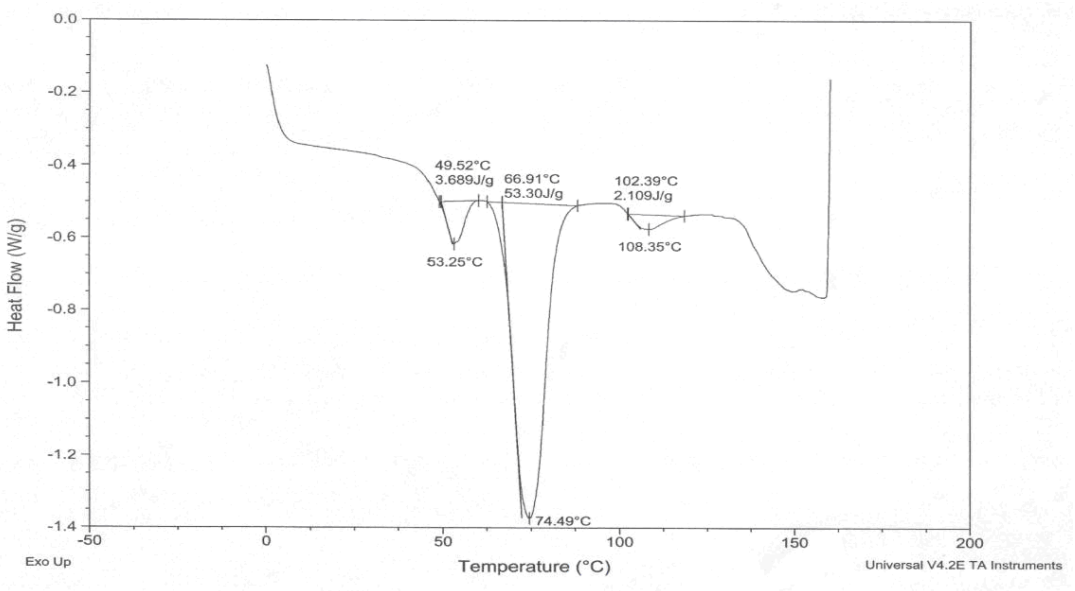


Figure 4.6. DSC thermogram of DPPC.

4.2.1.4. FTIR Spectroscopy Determination of Phospholipid

As given in Section 3.4.1.4, FTIR spectrum of DPPC was given in Figure 4.7. and the comparison of its chemical structure was given in Figure 4.8.

It was observed at the FTIR spectrum of DPPC that the carbonyl tension vibration of esters was obtained in between 1690-1760 cm^{-1}, the mild tension vibration of amine (N-H) was obtained in between 3300-3500cm^{-1}, the tension vibration of amide (C-N) was obtained in between 1180-1360 cm^{-1} and the strong aliphatic tension vibrations belong to alkans (C-H) was obtained at 2800-2970 cm^{-1} (297).

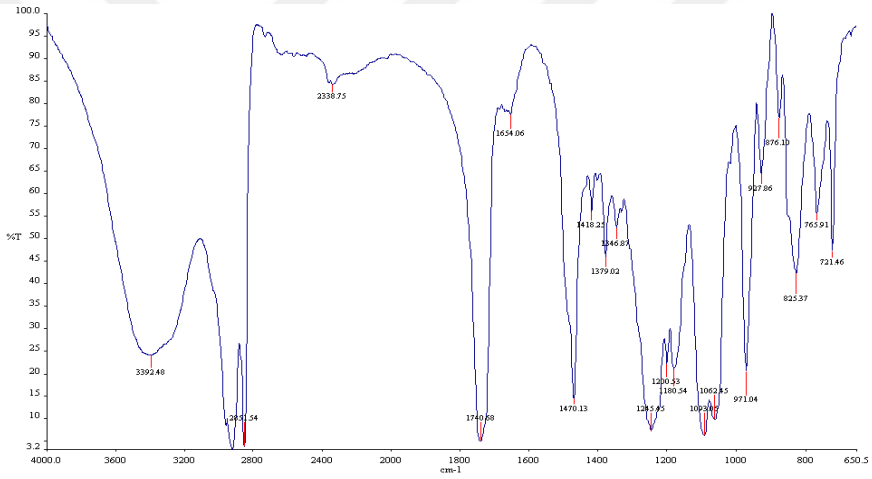


Figure 4.7. FTIR spectrum of DPPC.

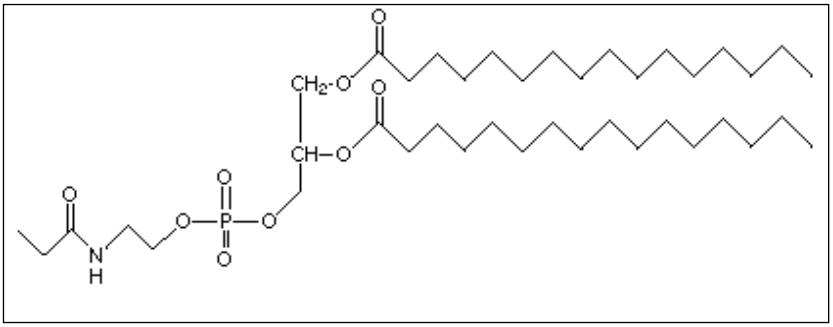


Figure 4.8. The molecular structure of DPPC.

4.2.2. Physicochemical Controls of Non-Ionic Surfactant

4.2.2.1. Identification of HLB Value of Non-Ionic Surfactant

By using the formula given in Section 3.4.2.1, HLB value of SUR II was calculated.

SUR II: (230/470) x 20 = 9,79 was obtained.

The HLB value of SUR II was found between 8-18 which designated that SUR II is in hydrophilic property. This value was found in accordance with the literature (271,298,299).

4.2.2.2. Identification of Surfactant by FTIR Spectroscopy Analysis

FTIR spectrum of SUR II was performed as explained in Section 3.4.2.2. The spectrum of SUR II was given in Figure 4.9.

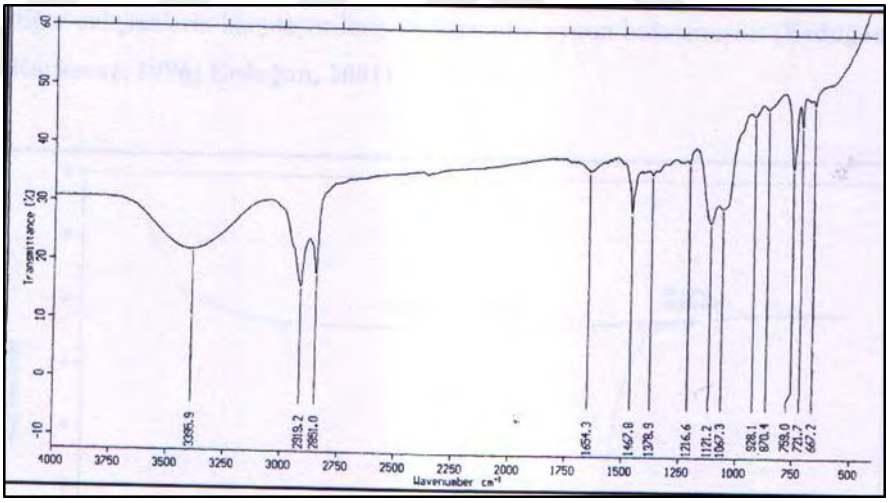


Figure 4.9. FTIR spectrum of SUR II.

In FTIR spectrum of SUR II, the tension vibration of hydroxy group (O-H) was obtained at 3396 cm^{-1}, the tension vibration of C-O and the distortion inclination of O-H were obtained in between 3584-3650 cm^{-1}, the tension vibration of alkans (C-H) were obtained at 2919 cm^{-1}, the inclination of $–CH_2$ was obtained at 1467 cm^{-1}, the tention vibration of C-O-C was obtained at 1216 cm-1 and the tention vibration

of –(CH_2)n was obtained at 759 cm^{-1}. The results were found proper with that of the previous studies (271,297-299).

4.2.2.3. DSC Determination of Surfactant

Thermal analysis of SUR II was performed as given in Section 3.4.2.3. T_c of SUR II was obtained as 50,9°C. DSC thermogram of DSC was given in Figure 4.10.

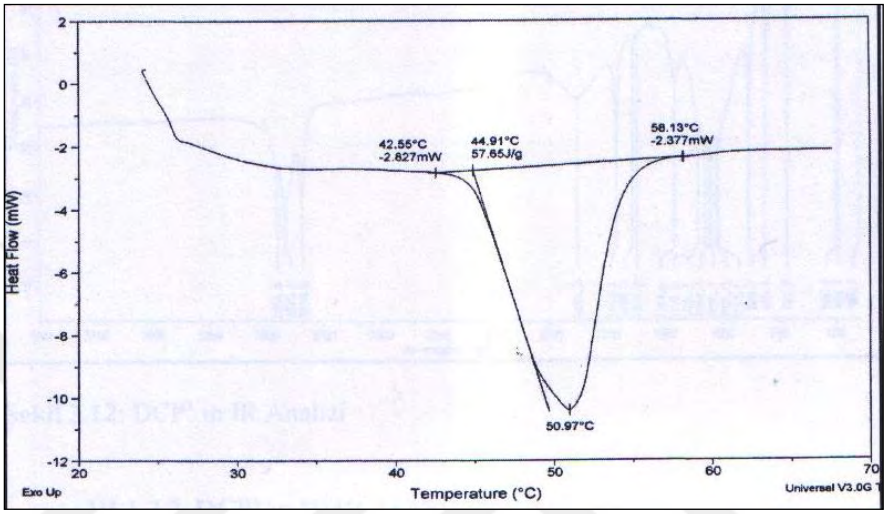


Figure 4.10. DSC thermogram of SUR II.

These results were observed compatible with the previous studies and the literature (271,299,300).

4.2.2.4. Melting Point Determination of Surfactant

The melting point determination of SUR II was performed as given in Section 3.4.2.4 and the melting point was found as 45ºC. This value was found parallel with the previous studies (301).

4.2.3. Physicochemical Controls of Stability Enhancing Ingredient

Cholesterol was employed as the bilayer condenser/stability enhancer in liposome and niosome dispersions.

4.2.3.1. TLC Determination of Cholesterol

TLC of cholesterol was performed as given in Section 3.4.3.1 and red-brown spots were observed in UV lamp at 254 nm. R_f value was calculated as 0.95 (Figure 4.11.). This R_f value was found in accordance with the literature (271).

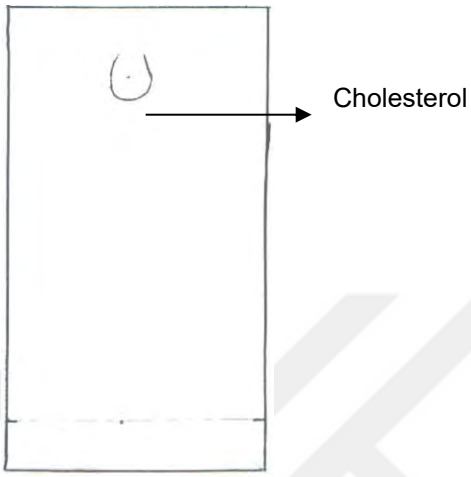


Figure 4.11. TLC chromatogram of cholesterol.

4.2.3.2. DSC Determination of Cholesterol

Thermal analysis of cholesterol was performed as given in Sections 3.4.3.2. The phase transition temperature and melting point of cholesterol were obtained as 45°C and 154°C, respectively. These results were observed compatible with the literature (300). DSC thermogram of DSC was given in Figure 4.12.

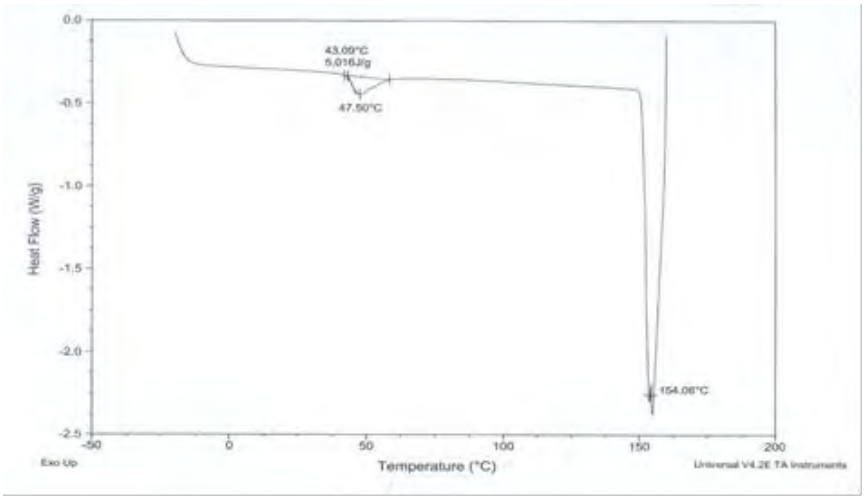


Figure 4.12. DSC thermogram of cholesterol.

4.2.3.3. FTIR Spectroscopy Determination of Cholesterol

FTIR spectrum of cholesterol was performed as explained in Sections 3.4.3.3. The spectrum and molecular structure of cholesterol were given in Figure 4.13. and Figure 4.14.

In FTIR spectrum of cholesterol, the tension vibration of hydroxy group (O-H) was obtained in between 3200-3600 cm^{-1}, the wide mild strenght tension vibration of cyclic and plane alkans (C-H) were obtained in between 3010-3100 cm^{-1}, the tension vibration of alkene (C=C) was obtained in between 1610-1680 cm^{-1} (297).

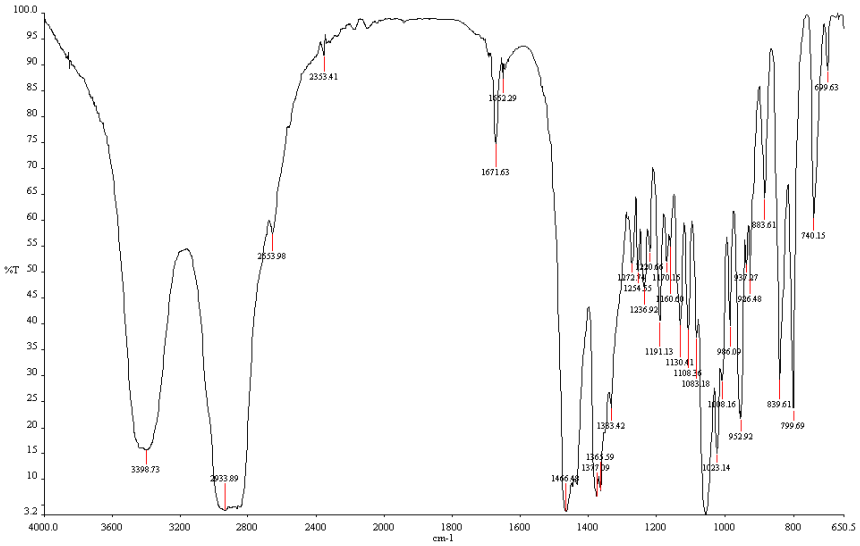


Figure 4.13. FTIR spectrum of cholesterol.

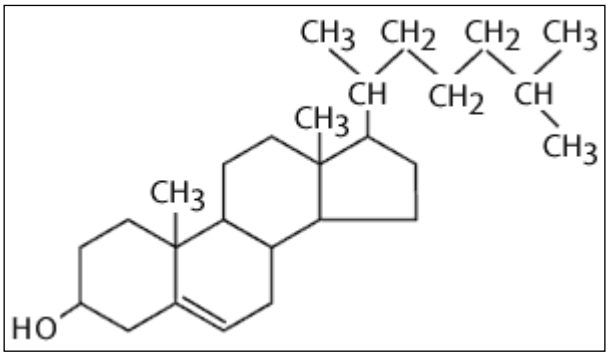


Figure 4.14. The molecular structure of cholesterol.

4.2.3.4. Determination of Melting Point of Cholesterol

The melting point determination of cholesterol was performed according to the procedure given in Section 3.4.3.4 and the melting point of cholesterol was determined as 148-149°C. This value was found compatible with the literature (271,302).

4.2.4. Physicochemical Controls of Coating Agent

PEG2000-DSPE was used as coating and linker agent.

4.2.4.1. TLC Determination of PEG2000-DSPE

After TLC analysis performed as given in Section 3.4.4.1, the plates were observed under UV lamp at 254 nm after heated in an incubator for 5-10 min. At 170ºC and developed substance was observed as purple colored spots. R_f value was calculated as 0,75 for PEG2000-DSPE at the chromatogram. This value was observed compatible with the literature (303). The chromatogram of PEG2000-DSPE was given in Figure 4.15.

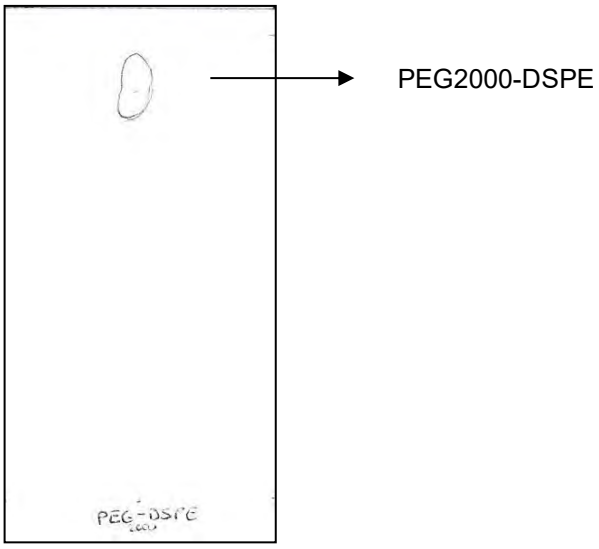


Figure 4.15. TLC chromatogram of PEG2000-DSPE.

4.2.4.2. FTIR Spectroscopy Determination of PEG2000-DSPE

FTIR spectrum of PEG2000-DSPE was obtained as given in Section 3.4.4.2 and FTIR spectrum and chemical structure of PEG2000-DSPE were given in Figure 4.16. and Figure 4.17.

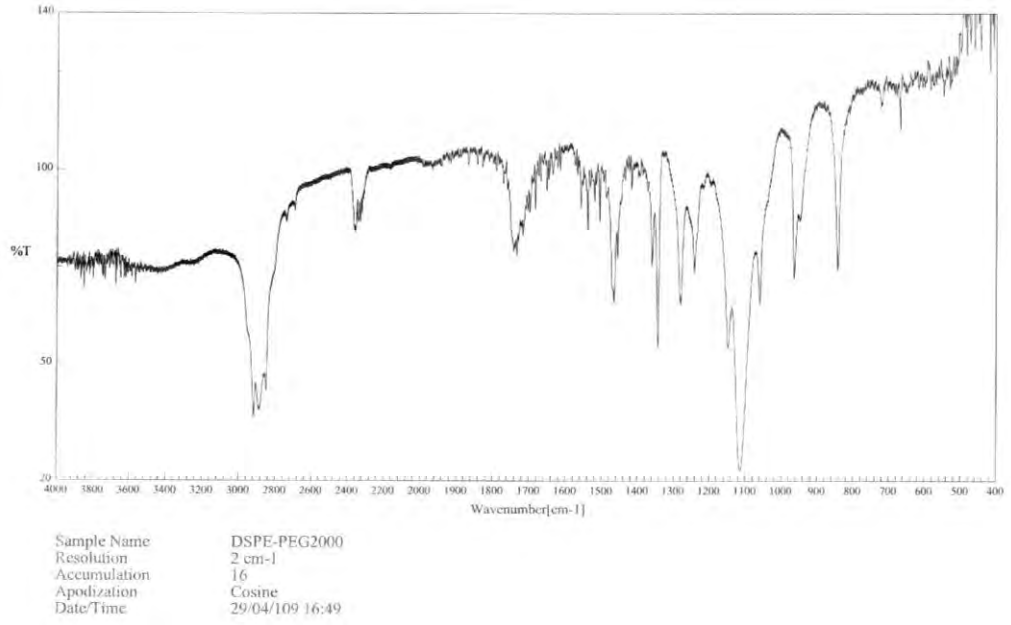


Figure 4.16. FTIR spectrum of PEG2000-DSPE.

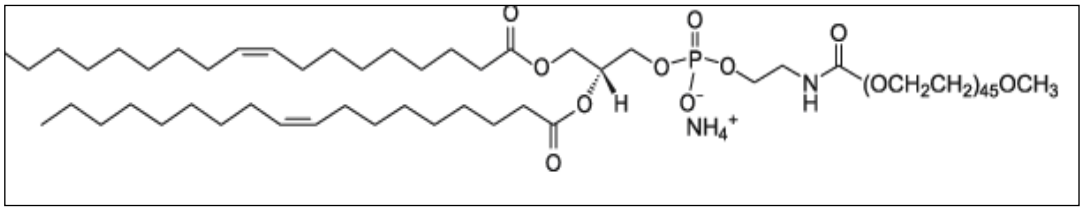


Figure 4.17. The chemical structure of PEG2000-DSPE.

In the FTIR spectrum of PEG2000-DSPE, the tension vibration of amine (N-H) was obtained in between 3500-3800 cm^{-1}, the tension vibration of aliphatic alkans (C-H) was obtained in between 2850-3000 cm^{-1}, the tension vibration of alkene (C=C) was obtained in between 2260-2155 cm^{-1}, the tension vibration of carbonyl of ester was obtained in between 1725-1745 cm^{-1}, the tension vibration of phosphorous compound (P=O) was obtained in between 1250-1300 cm^{-1} and the tension vibration of (P-O) was obtained in between 1030-1050 cm^{-1} (297).

4.2.5. Physicochemical Controls of Charge Inducer

SA was used as positive charge inducer.

4.2.5.1. Identification of SA by FTIR Spectroscopy

FTIR spectrum of SA was obtained as given in Section 3.4.5.1 and FTIR spectrum of SA was given in Figure 4.18.

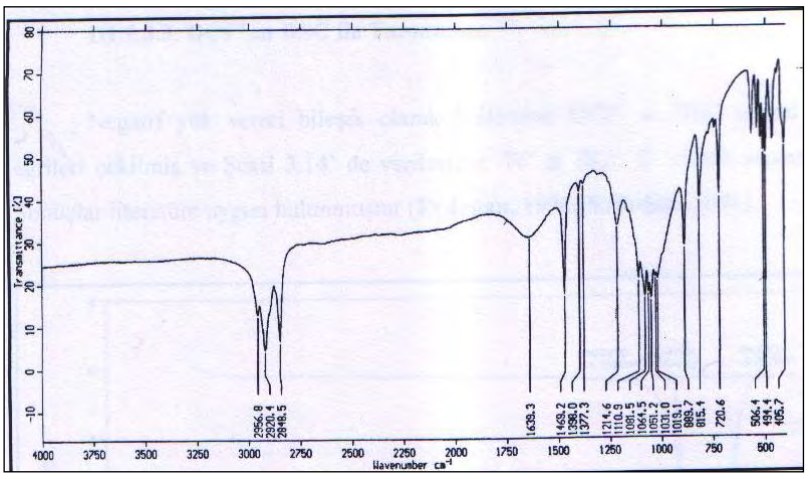


Figure 4.18. FTIR spectrum of SA

In the FTIR spectrum of SA, the tension vibration of aliphatic alkans (C-H) was obtained at 2956, 2920 and 2848 cm^{-1} and the inclination tension vibration of C-CH_3 was obtained at 1469 cm^{-1}, the tension vibrations of P=O were obtained at 1110, 1081, 1064, 1051, 1031 and 1019 cm^{-1} and the tension vibrations of P-O-P were obtained at 889, 815 and 720 cm^{-1}. FTIR spectrum of SA was found proper with the previous studies (271,297-299).

4.2.5.2. Identification of SA by DSC

Thermal analysis curve of SA was obtained by DSC according to the procedure previously described in Section 3.4.5.2. DSC thermogram of SA was given in Figure 4.19.

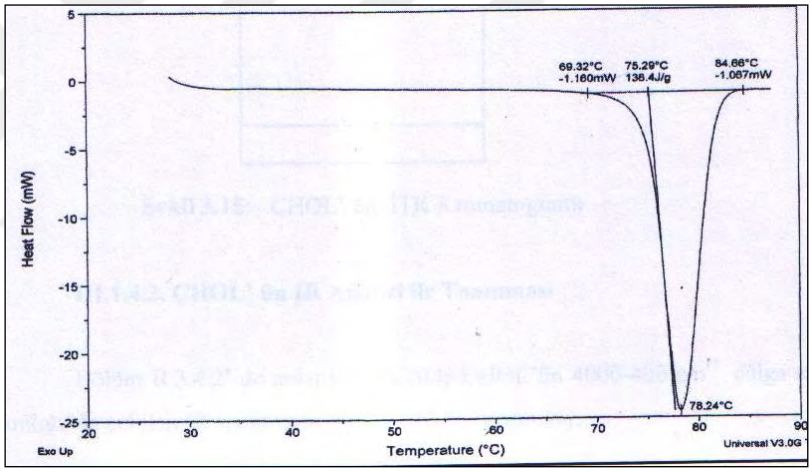


Figure 4.19. DSC thermogram of SA.

As seen from DSC thermogram of SA, Tc value of SA was obtained at 78,2ºC. Results were found proper with the previous studies and the literature (271,298-300).

4.3. STUDIES on LIPOSOME and NIOSOME DISPERSIONS

This section was divided into two parts: i. Results related with preliminary formulation studies on liposome and niosome dispersions, ii. Results related with characterization studies on liposome and niosome dispersions.

4.3.1. PRELIMINARY FORMULATION STUDIES

- Determination of DTPA-PE Synthesis

To radiolabel nanosized, PEGylated, Pramipexole encapsulated, liposomal and niosomal theragnostic liposomes DTPA-PE was synthesized as given in Section 3.5 to radiolabel with ^{99m}Tc and to observe therapeutic efficacy. The synthesis of DTPA was observed with TLC detection and FTIR spectrum.

- ***TLC Detection of DTPA-PE***

The TLC of DTPA-PE was performed as given in Section 3.5 and R_f value of DTPA-PE was calculated as 0.45 on the chromatogram by observing the spot under the UV lamp at 254 nm and found in accordance with the literature (273,275). The TLC chromatogram was given in Figure 4.20.

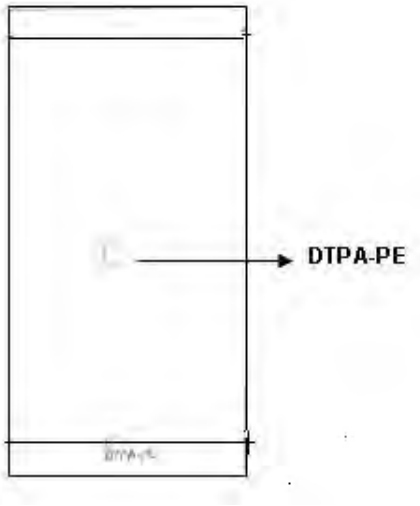


Figure 4.20. The TLC chromatogram of DTPA-PE.

- ***FTIR Spectrum of DTPA-PE***

FTIR spectrum of DTPA-PE was obtained as given in Section 3.5. The molecular structure of DTPA-PE and FTIR spectrum of DTPA-PE were given in Figure 4.21. and Figure 4.22., respectively. It was observed from the spectrum that (C-H) streching vibrations of aliphatic alkanes in between 2850-3000 cm^{-1}, carbonyl

(C=O) vibrations of esters in between 1735-1750 cm^{-1}, (N-H) contortion vibrations of secondary amine in between 1640-1560 cm^{-1}, (C=O) streching vibrations of secondary amine carbonyl in between 1630-1680 cm^{-1}. It was also found that (C-O) streching vibrations of ester and amide in between 1050-1300 cm^{-1} and (P-O) streching vibrations of phosphorous in between 1030-1050 cm^{-1} (275,297).

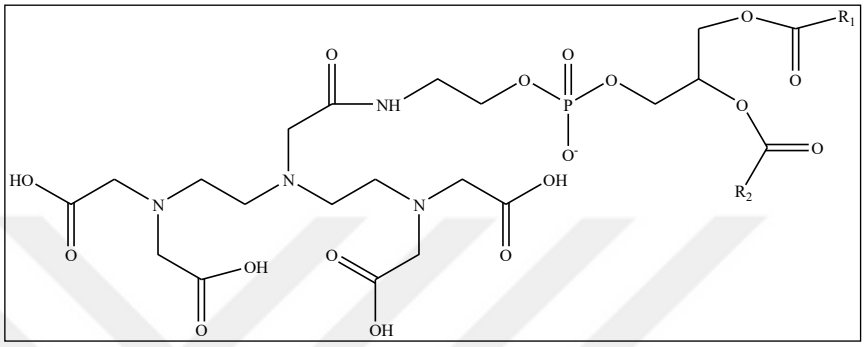


Figure 4.21. The molecular structure of DTPA-PE.

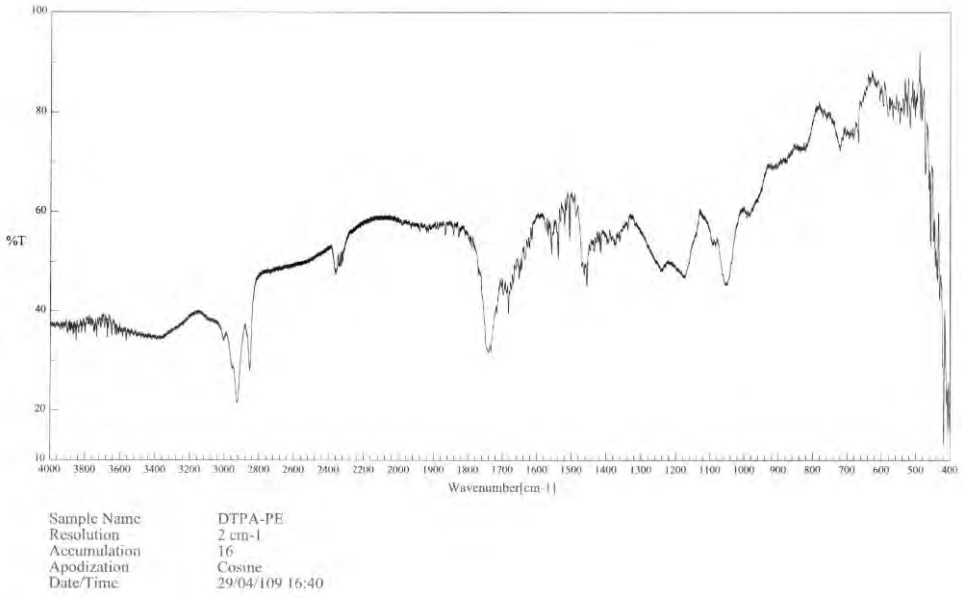


Figure 4.22. FTIR spectrum of DTPA-PE.

- ***Preliminary Studies About Synthesis of Dopamine D2/D3 Receptor or DAT Specific Ligand for Active Targeting of Liposome and Niosome Dispersions***

It was thought to synthesize the dopamine D2/D3 receptors within striatum and substantia nigra or DAT specific radioligand and modify it with neutral and positively charged liposomal or niosomal drug delivery systems with a linker to actively target the theragnostic delivery sytems for both imaging and therapy of PD. This part was thought and planned to be performed at François Rabelais de Tours University, Tours/France as the co-directed cotutelle doctorate thesis. All the D2/D3 receptors and DAT radioligands which were given at Section 3.5 in Table 3.1 and Table 3.2 were studied and searched for the ability to modify and link to liposomal and niosomal systems.

However, it was evaluated by the valuable radiochemist (Assoc.Prof.Dr. Johny Vercouillie) of Tours University Molecular Imaging and Brain lab. and by many other eminent scientist/radiochemists/chemists from different Universities that it is very hard to link these radioligands to the drug delivery systems. Even if it can be possible, three dimentional structure of the radioligand could be changed after modification or within the organism which can cause significant loss in the affinity to the target site within the brain.

All the radioligands given at Section 3.5 in Table 3.1 and Table 3.2 were investigated for their ability to modify to drug delivery systems one by one however, it was observed that they are improper for modification. As an example, ^{123}I-IBZM was selected to study for modification and computer-aided conformational chemistry was studied for this compound designating its molecular structure (Fig. 4.23.) and enterance into D2/D3 receptor pocket (Fig. 4.24. and Fig. 4.25.).

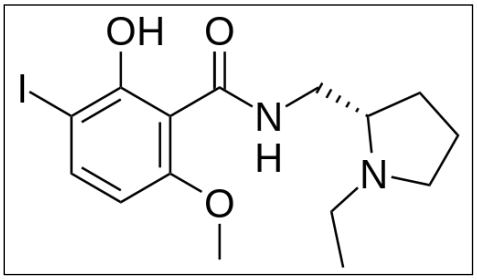


Figure 4.23. The molecular structure of ^{123}I-IBZM.

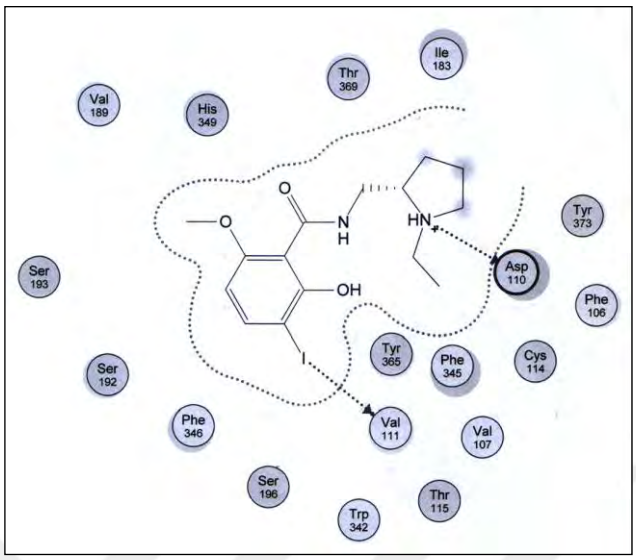


Figure 4.24. The interaction of ^{123}I-IBZM with dopamine D2/D3 receptor with the use of computer-aided conformational chemistry modelling.

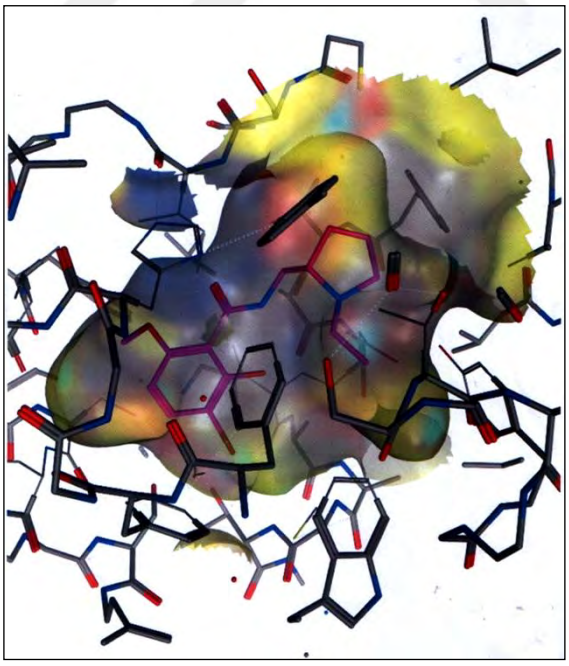


Figure 4.25. Three dimentional conformational structure of the interaction of ^{123}I-IBZM with dopamine D2/D3 receptor with the use of computer-aided conformational chemistry modelling.

These expert scientists' opinion were in agreement with each other that the modification of ^{123}I-IBZM radioligand to liposomal and niosomal delivery systems is mostly impossible with chemical synthesis methods. Therefore, it was aimed to prepare passively targeted, PEGylated, Pramipexole encapsulated liposomal and niosomal drug delivery systems and observe their efficacy by rotational behavior tests and autoradiography as a radioactive monitoring method for therapeutic efficacy.

4.3.2. Formulation and Characterization of Nanosized, PEGylated, Pramipexole Encapsulated Liposome and Niosome Dispersions

In order to easily compare and evaluate the characterization data of liposomal and niosomal formulations, these results will be given together in this section.

4.3.2.1. Mean Particle Size and Size Distribution and Zeta Potential of Liposome and Niosome Dispersions

The mean particle size, polydispersity index and zeta potantial of the prepared nanosized, Pramipexole encapsulated, PEGylated neutral and positively charged liposomal and niosomal formulations were measured as given in Section 3.6.1.2.1. Results are given in Table 4.7.

Table 4.7. The mean particle size, polydispersity index and zeta potantial of the prepared neutral and positively charged liposomal and niosomal formulations.

FORMULATIONS	MEAN PARTICLE SIZE (nm)	PDI	ZETA POTENTIAL (mV)
DPPC:Chol:PEG2000-PE	122 ± 1,3*	0,12	-10,6 ± 0,9*
DPPC:Chol:PEG2000-PE:SA	165 ± 1,7	0,14	-5,71 ± 1,5
SURII:Chol:PEG2000-PE	103 ± 1,2*	0,13	-13,8 ± 0,7*
SURII:Chol:PEG2000-PE:SA	159 ± 1,8	0,17	-7,7 ± 1,3

***(p<0.05),** n=6, PDI= Polydispersity Index

When the mean particle size and zeta potantial of liposomal and niosomal formulations were compared, no statistical difference was observed with the neutral liposomes and niosomes ($p>0.05$) however, a statistically significant alteration was observed in the mean particle size and zeta potential of positively charged liposomes and niosomes when compared with neutral liposomes and niosomes, respectively ($p<0.05$).

4.3.2.2. Encapsulation Efficiency of Liposome and Niosome Dispersions

The encapsulation efficiency of nanosized, Pramipexole encapsulated, PEGylated neutral and positively charged liposomal and niosomal formulations were evaluated as given in Section 3.6.1.2.2 (304). Encapsulation efficiency (%) was calculated and the results are given in Table 4.8.

Table 4.8. The encapsulation efficiency (%) of nanosized, Pramipexole encapsulated, PEGylated neutral and positively charged liposomal and niosomal formulations.

FORMULATIONS	ENCAPSULATION EFFICIENCY of PRAMIPEXOLE (%)
DPPC:Chol:PEG2000-PE	9.42 ± 0.01*
DPPC:Chol:PEG2000-PE:SA	5.94 ± 0.02
SURII:Chol:PEG2000-PE	10.51 ± 0.01*
SURII:Chol:PEG2000-PE:SA	6.84 ± 0.02

***(p<0.05)**, n=6.

No significant difference was observed between neutral liposome and niosome formulations ($p>0.05$). However, a statistically significant increase was observed in the encapsulation of both neutral liposomes and niosomes when compared with positively charged ones ($p<0.05$).

4.3.2.3. Liposomal Phospholipid Amount

The phospholipid amount of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged liposomal formulations was determined with Rauser method as given in Section 3.6.1.2.3 and the results are given in Table 4.9.

Table 4.9. The phospholipid amount and phospholipid efficiency of nanosized, Pramipexole encapsulated, PEGylated neutral and positively charged liposomal formulations.

FORMULATIONS	PHOSPHOLIPID AMOUNT (μmol lipit.mL^{-1})	PHOSPHOLIPID EFFICIENCY (%)
DPPC:Chol:PEG2000-PE	90.43 ± 2.13	91.6 ± 1,5
DPPC:Chol:PEG2000-PE:SA	81.56 ± 3.21	82,68 ± 2,7

The phospholipid loss in DPPC:Chol:PEG2000-PE liposomal formulation was observed higher when compared with DPPC:Chol:PEG2000-PE:SA liposomal dispersions due to the preparation process and experimental conditions; however, this difference was found statistically insignificant ($p>0.05$).

4.4. Release Kinetics of Nanosized, Pramipexole Encapsulated Neutral and Positively Charged Liposome and Niosome Dispersions

The release profile and kinetics of Pramipexole encapsulated liposomal and niosomal dispersions were investigated under this heading.

4.4.1. Release Kinetics of Pramipexole Encapsulated Liposome Dispersions

The release studies of Pramipexole from nanosized, PEGylated, neutral and positively charged liposomes were performed as given in Section 3.7.1. The comperative cumulative release (%) values for Pramipexole encapsulated, neutral and positively charged liposomes are given in Figure 4.26 and Table 4.10.

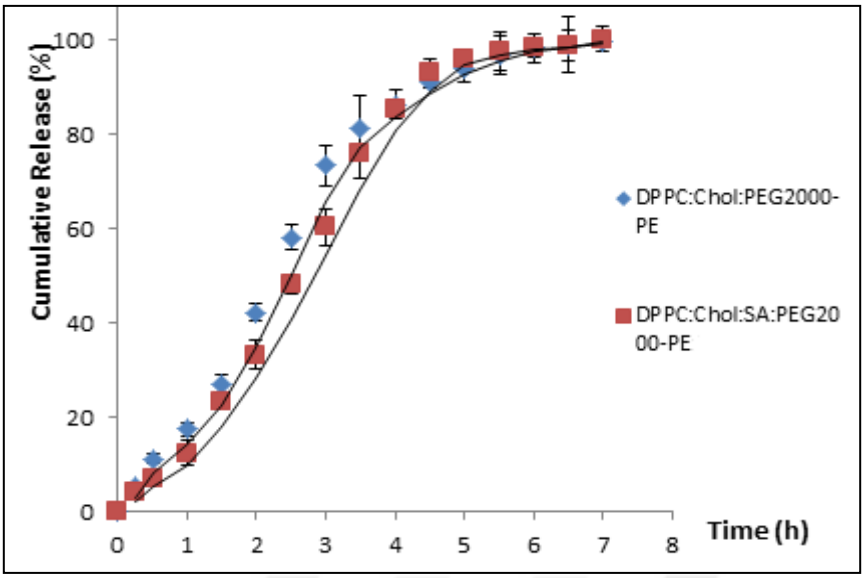


Figure 4.26. In vitro release of Pramipexole from neutral and positively charged liposome formulations in Tris (20 mM, pH 7.4) buffer (n=6).

Table 4.10. The evaluation of in vitro release kinetics of Pramipexole encapsulated neutral and positively charged liposome formulations in Tris (20 mM, pH 7.4) buffer.

Formulations	Zero Order			First Order			Higuchi		
	m	a	R^2	m	a	R^2	m	a	R^2
PPX-DPPC	51,2343	-24,9620	0,9558	-0,7222	5,2397	0,9762	15,1660	11,7994	0,8987
PPX-DPPC:SA	56,4173	-36,3841	0,9538	-0,7614	5,3888	0,9651	17,6794	1,4763	0,9426

*(m= slope, a=intercept, R^2=determination coefficient).

As seen from the Figures 4.26 and Table 4.10, the release kinetics of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged liposomes fitted to the first-order release kinetics.

4.4.2. Release Kinetics of Pramipexole Encapsulated Niosome Dispersions

The release studies of Pramipexole from nanosized, PEGylated, neutral and positively charged niosomes were performed as given in Section 3.7.2. The comparison of cumulative release (%) values for Pramipexole encapsulated, neutral and positively charged niosomes are given in Figure 4.27 and Table 4.11.

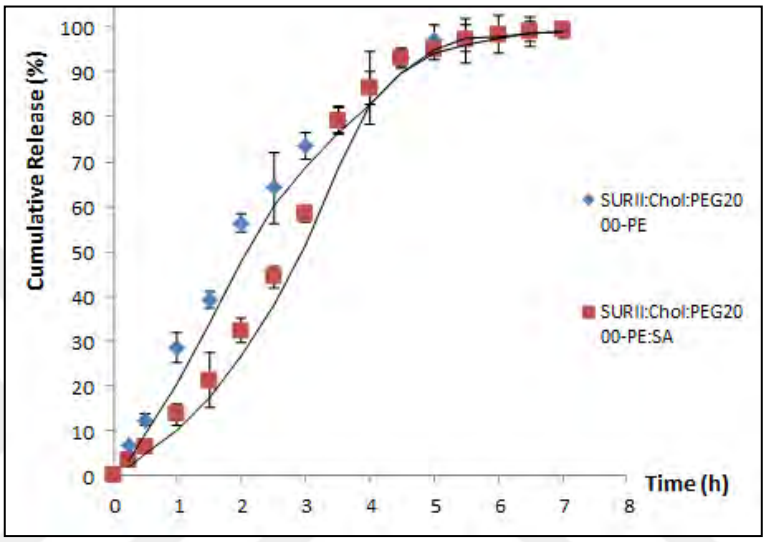


Figure 4.27. In vitro release of Pramipexole from neutral and positively charged niosomal formulations in Tris (20 mM, pH 7.4) buffer (n=6).

Table 4.11. The evaluation of in vitro release kinetics of Pramipexole encapsulated neutral and positively charged niosome formulations in Tris (20 mM, pH 7.4) buffer.

Formulations	Zero Order			First Order			Higuchi		
	m	a	R^2	m	a	R^2	m	a	R^2
PPX-SURII	48,0721	-16,1013	0,9674	-0,7346	5,1440	0,9676	14,0523	19,0160	0,8870
PPX-SURII:SA	54,5823	-34,5161	0,9463	-0,7485	5,3824	0,9535	16,4019	3,7868	0,9170

*(m= slope, a=intercept, R^2=determination coefficient).

As seen from the Figures 4.26, 4.27 and Tables 4.10, 4.11, depending on the high determination coefficient (R^2), the release kinetics of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged liposomal and niosomal formulations fitted to the first-order release kinetics.

For a better comparison, a cumulative release (%) graph was given in Figure 4.28 for both neutral and positively charged, nanosized, Pramipexole encapsulated, PEGylated liposomes and niosomes.

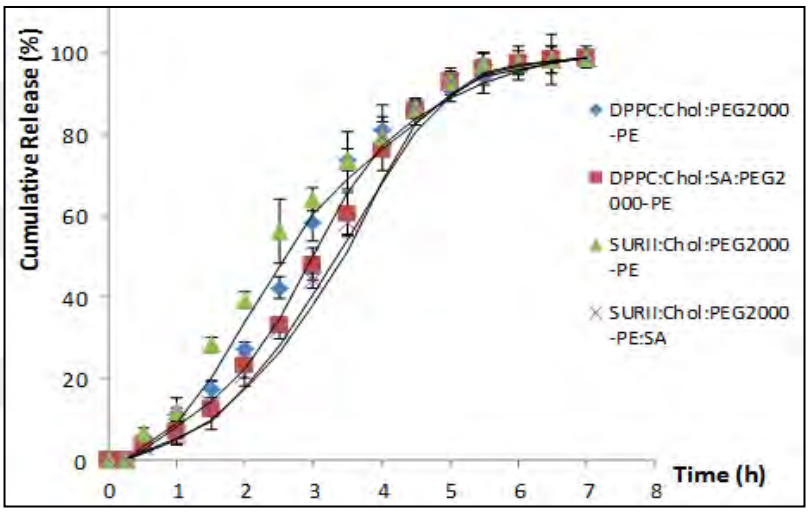


Figure 4.28. In vitro release of Pramipexole from neutral and positively charged liposomal and niosomal formulations in Tris (20 mM, pH 7.4) buffer (n=6).

Both neutral and positively charged liposomes and niosomes showed first-order release kinetics. Different charged liposome formulations containing DPPC as phospholipid showed slightly better in vitro Pramipexole release rate. They may be more efficient than niosome formulations however, this difference was found statistically insignificant ($p>0,05$).

4.5. In Vitro BBB Cell Co-Culture Studies

In vitro BBB penetration of nanosized, PEGylated, Pramipexole encapsulated neutral and positively charged liposomal and niosomal dispersions were carried out in BBB Cell Co-Culture model in Transwell® plate composed of astrocyte at the bottom and endothelial cells at the top part as given in Section 3.8. The fluorescence microscopy images were taken at 0 and 2 h after application of nanosized, Rhodamine labeled, Pramipexole encapsulated, neutral and positively charged liposome and niosome formulations on the Transwell® plates (Fig. 4.29).

As observed from microscopy images (Fig. 4.29), significantly high fluorescence signal was observed at cell cultures incubated with all formulations at 120 min designating BBB penetration at BBB Cell Co-Culture model. Especially, significantly higher fluorescence signal was obtained from the cells administered neutral, nanosized, Rhodamine labeled, Pramipexole encapsulated liposomes and niosomes at 120 min.

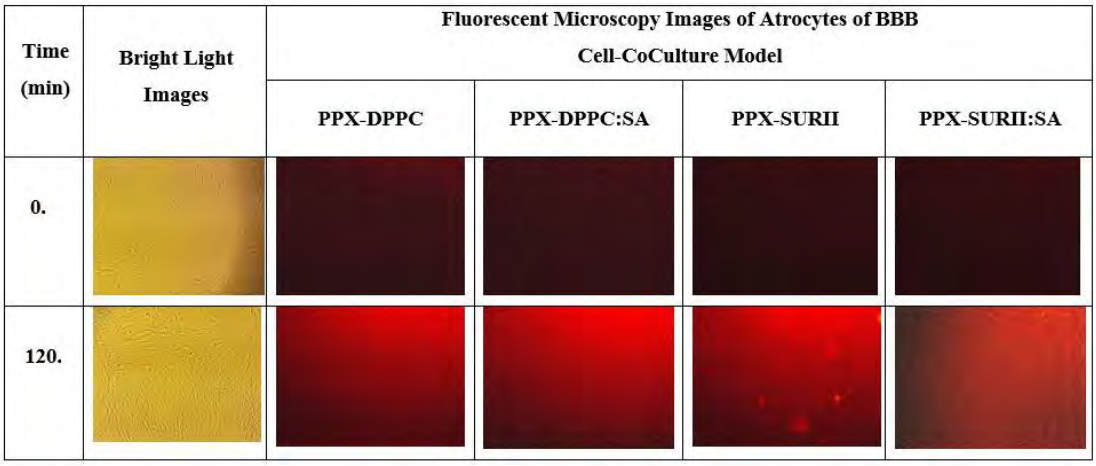


Figure 4.29. Bright light images and fluorescence microscopy images designating penetration of BBB Cell Co-Culture model of neutral and positively charged liposomes and niosomes at different time points (Magnification: x 40).

In order to observe the significance of penetration through BBB Cell Co-Culture model, releative fluorescence intensity was also measured after sampling at the bottom part of model to evaluate BBB penetration of nanosized, PEGylated, Pramipexole encapsulated neutral and positively charged liposomal and niosomal dispersions by spectrofluorometry at excitation=560 nm and emmission=583 nm wavelength at 0, 30, 60, 90, 120, 150 and 180 min (Figure 4.30).

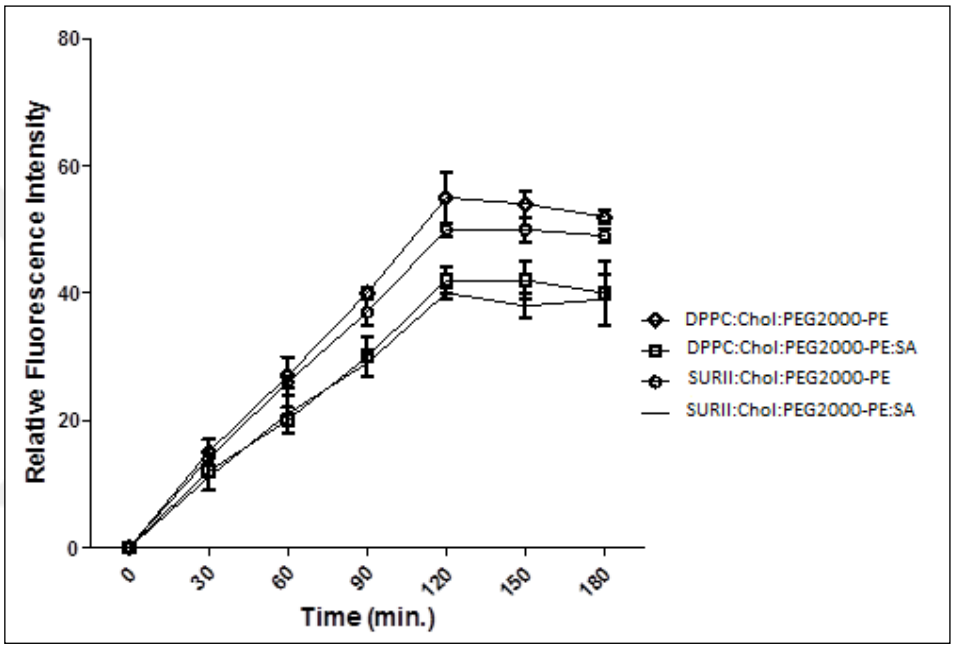


Figure 4.30. Relative fluorescence intesities designating penetration of BBB Cell Co-Culture model of neutral and positively charged liposomes and niosomes at different time points (n=6).

All formulations exhibited a significant increase in relative fluorescence intensity at 120 min. when compared with 30, 60 and 90 min. ($p<0,05$). Especially neutral, nanosized, Rhodamine labeled, Pramipexole encapsulated liposomes and niosomes exhibited a significant increase in relative fluorescence intensity at 120 min. when compared with 30, 60 and 90 min. ($p<0,05$) which designating BBB

penetration at BBB Cell Co-Culture model. No significant increase was observed in relative fluorescence intensity from 120 to 180 min. ($p>0,05$).

4.6. The Decision of Optimum Formulations for In Vivo Animal Studies

Due to the smaller mean particle size, proper zeta potential, larger encapsulation efficiency, higher phospholipid amount and better release kinetics, neutral liposomes and niosomes were selected as optimum formulations to apply to in vivo studies (Table 4.12.). Therefore, therapeutic efficacy of nanosized, Pramipexole encapsulated, neutral liposome and neutral niosomes were evaluated and monitored in PD model developed rats to compare with premipexole solution and Tris buffer.

Table 4.12. The mean particle size, polydispersity index and zeta potantial of optimum neutral liposomal and niosomal formulations.

FORMULATIONS [Molar Comp. (%)]	MEAN PART. SIZE (nm)	PDI	ZETA POTENTIAL (mV)	ENCAPS. EFF. of PRAMIPEXOLE (%)	PHOSPH. AMOUNT (µmol lipit.mL^{-1})	PHOSPH. EFF.(%)
DPPC:Chol: PEG2000-PE [10:5:1]	122 ± 1,3	0,12	-10,6 ± 0,9	9,42 ± 0,01	90,43 ± 2,13	91,6 ± 1,5
SURII:Chol: PEG2000-PE [10:5:1]	103 ± 1,2	0,13	-13,8 ± 0,7	10,51 ± 0,01	-	-

4.7. 6-OHDA Induced Unilateral Partial Striatal Lesion of Rats

24 rats were selected as succesful PD model after application of unilateral 6-OHDA due to the rats performed equal and more than 8 turns/min in rotometer test at 7 dpl.

4.8. Application of Liposomes and Niosomes

6-OHDA lesioned rats performing 8 or more ipsilateral turns/min were administered nanosized, PEGylated, Pramipexole encapsulated neutral liposomes (liposomes), nanosized, PEGylated, Pramipexole encapsulated neutral niosomes (niosomes), Pramipexole solution and Tris buffer (20mM, pH=7,4) as control group i.p. till 21 dpl. All formulations contain the same amount of Pramipexole (0,5 $mg.mL^{-1}$) to compare the efficacy.

Two rats were tried to administer nanosized, PEGylated, Pramipexole encapsulated neutral liposomes (liposomes) and nanosized, PEGylated, Pramipexole encapsulated neutral niosomes (niosomes) i.v. designated occlusion and inflammation at tail vein due to the chronic administration, so this group was removed from experimental protocol.

4.9. Monitoring of Therapeutic Efficacy

4.9.1. Rotometer Test

Therapeutic efficacy of Pramipexole encapsulated liposomes and niosomes were evaluated by the number of ipsilateral turns in rotometer test as given in Section 3.10.3.3, Section 3.10.3.4 and Section 3.13. The difference between rotometer test results for 6-OHDA lesion PD model rats i.p. administered nanosized, PEGylated, Pramipexole encapsulated neutral liposomes, niosomes, Pramipexole solution and Tris buffer (20mM, pH=7,4) as control group at 7 dpl, 14 dpl and 21 dpl after amphetamine sulphate injection (3 $mg.kg^{-1}$, i.p.) are given in Figure 4.31.

Regarding the delay after lesion, no difference in the number of ipsilateral turns was observed between 7, 14 and 21 dpl in the control and PPX groups. However, a slight increase was observed in Pramipexole encapsulated neutral liposome group at 21 dpl ($p<0.0421$ vs controls) and in Pramipexole encapsulated neutral niosome group at 14 and 21 dpl ($p<0.0362$ and 0.0459, respectively).

At 7 and 14 dpl, the number of turns was similar in 4 groups. However, at 21 dpl a slight but significant increase was observed in the Pramipexole encapsulated neutral niosome compared to the control goup ($p<0.0294$).

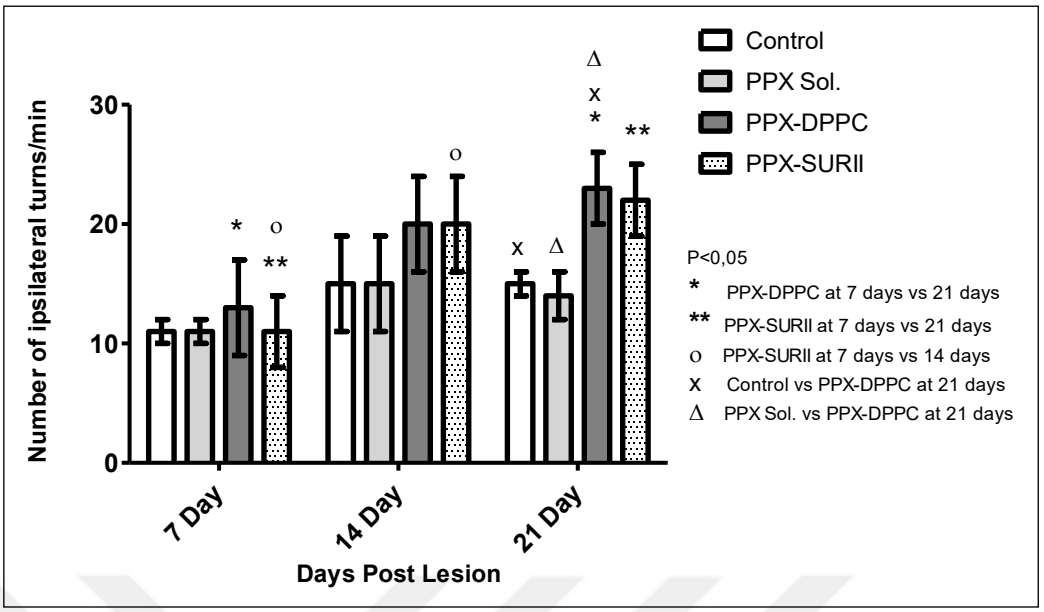


Figure 4.31. The mean ± SD values of number of ipsilateral turns in min. at 7, 14 and 21dpl after i.p. administration of nanosized, PPX-DPPC liposomes, PPX-SURII niosomes, pramipexole solution and control (n=6 in each group).

4.9.2. Autoradiography Studies

In order to observe the therapeutic effect of nanosized, pramipoexole encapsulated, liposomes and niosomes more accurately, DAT autoradiography was performed after a period of 21 days of therapy following sacrifice. DAT ^{125}I-PE2I autoradiography was performed as given in Section 3.14 to designate the radioactivity accumulation in both ipsilateral and intact part of the brain sections. The percent loss in DAT specific radioligand [^{125}I]PE2I binding values in the lesioned rats were calculated by comparing the lesioned to intact striatum.

Some of autoradiograms are given in Figure 4.32. representing the accumulation of radionuclide in striatum.

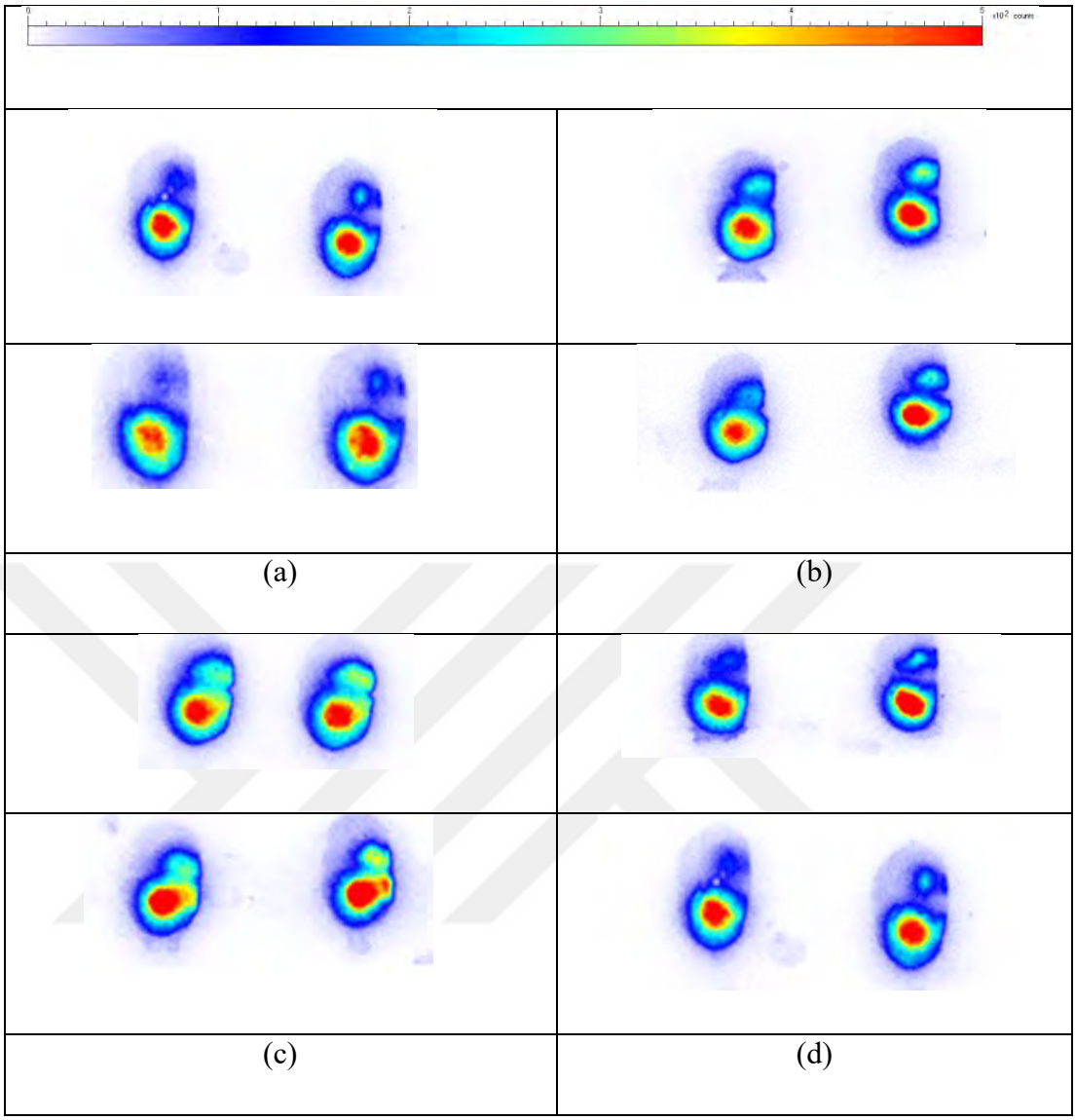


Figure 4.32. Autoradiograms of brain striatum of (a) Pramipexole encapsulated, PEGylated, neutral liposomes, (b) Pramipexole encapsulated, PEGylated, neutral niosomes, (c) Pramipexole solution and (d) Tris buffer (20 mM, pH:7,4) i.p. administered 6-OHDA lesioned rats (while upper parts representing ipsilateral (lesioned part) of the striatum, bottom parts represent controlateral parts of the striatum of the brain (control)).

The percent loss in the accumulation of DAT specific radioligand [^{125}I]PE2I in the lesioned part was calculated and results are given in Figure 4.33.

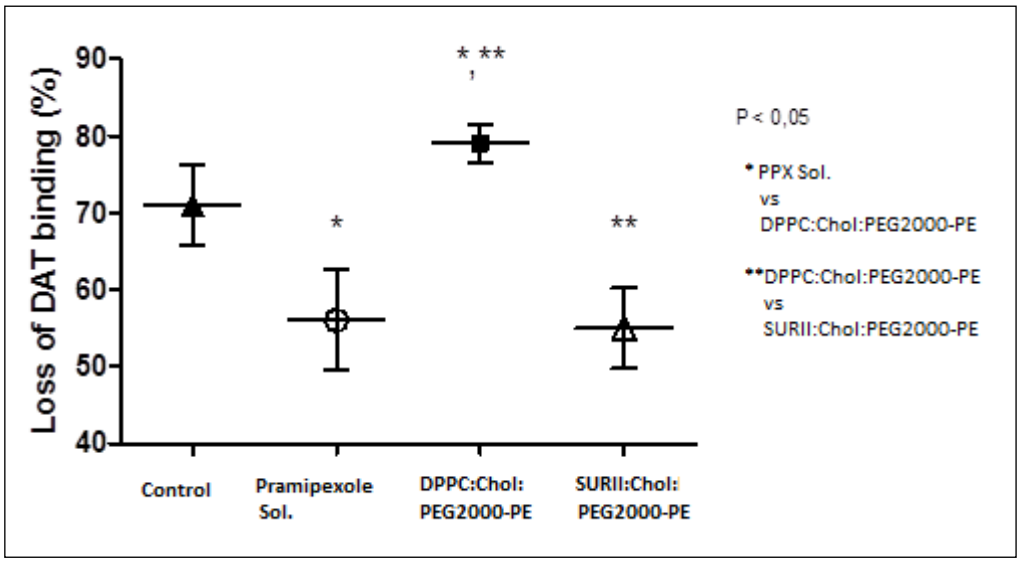


Figure 4.33. The percent loss in the radioactivity accumulation in the lesioned part of 6-OHDA partial lesioned PD model rats after i.p. administration of nanosized, Pramipexole encapsulated DPPC:Chol:PEG2000-PE liposomes, SURII:Chol:PEG2000-PE niosomes, Pramipexole solution and Tris buffer (control) (n=4).

The effect of formulations can be observed from the decrease in the loss of binding [(Non-lesioned part-lesioned part)/Non-lesioned part] (%) at the ipsilateral (lesioned) side. The percent loss of DAT binding was found signifcantly lesser with both neutral niosomes and Pramipexole solution than untreated group (control) and neutral liposomes ($p<0,05$) which attenuates pramipexole niosomes a tendency of potential therapeutic efficacy. Niosomes gave better results than Pramipexole solution ($p>0,05$). While the increase in the percent loss of accumulation in the lesioned part compared with the nonlesioned part was found higher after therapy with i.p. Pramipexole encapsulated DPPC:Chol:PEG2000-PE liposome administration, it was found lesser with SURII:Chol:PEG2000-PE niosome therapy ($p<0,05$). The therapy effect of both Pramipexole encapsulated SURII:Chol:PEG2000-PE and Pramipexole solution found similar in autoradiograms. Both SURII:Chol:PEG2000-PE and Pramipexole solution therapy gave better results than control Tris buffer (20 mM, pH:7,4) in autoradiography studies ($p<0,05$).

Becasuse the administration protocol of pramipexole tablets in clinics begins with three times in a day of 0,125 mg (0,375 $mg.day^{-1}$) premipexole during the first week then it can be increased till maximum 4,5 $mg.day^{-1}$ (305,306). The ascending dosage schedule of pramipexole tablets for Parkinson's Disease is given in Table 4.13 (305).

Table 4.13. Ascending Dosage Schedule of Pramipexole tablets for Parkinson's Disease (305).

Week	Dosage (mg)	Total Daily Dose (mg)
1	0.125 three times a day	0.375
2	0.25 three times a day	0.75
3	0.5 three times a day	1.50
4	0.75 three times a day	2.25
5	1 three times a day	3.0
6	1.25 three times a day	3.75
7	1.5 three times a day	4.50

The pramipexole dose which was given with pramipexole encapsulated, nanosized, PEGylated liposomes and niosomes is 0,043 $mg.day^{-1}$ (Briefly the encapsulated pramipexole dose of liposomes and nisomes are similar and about 0,5 $mg.mL^{-1}$. The pramipexole dose in pramipexole solution is the same as 0,5 $mg.mL^{-1}$. We administered rats 3 days a week about 0,2 mL of formulations each so the administered volume to a single rat is 0,6 $mL.week^{-1}$. The pramipexole dose administered to a single rat is 0,3 $mg.week^{-1}$ which corresponds to a dose of 0,043 mg. day^{-1}). This dose is approximately 9 times lesser than the minimum starting dose of conventional pramipexole tablets routinely used in Neurology clinics.

5. DISCUSSION

5.1. The Evaluation of Pramipexole Encapsulated, PEGylated, Liposome and Niosome Formulations

5.1.1. Selection of Active Ingredient

As active ingredient, Pramipexole dihydrochloride monohydrate was encapsulated in the core of both liposome and niosome dispersions as an antiparkinsonian agent. Pramipexole is a non-ergot derived D2 and D3 receptor agonist and it has a selective activity at dopamine receptors belonging to the D2 receptor subfamily (D2, D3, D4 receptor sub-types) with preferential affinity for D3 receptor subtype. Additionally, it is approved as monotherapy in early PD and as adjunctive therapy to levodopa in patients with advanced disease experiencing motor effects because of diminished response to levodopa. Initiation to a treatment protocol with a dopamine agonist is better for slowing down initiation of dyskinesia, wearing off. It appears advantageous to use a dopamine agonist such as Pramipexole in long-term treatment protocol as monotherapy or adjunctive therapy to levodopa for decreasing not only adverse effects but also treatment cost (307). Pramipexole can inhibit neurotoxicity of levodopa (308) via neuroprotection effect toward nigral dopamine neurons in vivo and results in protection of dopamine neurons from toxicity of methamphetamine (309), 6-OHDA, MPTP (310) and acute hypoxia or ischemia (57,309). Pramipexole was found effective in inhibiting free radical-mediated lipid peroxidation and protecting MPTP induced nigral dopaminergic injury (57,311). Although Pramipexole is used in clinics for PD therapy as either monotherapy or adjunctive therapy to L-DOPA currently its liposomal and niosomal formulations have never been studied before.

The reason of Pramipexole selection depends on its quite high solubility which is more than 20 % in water, about 8 % in methanol and 0.5 % in ethanol (265). Pramipexole dihydrochloride monohydrate is classified as BCSC1 substance

depending on high solubility and high permeability properties (312). This property is very useful for obtaining high encapsulation in liposomes and niosomes. Another reason of this choice is that there is no research about liposomal and niosomal formulations of Pramipexole. Only oral formulations are available in markets for PD therapy. Liposomal and niosomal formulations may be a potential alternative to obtain similar therapeutic effects with encapsulating lesser doses. Additionally, the prevalence of the observation of side effects with Pramipexole administration is very rare or none. It is mostly a very safe drug but it can cause some potential side effects in some patients like allergic reactions such as hives, dizziness or drowsiness, difficulty in breathing, swelling of the face, lips, tongue, or throat and sometimes nausea, sweating, feeling light-headed, fainting; hallucinations, muscle pain. But, these adverse reactions can be seen in a very limited population. Although pramipexole designates side effects very rarely, the observation of possible side effects may be significantly decreased by the administraion of lesser doses of pramipexole with liposomes and niosomes. Depending on its high water solubility it was successfully encapsulated in the aqueous core of PEGylated liposome and niosome dispersions for PD treatment and by this way it was aimed to reduce side effects when compared with Pramipexole solution which was routinely used in neurology clinics of hospitals. Depending on encapsulation in these systems it was aimed to enhance brain penetration. Another very significant property is to diminish dose administration frequency and to increase patient compliance depending on higher drug encapsulation in lipoosme and niosome vesicles.

A variety of studies were carried out in different formulations of Pramipexole. To evaluate the effect of Alzet minipumps implants, continuous release formulation of Pramipexole as s.c. injection was compared in rats (313). As another study, Pramipexole microsphere formulation was formulated by Wakode et al (314). Diffusion controlled, prolonged release about 24 h was obtained (314) which shows the effectiveness and usefullness of different formulations of pramipexole.

5.1.1.1. Tests on Pramipexole and Evaluation of UV Spectrum and Standart Line of Pramipexole

All the tests related with the properties of Pramipexole (as the salt of dihydrochloride monohydrate) was admitted depending on its analytical sertificate obtained from the drug company (Abdi Ibrahim Ilaç San.).

To evaluate the encapsulation efficiency of liposome and niosome formulations, the UV spectrum and standart line and line equations were calculated in both Tris buffer (20 mM, pH:7.4) and ethanol as given in Section 3.3.2.1 and Section 3.3.2.2, respectively. UV spectra of Pramipexole were obtained in Tris buffer (20 mM, pH 7.4) in the mean of 6 series as explained in Section 3.3.2.1 and λ_{max} was observed at 262 nm. UV spectrum in Tris buffer (20 mM, pH:7.4) is crutial in the determination of the released amount of Pramipexole from liposome and niosome vesicles which was evaluated by dialysis method. Analytical method validation was also performed to evaluate the reliability of the method of determination of the amount of the active ingredient within the way of aimed usage.

UV spectra of Pramipexole were obtained in ethanol as the mean of 6 series as explained in Section 3.3.2.2 and λ_{max} was observed at 262 nm. UV spectrum and standart line in ethanol is important to determine the encapsulation amount. While the determination of encapsulation efficieny (%) is significant in the determination of the characterization of liposomal and niosomal dispersions. The investigation of released amount is significant in the determination of the release profile of these drug delivery systems which may cause a significant advantage when compared with conventional drugs in PD treatment.

5.1.2. DRUG DELIVERY SYSTEM DESICION

Nanosized, Pramipexole encapsulated, PEGylated liposome and niosome formulations were formulated, characterized and evaluated for the therapeutic efficacy in PD treatment. The major problem of the use of conventional drugs in PD treatment is their lesser amount of drug penetration from BBB and reaches to the

brain. BBB is a barrier which is formed by blood vessel endothelial and is a dynamic block which can be affected by physical, humoral and neural stimuli. In brain blood vessel endothelium, there are tight junctions which prevents change of solid and aquoeous substance freely on the contrary to peripheral tissue endothelium. In the presence of BBB, more than 98% of the active ingredients can not penetrate into the brain. The penetration degree of BBB depends on oil dissolution, molecular weight, charge and the degree of binding to serum proteins. Another way of penetration into the BBB is the use of nanoparticles as drug carrier systems. Although nanotechnology is generally related with materials and devices that are smaller than 100 nm, the avarage radius of nanoparticles is in nanometer sizes such as about 100-400 nm. To obtain therapeutic effect in brain, dose is increased which it increases the frequency of side effects. Therefore, the solutions are searching for increasing drug amount without increasing drug dose. Nanosized drug delivery systems have important advantages in this issue (8).

The potential applications of nanotechnology to drug field comprises subjects such as improvements and potential applications of drug delivery systems and diagnostic devices and gene therapy. So many benefits are obtained by the application of nanotechnology to pharmaceutical field. Nanosystems that are used in nanomedicine comprises liposomes, niosomes, micelles, nanospheres, polymeric delivery systems, dendrimers, emulsions, nanoparticles and nanocapsules etc. Apart from other conventional drugs, the superiority of these drug delivery systems using benefits of nanotechnology basically depends on small particle size. Nanosized drugs can cause more rapid and efficient effect even in lower administered concentrations (8-11).

Among all drug delivery systems, liposomes and niosomes have been studied for many years depending on their different advantages due to the use as drug delivery system by Bangham et al in 1965 (99). Liposomes are synthetic analogous of natural membranes and composed of phospholipids. They are generally composed of one or more consantric vesicles containing lipid bilayers that are seperated by aqueous buffer compartments. Niosomes are nonionic surfactant vesicles in which hydrophobic and hydrophilic active pharmaceutical ingredients can be encapsulated

(14). The composition of niosomes are very similar to that of liposomes, however, the substances used for the preparation of niosomes, non-ionic surfactants, give them a more stable structure and more ability to penetrate through BBB.

Liposome and niosome dispersions were selected for delivery of Pramipexole to brain for the therapy of PD for some reasons such as;

a) Liposomal and niosomal systems of Pramipexole was never studied before,

b) Liposome and niosome dispersions are very safe, biocompatible, biodegredable and non-toxic and additionally liposomes increase bioavailability of drugs,

c) Due to the membrane properties of liposomes and niosomes, these drug delivery systems can be targeted to the desired tissue such as tumor and brain by altering surface properties (315-318),

d) Depending on the phospholipid structure of liposomes and surfactant structure of niosomes Pramipexole can be effectively delivered to the brain in which these causes flexibility to these systems for BBB penetration,

e) Non-ionic surfactant within the niosomes can facilitate BBB penetration depending on causing any disruption in BBB tight junction or endothelials,

f) Controlled release of Pramipexole can be achieved by encapsulating Pramipexole within liposome and niosome dispersions,

g) Higher Pramipexole dose can be delivered to the brain by liposomal and niosomal formulations without causing any side effects or lesser side effects which can be seen with the long term use of conventional oral dosage forms such as some motor complications like abnormal unintended movements and shortening response to each dose (wearing off phenomenon),

h) Dosing frequency can be diminished by the use of liposomal and niosomal formulations with prolonged and controlled drug delivery and by this way patient complience can be increased

i) An alternative delivery system of Pramipexole can be obtained other than oral delivery as routinely used in neurology clinics.

5.1.3. Selection and Physicochemical Controls on Other Ingredients within Liposome and Niosome Dispersions

5.1.3.1. The Selection of Phospholipid and Surfactant

It is significant to pay attention that phospholipids in **liposomes** and surfactants in **niosomes** should have a definite and proper phase transition temperature in which liposomes and niosomes formed vesicles by performing phase transition in a characteristic gel-liquid phase transition temperature.

Phase-transition temperature is a function of acyl chain in the structure of phospholipids in **liposomes** and the phase transition temperature designated a temperature increase about 15ºC by adding every methylene group at position 2. The presence of acidic phospholipid head groups within the structure such as unsaturated acyl chains, branching, phosphatidyl serine and phosphatidyl glycerole head groups may cause a decrease in phase transition temperature (121). This property gives liposomes to exert in vitro and in vivo behaviour preciously.

DPPC as gel state phospholipid was decided as phospholipid in the structure of liposome formulations. DPPC is selected which is a saturated synthetic phospholipid. The unsaturation within the structure was disappeared in gel state DPPC and by this way gel state phospholipids are more resistant to oxidation when compared with liquid-crystalline type phospholipids.

The phase transition temperature of phospholipid DPPC is about 41°C. The phase transition temperature of phospholipids is very significant because it was estimated that it has a crutial role in physicochemical properties of liposomes such as particle size and encapsulation efficiency and as a result of this it has an impact on its blood circulation time and in vivo targeting (267). The results obtained from characterization studies such as particle size, encapsulated amount and liposomal phospholipid amount were found very convinient for liposomes.

The same issue was also mentioned for **niosomes**. SUR II was selected as non-ionic surfactant for the formulation of nanosized, PEGylated, Pramipexole encapsulated niosomes. SUR II is a gel type surfactant with a phase transition temperature of 50,9 ºC. The HLB value of SUR II was found as 9,79 which

designated that SUR II has hydrophilic property. The phase transition temperature of non-ionic surfactant is very significant because similar to liposomes, it was estimated that it has a crutial role in physicochemical properties of niosomes such as particle size and encapsulation efficiency and as a result of this, it has an impact on its blood circulation time and in vivo targeting (267). The results obtained from characterization studies such as particle size, encapsulated amount and liposomal phospholipid amount were found very proper for the formulation of passively brain targeted nanosized, Pramipexole encapsulated, PEGylated niosomal formulations for the therapy of PD.

5.1.3.2. Addition of Cholesterol to the Formulation

Cholesterol is added to the drug delivery vesicular systems to optimize the permeability of the bilayer. While cholesterol contributes to a slide fluidity in the formulation of gel type vesicles, it mostly contributes to regulatory effect in the formulation of liquid type vesicles. Thus, cholesterol decreases the leakage of encapsulated drugs by decreasing the permeability of liquid type phospholipid bilayer and increasing the rigidity (267).

A variety of advantages was mentioned in the literature about the use of cholesterol in the preparation of vesicular systems. Cholesterol has very significant effect by decreasing of leakage of hydrophilic drug encapsulated inside the aquous core liposomes and increasing in vivo stability of liposomes by optimization of rigidity of lipid bilayer.

Plasma proteins give damage to the liposome integrity and membrane composition by conjugating with phosphatidylcholine within liposome composition. Cholesterol prevents interaction of high density lipoproteins with phospholipids. The composition of cholesterol is also very crutial for the formulation of liposomes and niosomes. It was observed that phase transition was disappeared completely when cholesterol ratio reaches about 33% (119,121).

Cholesterol was used in all nanosized, PEGylated, Pramipexole encapsulated liposome and niosome formulations in proper ratios due to the advantages of cholesterol which were given above.

5.1.3.3. Addition of PEG2000-DSPE

One approach to increase the blood circulation of pharmaceutical nanocarriers is the chemical modification with a hydrophilic polymer such as PEG. PEG is one of the mostly desired polymer, nowadays. An increase in the surface charge and hydrophilicity by PEGylation of nanoparticles can be achieved and by this way an increase in the repulsive interaction within the polymer coated nanocarrier and blood components can be obtained. By the effect of steric stabilization, rapid removal of liposomes from blood-circulation by opsonization with RES systems such as plasma proteins and macrophages can be achieved (142,319).

Anticancer drugs with low dissolution designated increased amount of accumulation in tumor tissue with EPR effect with angiogenesis and enhanced leakage from vessels to tissue by PEGylation (123,133,147).

Lukyanov et al (149) prepared micelles conjugated with PE attached PEG chains having different molecular length and after In-111 labeling they observed tumor accumulation of these PEG chains. As a result, different PEG-PE conjugates prepared with PEG blocks with a molecular weight of 750-5000 Da contribute to formulate very stable micelles having low critical micel concentration which designates high in vivo tumor accumulation.

Surface modification was performed by PEG2000-DSPE coating in all liposome and niosome fomulations comprising DPPC as phospholipid and SUR II as non-ionic surfactant, respectively. By this way, it was aimed passive tumor targeting by EPR effect in tumor tissue with preventing opsonization by RES cells and increasing blood circulation time. DSPE provides conjugation of PEG on liposome bilayer.

The molecular weight of PEG should be proper for surface coating and hydrolization to prevent or decrease RES uptake and rapid removal from blood circulation (274). PEG2000-DSPE was added both liposome and niosome dispersions in a proper amount.

5.1.3.4. Addition of Positive Surface Charge

Steraylamine (SA) is a positively charged ingredient to provide positively charged formulations. SA was added both liposomal an niosomal dispersions in a proper amount to evaluate both the characterization, release kinetics and passive targeting of liposome and niosome dispersions and compare its effect with non-charged (neutral) nanosized, Pramipexole encapsulated, PEGylated liposome and niosome dispersions.

Charge inducer ingredient provides to perform electrostatic interactons within vesicles and electrostatic charge potential on the vesicle surfaces. This may contribute to the physical stability of liposomal and niosomal formulations by increasing the distance within the vesicles. By this way, repulsion can be observed within different bilayers and the possibility of formation of aggregation may be disappeared (320,321).

5.1.3.5. Chelating Agent

In preliminary studies, the liposome and niosome dispersions were radiolabeled with Tc-99m as to perform SPECT imaging in vivo to evaluate both imaging and therapy of PD. DTPA-PE was used as chelating agent to attach Tc-99m on the surface of liposome and niosome formulations. For the purpose of incorporating radionuclide DTPA-lipid conjugate DTPA-PE was synthesized in our lab. as given in Section 2.5 with the method performed by Grant et al (273).

Radionuclides used for scintigraphic imaging such as Tc-99m, In-111 and contrast agents used for MRI such as Gadolinium (Gd) can be incorporated in the structure of liposomal vesicles with a two basic approaches up to now. As the first approach, contrast agent or radionuclide loaded DTPA was encapsulated inside the core of liposomes (275,322).

As an alternative approach, DTPA or a different chelating agent was modified with a hydrophilic group by a chemical process and conjugated at the surface of lipid bilayer of liposomal vesicles at the time of or after the preperation (323-325). One of the most commonly preferred hydrophilic group is phosphatidyl ethanolamine (PE). Chelating agent DTPA conjugates to the amino head group with –COOH group and by this way while PE was incorporated with the lipid bilayer, contrast material or

radionuclide was incorporated with DTPA (273,275). Besides PE, SA and stearylalcohol are also used for this purpose. However, depending on the neurotoxic effect of stearylamine (SA) and stearylalcohol, these molecules are not prefered. Although, DTPA is the most commonly used metal chelator agent nowadays, the most significant disadvantage is the limited loading capacity of metal groups. One metal group is loaded on the liposome surface on every chelating agent. DTPA-PE was synthesized and added to the composition of nanosized, Pramipexole encapsulated, PEGylated liposomes and niosomes to radiolabel with Tc-99m in preliminary studies for both imaging and therapy of PD. However, due to the reasons given above theragnostic systems were quited and passive targeted liposome and niosome dispersions were formulated and evaluated for the therapy of PD.

5.1.4. Physicochemical Controls on Ingredients

TLC, DSC and FTIR spectroscopy determination for phospholipid and surfactant within both liposome and niosome formulations gave important information about the composition of formulations. These analysis were performed as given in Section 3.4. These techniques depend on the identification and characterization of ingredients. Melting point determination was made for surfactant and cholesterol as given in Section 3.4.2.4 and Section 3.4.3.4.

TLC is a chromatography technique depends on the seperation of substances due to their polarity and non-polarity (326). By the use of TLC, compounds in a mixture can be identified and their purity can be determined (327). In case of TLC, R_f values of all the ingredients within the liposomal and niosomal formulations including phospholipid, cholesterol, PEG2000-DSPE were found very proper and compatible with the literature (271,275,294,295,303). Additionally, the synthesized chelating agent DTPA-PE used for the preliminary formulation studies was also found proper with the literature designating the success of the sythesizing process (273,275).

The Hydrophilic - Lipophilic Balance (HLB) value is very crutial for surfactants. HLB is the ratio of oil and water-soluble constituents of a non ionic surfactant. The HLB value can be used to predict the surfactant properties of a molecule or a formulation (268,269). While the lower the HLB, the more lipophilic

or oil soluble surfactant becomes, the higher the HLB, the more water soluble or hydrophilic surfactant becomes (328). The HLB value of SUR II was evaluated as given in section 2.4.2.1 and it was calculated as 9,79 which designated that SUR II has hydrophilic property. This value was found paralell with the literature (271,298,299). The melting temperature of SUR II was found as 45ºC. which found in accordance with the previous studies (301).

DSC is another identification technique to asess the purification of the ingredients. DSC is a thermoanalytical technique and as a mechanism of this technique the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. DSC can be used to measure a number of characteristic properties of a sample. Using this technique it is possible to observe fusion and crystallization events as well as glass transition temperatures (Tg) and phase trensition temperatures (Tm). DSC is also useful to study oxidation, as well as other chemical reactions to define the purification of substances (329-331). DSC thermograms of phospholipid (DPPC), non-ionic surfactant (SUR II), cholesterol and positive charge inducer (SA) were determined. Depending on the DSC thermograms and thermal analysis slopes, the phase transition temperatures of DPPC, SUR II were observed at about 41°C and 50,9ºC, respectively which were consistant with the literature (271,275,296,298,299). Dependig on the DSC thermograms of cholesterol and SA, the phase transition temperatures were obtained as 45°C and 78,2ºC, respectively. These results were observed compatible with the literature (271,275,298-300).

FTIR spectroscopy is another crutial chemical technique for the purpose of identifying the substances and their quality conrol. FTIR energy can be emitted or absorbed by molecules when they change their rotational-vibrational movements (332). FTIR spectroscopy is a simple and reliable technique widely used in both organic and inorganic chemistry, in research and industry. FTIR spectroscopy is a technique used to identify molecules by analysis of their constituent bonds. Each chemical bond in a molecule vibrates at a frequency characteristic of that bond. Its principle depends on the absorption spectroscopy and it can be used to identify and study chemicals (333). As seen from the FTIR spectra of DPPC, SUR II, cholesterol and SA, the tension vibrations and other bending and streching attitutes are found

proper with the structure of these substances and also found in accordance with the literature (271,275,297-299).

5.2. Preliminary Studies Related with the Formulation of Theragnostic Liposome and Niosome Dispersions

At the beginning of the research and before determination of the optimum formulation approach, several preliminary studies on formulations were performed as given in Section 3.5 after following an intensive literature survey about the studies related with BBB delivery of liposomal and niosomal dispersions about two years. At the beginning of the research, it was planned to formulate theragnostic drug delivery systems for both the diagnosis and therapy of PD, equally. Recent studies about theragnostics are very popular especially in the field of oncology due to the ability to provide therapy monitoring at the same time with therapy application (334-337). We intended to formulate theragnostic liposome and niosome formulations for both the diagnosis and therapy of PD.

As preliminary studises, it was tried to formulate premipexole encapsulated, PEGylated, nanosized theragnostic liposome and niosome formulations which were dopamine D2/D3 receptor or dopamine transporter (DAT) specific ligand modified to obtain active targeting and ^{99m}Tc radiolabeled systems to obtain SPECT molecular imaging and diagnosis in preliminary formulation studies. ^{99m}Tc radiolabeling was performed with the help of DTPA-PE attachment which its sythesis has been given in Section 3.5 (273,274). The synthesis of DTPA was evaluated with TLC detection and FTIR spectrum.

The synthesis, modification and anchoring of dopamine D2/D3 receptor or DAT specific ligand onto these liposomal and niosomal drug delivery systems which were thought and planned to perform at François Rabelais de Tours University, Tours/France. All the D2/D3 receptors and DAT radioligands which were given at Section 3.5 in Table 3.1 and Table 3.2 were studied and detected for the ability to modify and link to liposomal and niosomal systems.

It was evaluated by different valuable radiochemists and chemists in France, Turkey and Netherlands and it was concluded that the conjugation and binding of these radioligands to the drug delivery systems is very hard to perform. Even if it

could be possible, three dimentional structure of the radioligand could be changed after modification or in the organism which can cause significant loss in the affinity to the target site in the brain.

These expert scientists' opinion were in agreement with each other that the modification of ^{123}I-IBZM radioligand or other radioligands to liposomes and niosomes is mostly impossible with chemical synthesis methods. Because these drug delivery systems are very sensitive to the high temperature which may be applied within the synthesis process and also three dimentional structure of the radioligand will most probably change after the modification. Therefore, it has to be cancelled to prepare actively targeted theragnostic drug delivery sytems for both imaging and therapy of PD. Therefore we had to modify our aim and later it was aimed to prepare nanosized, PEGylated, Pramipexole encapsulated liposomal and niosomal drug delivery systems and observe their efficacy by rotational behavior tests and autoradiography as a radioactive monitoring method for therapeutic efficacy, solely.

5.3. Formulation Studies Related with Nanosized, Pramipexole Encapsulated, PEGylated, Liposome and Niosome Dispersions

Pramipexole encapsulated, nanosized, PEGylated neutral and positively charged liposome formulations were prepared according to the preparation method performed by Oku et al (276) according to the proper phospholipid compositions for brain penetration. As similar to liposomes, niosomes were also prepared according to the same procedure because niosomes are very similar to liposomes; the only difference is the non-ionic surfactant which is used in the formulation instead of phospholipid.

5.3.1. Characterization of Nanosized, Pramipexole Encapsulated, PEGylated, Liposomal and Niosomal Dispersions

In this section, the characterization of both neutral and positively charged, Pramipexole encapsulated, PEGylated liposome and niosome dispersions were evaluated as given in Section 3.6.1.2 and Section 3.6.2.2 and the results were discussed from particle size, zeta potential, encapsulation efficiency and liposomal phospholipid amount viewpoints.

- ***Particle Size and Size Distribution and Zeta Potentials***

The particle size and size distribution of liposome and niosome dispersions are very crutial for increasing blood-circulation time and especially in the case of diseases related with damaged vessel integrity to enhance drug delivery accumulation and targeting in the desired disease area by EPR effect such as tumor, brain infection and inflammation. The particle size and size distribution are affected from a variety of factors such as preparation method of liposomes and niosomes and bilayer composition, ingredients molar composition and the molecular weight of the encapsulated molecule (275).

Nanosized, PEGylated, Pramipexole encapsulated, neutral and positively charged liposome and niosome formulations were prepared according to the procedure given in Section 3.6.1.1 and Section 3.6.2.1 according to the preparation method performed by Oku et al (276) according to the proper ingredient compositions for brain penetration. Afterwards, liposome and niosome dispersions were extruded for the purpose of reducing and homogenizing the particle size. Extrusion method was used because this method is known and used efficiently to reduce particle size of vesicular delivery systems (275). The particle size, size distribution and zeta potentials of all liposomal and niosomal dispersions were performed as given in Section 3.6.1.2.1 and Section 3.6.2.2.1 and the results were given in Table 4.7. As seen from Table 4.7, all the formulations exhibited nanosized distribution of particle size which were about 100 nm in size. Although, there were some controversial opinions, drug delivery systems having a particle size about or near to 100 nm found very proper to penetrate from the membranes (8,9). As seen from Table 4.7, the particle size of neutral liposome and niosome formulations was found smaller than positively charged formulations. This difference was found statistically significant ($p<0.05$). This increase in the particle size may be related with the addition of positive charge to the liposomal and niosomal dispersions. When compared the particle size of neutral liposomes and niosomes, the particle size of neutral liposomes found larger than neutral niosomes, however, this difference was found statistically insignificant ($p>0.05$). The same situation was also valid for positively charged formulations. When compared the particle size of positively

charged liposomes and niosomes, the particle size of positively charged liposomes found larger than positively charged niosomes, however, this difference was statistically insignificant ($p>0.05$).

Particle size distribution can be observed by the value of polydispersity index (PI) in which small values are a clue of the homogenity of liposome and niosome dispersions. This value should be in between 0 and 1 for homogenous formulations. When the values in the Table 4.7 were evaluated, it was observed that all the PDI values are about 0,1 which is a very convinient and a small value designating the homogenity of liposome and niosome formulations.

When zeta potential of liposomal and niosomal formulations were compared, no statistical difference was observed within the neutral liposomes and niosomes ($p>0.05$) however, a statistically significant alteration was observed in zeta potential of positively charged liposomes and niosomes when compared with neutral liposomes and niosomes, respectively ($p<0.05$). The high absolute value of zeta potential shows a good stability of colloidal dispersions thus neutral liposomes and niosomes have a better zeta potential when compared with the positively charged ones. The mean particle sizes of all formulations are about nanosizes however, neutral liposomes and niosomes exhibited smaller particle sizes when compared with positively charged ones.

Due to the smaller particle size and proper, higher absolute values of zeta potential, neutral liposome and niosome dispersions were found convinient and optimum formulations were obtained.

- ***Encapsulation Efficiency of Liposome and Niosome Dispersions***

Encapsulation amount and encapsulation efficiency of Pramipexole are important to increase therapeutic efficacy for PD treatment by increasing dose amount reached in a single liposome or niosome vesicle.

Due to gel-state phospholipid structure of DPPC, it produces liposomes having high phase transition temperature and rigid phospholipid bilayer structure. This provides high encapsulation efficiency when compared with liposomes composed of liquid-state phospholipids (275).

The encapsulation efficiency of nanosized, PEGylated, Pramipexole encapsulated, neutral and positively charged liposomal and niosomal formulations was performed as given in Section 3.6.1.2.2 and Section 3.6.2.2.2 and the results were given in Table 4.8.

Niosome dispersions showed slightly higher encapsulated efficiency when compared with liposome dispersions, however, this difference was found statistically insignificant ($p>0.05$). No significant difference was observed in between neutral liposome and niosome formulations ($p>0.05$). However, a statistically significant increase was observed in the encapsulation of both neutral liposomes and niosomes when compared with positively charged ones ($p<0.05$). The decrease in the encapsulation efficiency may be related with deterioration of vesicle bilayers with the addition of positive charge (SA). Depending on the enhancement in the encapsulation efficieny, neutral liposome and niosome dispersions were found proper and optimum formulations were obtained.

- ***Liposomal Phospholipid Amount***

The effect of liposome preparation method to formulation efficiency can be determined with liposomal phospholipid amount. The phospholipid loss depends on extrusion process due to "*Onion Peeling Effect*" during the preparation process (271,338). Especially for the formulations encapsulating the lipophilic substances in phospholipid bilayer, liposomal phospholipid amount is a significant parameter and this should be controlled in characterization studies.

As mentioned before, liposomal and niosomal dispersions were extruded after preparation process to reduce particle size and obtaining homogenity. After a series of extrusion process, phospholipid loss is observed in liposomal preparations depending on removal of external lipid layer and the radius of vesicles will be decreased (275).

The liquid state liposomes were compared with the gel state lipsomes such as DPPC containing ones, it showed higher phospholipid loss after extrusion process due to lesser elasticity facilitating to penetrate through pores of membrane filters used in extrusion process under a certain pressure (99,271,338).

The phospholipid amount of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged liposomal formulations was determined with Rauser method as given in Section 3.6.1.2.3 and Figure 3.3 and the results were given in Table 4.9. The liposomal phospholipid loss in nanosized, Pramipexole encapsulated, positively charged DPPC:Chol:PEG2000-PE:SA liposomal formulation was observed higher when compared with nanosized, Pramipexole encapsulated, neutral DPPC:Chol:PEG2000-PE liposomal dispersions due to the preparation process, experimental conditions and addition of positive charge. However, this difference was found statistically insignificant ($p>0.05$). Due to the less phospholipid amount, neutral liposomes were selected as the optimum formulation for liposome dispersions.

5.3.2. Release Kinetics of Nanosized, Pramipexole Encapsulated, PEGylated Liposomal and Niosomal Dispersions

In this section, the characterization of both neutral and positively charged, Pramipexole encapsulated, PEGylated liposome and niosome dispersions were evaluated such as particle size, zeta potential, encapsulation efficiency and liposomal phospholipid amount.

- ***Release Kinetics of Nanosized, Pramipexole Encapsulated, PEGylated Liposomal Dispersions***

The release profile of Pramipexole encapsulated, nanosized, PEGylated liposomes was performed according to the dialysis method as given in Section 3.7.1 and the results related with the cumulative release (%) values and release kinetics of Pramipexole from liposome vesicles were given in Figure 4.26 and Table 4.10. Depending on higher determination coefficient (R^2), the release kinetics of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged liposomes exhibited first-order release kinetics. These results are compatible with the literature (294,339,340).

It was observed that liposomes containing phospholipids with high phase transition temperature like DPPC, generally form more-stable formulations showing

better performance in keeping of liposomal integrity, encapsulated drug amount and significant increase in drug-circulation life-times (341). A similar result was also observed by Bally et al. (342) with doxorubicin containing liposomes.

Both neutral and positively charged liposomes exhibited first-order release kinetics which is processed due to the concentration gradient (from high to lower concentration) and fits to Fick's law of diffusion. Neutral and positively charged, Pramipexole encapsulated, liposomes showed similar release profiles and the difference in between the formulations was found statistically insignificant ($p>0,05$). The release kinetics of liposomal formulations found in agreement with the literature (343). For first-order release kinetics, under constant conditions, the reaction rate is proportional with the concentration of the ingredients entering reaction medium and half-life is free from initial concentration. There is no need to energy and carrier. Reaction rate depends on the concentration of only one ingredient among other ingredients at first-order reactions (344).

- ***Release Kinetics of Nanosized, Pramipexole Encapsulated, PEGylated Niosomal Dispersions***

The release profile of Pramipexole encapsulated, nanosized, PEGylated niosomes was performed according to the dialysis method as given in Section 3.7.2 and the results related with the cumulative release (%) values and release kinetics of Pramipexole from niosome vesicles were given in Figure 4.27 and Table 4.11. Depending on higher determination coefficient (R^2), the release kinetics of nanosized, Pramipexole encapsulated, PEGylated, neutral and positively charged niosomes exhibited first-order release kinetics. Neutral and positively charged, Pramipexole encapsulated niosomes showed similar release profiles and the difference within the formulations was found statistically insignificant ($p>0,05$). The release kinetics of niosomal formulations were found in accordance with the literature (294,339,340).

Due to the confirmation criterion of release kinetics, both neutral and positively charged Pramipexole encapsulated niosomes exhibited first-order release kinetics. In vitro drug-release behavior from niosome dispersions has been found to be the

function of formulation variables, which led to different physical properties (345). Our results were found parallel with the literature (294,295,339). A similar result was also observed by Bally et al. (342) with doxorubicin containing liposomes.

It was observed from Figure 4.28 that both neutral and positively charged **liposomes** and **niosomes** showed first-order release kinetics. This depends on the concentration gradient (from high to lower concentration) and fits to Fick's law of diffusion. Neutral and positively charged, Pramipexole encapsulated, nanosized, PEGylated liposomes and niosomes exhibited similar release profiles which are in accordance with the literature (343).

5.4. In Vitro Cell Co-Culture Studies

A variety of in vitro models developed with the purpose of mimicking critical functionalities of BBB and to predict the permeability of drugs or drug delivery sytems into BBB (Fig. 4.29). However, the problem is the development and determination of a proper model retaining BBB characteristics and meeting on the studied issue of the drug delivery system (279).

Generally in vitro permeability assays are carried out in multi-well plates. In this system two compartments simulating the blood (apical) and brain (basolateral) sides are separated by a microporous filter. Cells are seeded on this filter to develop a cell monolayer contacting with different culture media in each compartment. The filters generally include the Transwell™ polycarbonate or polyethylene terephthalate inserts.

BBB cell culture model was developed after the first isolation of cerebral microvessels by growing CECs out of brain capillaries under adequate culture conditions (346). Several cell culture model has been studied to reconstitute BBB in vitro for evaluating the drug permeation rate across the tight layer of CECs from the blood into the brain. According to Tóth et al. (346), eight in vitro cell-based models of BBB were patented between 1990 and 2010. These systems incorporated a variety of cells of non-cerebral origin or CECs from primary cultures or cell lines, in monoculture, co-culture or triple co-culture configurations with other cells of the neurovascular unit.

Due to the cost-effectiveness, high throughput screening and high, versatility of in vitro culture models of BBB, it is desired to perform ferquently as a first step of in vivo experiments. However, due to the cell culture environmental conditions, it has some diversities when compared with in vivo conditions such as different expression of relevant cell biological transporters, ligands and enzymes (347) Up to date, no specific in vitro BBB model has been chosen as the ‘‘gold standard’’ by pharmaceutical industries. Therefore more reliable BBB models should be developed and amended with the purpose of displacing in vivo experiments (279,348).

BBB penetration of Rhodamine labeled, nanosized, PEGylated, Pramipexole encapsulated neutral and positively charged liposomal and niosomal dispersions was evaluated in BBB Cell Co-Culture model by inspection of fluorescent microscope images and measuring relative fluorescence intensity obtained by fluorospectroscopy as given in Section 3.8.2.

The increase in the fluoresecent intensity in fluorecent images was evaluated to penetrate better. While the fluorescence intensity was found higher in all formulations at 120 min, neutral liposomal and niosomal formulations showed higher fluorescence intensity when compared with positively charged ones.

The relative fluorescence intensities in all formulations exhibited an increase at 120 min. when compared with 30, 60 and 90 min. ($p<0,05$). No significant increase was observed in relative fluorescence intensity from 120 to 180 min. ($p>0,05$). The difference between relative intensities of liposomal and niosomal formulations was found insignificant ($p>0,05$). According to both fluorescent microscope images and relative fluorescence intensity obtained by fluorospectroscopy, it can be concluded that all formulations penetrate BBB Cell Co-Culture and maximum penetration was observed at 120 min.

5.5. The Selection of Optimum Liposome and Niosome Formulations for In Vivo Animal Studies

As given in Section 4.6, after evaluation of the mean particle size, zeta potential, encapsulation efficiency, liposomal phospholipid amount and release

kinetics of all formulations were evaluated. Pramipexole encapsulated neutral liposomes and niosomes were selected as optimum formulations. When liposomes and niosomes were compared depending on the charge, neutral ones were chosen as optimum formulations to carry out the in vivo studies. Smaller particle size, higher absolute value of zeta potential, larger encapsulation efficiency and better release kinetics were observed with neutral formulations when compared with positively charged ones. Additionally, it would also be better to continue in vivo studies with these neutral formulations depending on higher BBB penetration chance with the neutral ones when compared with charged particles. The common approaches to prevent RES uptake are formulating the particles with neutral surface charge, to coat their surface with different hydrophilic surfactants, such as polysorbates and PEG, and to use small size nanoparticles (e.g., <80 nm) (349,350). These properties attribute nanoparticles "stealth" effect avoiding RES escape and maintaining long circulation time and stability in blood to successfully target and cross the BBB (350,351).

Therefore, only optimum nanosized, neutral, Pramipexole encapsulated, PEGylated, liposome and niosome formulations were applied to animals to monitor therapeutic efficacy of Pramipexole encapsulated formulations.

5.6. 6-OHDA Induced Unilateral Partial Striatal Lesion of Rats

To perform early stage PD model in rats, partial unilateral 6-OHDA was administered to all rats to develop lesion as given in Section 3.10.2. **Partial lesion model** induces progressive and retrograde degeneration of dopaminergic neurons corresponding to an early symptomatic stage of PD (282,283). This model is relevant to test the therapeutic strategies, rather than other models such as intra-substantia nigra pars compacta (SNc) or intramedial forebrain bundle 6-OHDA lesions which generally cause a complete and immediate lesion that is not easily accessible to a therapeutic window (285).

Rats were selected due to the ipsilateral turns.min^{-1} at rotometer test at 7 dpl to apply therapy for the treatment of PD. Rats performing equal and more than 8 turns were selected for therapy.

It was reported that unilateral 6-OHDA rat model can be used widely in the assessment of the antiparkinsonian effects and neuroprotection of new pharmacological therapies as a preclinical model of PD (222-224). Rotometer test was used here for observing and evaluating the validity of 6-OHDA lesion administered PD model in rats after 7 dpl.

5.7. Monitoring and Evaluation of Therapeutic Efficacy of Liposomal and Niosomal Formulations

The therapeutic efficacy of Pramipexole encapsulated, nanosized, PEGylated, neutral liposome and niosome vesicles were evaluated by the rotometer test and autoradiography after therapeutic application as given in Section 3.13 and 3.14, respectively.

5.7.1. Rotometer Test

The rotational behaviour model can be used for observing the effect of new drugs for dopaminergic activity (352) and to further our understanding of basal ganglia function (286,353,354).

The rotometer test for 6-OHDA lesion PD model rats i.p. administered with nanosized, Pramipexole encapsulated neutral liposomes and niosomes, Pramipexole solution and Tris buffer (20mM, pH=7,4) as control group at 7 dpl, 14 dpl and 21 dpl after Amphetamine Sulphate injection (3 $mg.kg^{-1}$, i.p.) were performed as given in Section 3.13. Amphetamine is a CNS stimulant and psychostimulant drug. It stimulates the central nervous system (nerves and brain) by increasing the amount of certain chemicals in the body. This increases heart rate and blood pressure and decreases appetite, among other effects (355). It enhances the synaptic activity of three neurotransmitters - dopamine, serotonin and norepinephrine. Its effects are similar to those of cocaine (356).

The rotometer test results were given in Figure 4.31. The effect of formulations can be observed by measurement of mean numbers of ipsilateral turns/min.

Generally, a stable turn number was observed at 7, 14 and 21 dpl in control and PPX groups and a tendency to increase between 7 and 14 dpl in PPX-DPPC and

PPX-SURII and then stabilization was observed between 14 and 21 days. To compare between groups, a tendency of increase was observed at 14 and 21 dpl for both liposomes and niosomes vs control and PPX solution.

No statistically significant difference was seen within different days after therapy with either control or Pramipexole solution ($p>0,05$). An increase in ipsilateral turns for liposomes was observed at 21 dpl when compared with that of 7 dpl ($p<0,05$). Also, an increase in ipsilateral turns was observed for nanosized, Pramipexole encapsulated SURII:Chol:PEG2000-PE niosomes both at 14 and 21 dpl when compared with that of 7 dpl ($p<0,05$). The number of ipsilateral turns in min. was found higher at 21 dpl when compared with 7 dpl after i.p. administration therapy with nanosized, Pramipexole encapsulated DPPC:Chol:PEG2000-PE liposomes ($p<0,05$).

The therapy effect of neutral, Pramipexole encapsulated DPPC:Chol:PEG2000-PE liposome and SURII:Chol:PEG2000-PE niosome was observed very similar with Pramipexole solution by number of ipsilateral turns in min. It seems to be observed that insignificant results were achieved in rotameter study after administration of formulations. This insignificancy and similarity in rotometer test results in animal studies may be due to the nature of rotameter study. Rotameter test is probably less adapted for a partial lesion. When compared with autoradiography, rotameter test is less reliable and can be effected from different conditions such as small failures in sliding of amphetamin injection site and in performing 6-OHDA lesioned rats and light existance at experimental medium. Of course, the result of rotometer test is not very accurate by itself and should be supported with autoradiography studies which will be very precise to decide.

Similarly, a study performed by Papathanou et al in 2011 (357) related with the effect of different doses of L-DOPA and four dopamine agonists of different duration of action to induce Abnormal Involuntary Movements (AIMs) in 6-OHDA-lesioned rats and their ability to express established AIMs following prior exposure to L-DOPA. 6-OHDA-lesioned rats were treated with saline, L-DOPA/benserazide, apomorphine, ropinirole, Pramipexole or pergolide once daily for 15 days. In part of this study, different doses of drugs were administered to observe rotations after

Amphetamin Sulfate Injection. As supporting our rotational movement results, Papthanou et al observed that administration of increased dose of Pramipexole caused an increase in rotations. Another study supporting a similar situation of therapy with liposomal formulations not for PD but for epilepsy was performed in 2014 by Holtman et al (358). It was aimed to provide the delivery of glucocorticoids (effective in inflammation inhibition in epileptogenesis) better to the brain and decrease its severe adverse effects related with long-term glucocorticoid treatment by developing nanosized, PEGylated, glucocorticoid (10 $mg.kg^{-1}$ dose) encapsulated liposomal formulations. However, no therapy and no reduction in microglia activation were obtained with glucocorticoid liposomes after 6 weeks of therapy in post-status epilepticus rat model for temporal lobe epilepsy. According to a tudy performed by Arica et al (359), carbidoba/levadopa loaded microspheres were formulated for intracerebral treatment of PD. The efficiency of these microspheres was evaluated by altering of apomorphine-induced rotational behavior in 6-OHDA unilaterally lesioned rat model. carbidoba/levadopa loaded microspheres exhibited lower rotation scores than blank microspheres at 7dpl.

A study about observing the effect of L-DOPA and dopamine agonists such as SKF38393, Quinpirole and Pramipexole on dopamine neuron degeneration was performed by Jeon et al in 2007 (360) in the progressive hemiparkinsonian rat models with 6-OHDA administration. Behaviour tests with forepaw adjusting step test and TH-immunohistochemical staining after 9 weeks of i.p. administration showed that only high dose L-DOPA (100 $mg.kg^{-1}.d^{-1}$) decreased dopaminergic neurons significantly. They also did not observe any protective effect with dopamine agonists.

5.7.2. Autoradiography Studies

In order to observe the therapeutic effect of nanosized, pramipoexole encapsulated, liposomes and niosomes more accurately, DAT autoradiography was performed after a period of 21 days of therapy following sacrification. The autoradiography studies were performed according to the process given in Section 3.14 in order to determine the radioactivity accumulation in both ipsilateral and

intact part of the brain sections. To determine the efficacy of the Pramipexole encapsulated liposomes and niosomes more accurately and to compare their efficacy with Pramipexole solution and Tris as control, autoradiography was performed in PD developed rats at 21 dpl after decapitation for the purpose of evaluating the percent loss of the binding part values of the brain. The autoradiograms are given in Figure 4.32. The increase in the therapeutic efficacy of antiparkinsonian effect of formulations can be observed from the decrease in the loss of binding [(Non-lesioned part-lesioned part)/Non-lesioned part] (%) at the ipsilateral side. The percent loss in the radioactivity accumulation in the lesioned part of PD model rats after i.p. administration of nanosized liposomes, niosomes, Pramipexole solution and control is given in Figure 4.33.

The therapy effect of both Pramipexole encapsulated SURII:Chol:PEG2000-PE and Pramipexole solution was found similar in autoradiograms. The percent loss of DAT binding [(Non-lesioned part-lesioned part)/Non-lesioned part] (%) was found lesser with both Pramipexole encapsulated SURII:Chol:PEG2000-PE niosomes and Pramipexole solution than neutral DPPC:Chol:PEG2000-PE liposomes ($p<0,05$). Niosomes gave slightly better results than Pramipexole solution ($p>0,05$). Both, Pramipexole encapsulated SURII:Chol:PEG2000-PE and Pramipexole solution therapy gave better results than control Tris buffer (20 mM, pH:7,4) in autoradiography studies which shows that SURII:Chol:PEG2000-PE niosomes have significant PD therapeutic effect even ($p<0,05$).

With i.p. administration of nanosized, PEGylated, pramipexole encapsulated, neutral niosomes similar but slightly better therapeutic effect was achieved with pramipexole solution at same doses in 6-OHDA lesioned rats. If this dose is compared with conventional pramipexole tablets used for humans in Neurology clinics, we achieved beneficial therapeutic effect at approximately 9 times lesser doses. Becasuse the administration protocol of pramipexole tablets in clinics is three times of 0,125 $mg.day^{-1}$ during the first week then then it can be increased till maximum 4,5 $mg.day^{-1}$ (305,306). As mentioned before, the pramipexole dose which was given with pramipexole encapsulated, nanosized, PEGylated liposomes and niosomes is 0,043 $mg.day^{-1}$ in our study. This dose is

approximately 9 times lesser dose given with minimum dosed conventional pramipexole tablets. Nanosized, PEGylated, neutral, pramipexole encapsulated niosomes found beneficial, effective in PD therapy, BBB permeable even in 9 times lesser doses used in Neurology clinics.

6. CONCLUSION

CNS disorders are one of the first ordered disease to endorse their research in the diagnosis and therapy with several framework projects in Europe and all around the World. The huge gap in the issue of efficient CNS drug delivery and the success of PD therapy needed to be researched and investigated. For the therapy of PD, there is still a long way to go through. As being the first study in the literature, liposomal and niosomal formulations of Pramipexole have never been studied before.

The characterization of both neutral and positively charged, nanosized, Pramipexole encapsulated, PEGylated liposome and niosome formulations was found proper. Pramipexole encapsulated all formulations (DPPC:Chol:PEG2000-PE, DPPC:Chol:PEG2000-PE:SA, SURII:Chol:PEG2000-PE, SURII:Chol:PEG2000-PE:SA) exhibited nanosize and proper zeta potential. All formulations designated large encapsulation efficiency for Pramipexole (about 10%). Both, neutral and positively charged liposomes showed proper phospholipid content showing the quality of liposome formulations. Additionally, all formulations fitted to first-order release kinetics. BBB penetration of neutral liposome and niosome formulations was found better than positively charged ones at in vitro BBB Cell Co-Culture studies. However, this difference was found statistically insignificant.

Although the therapeutic efficacy of PPX-DPPC and PPX-SURII was found insignificant in rotameter test, nanosized, neutral PPX-SURII niosome designated similar even better effect than pramipexole solution in autoradiograhy studies in 6-OHDA lesioned rats. This pramipexole dose is approximately 9 times lesser doses applied with conventional pramipexole tablets for humans in Neurology clinics. Therefore we achieved a beneficial therapeutic effect at significantly lesser doses with nanaosized, neutral niosomes. It is most probably depend on the non-ionic surfactant properties of nisomes which can enhance BBB penetration properties. This beneficial effect may probably help reduction in possible side effects and prevent observation of drug resistance and adverse effects at PD patients. It will be very beneficial to try nanosized, pramipexole encapsulated, neutral liposomes in a large number of 6-OHDA lesioned animals to evaluate antiparkinsonian effect better. These results can also enlighten the further human studies for therapy of PD in the

future. Future studies may lead in developing commercial preparations for neurology clinics for effective therapy of PD with decreased side effects and frequency of administration which is very significant for patient's compliance to the therapy.

REFERENCES

1. Ronken, E., Van Scharrenburg, G. J. M. (2002). *Parkinson's disease.* Amsterdam: IOS Press
2. Milliyet, pembenar, Parkinsona hareketle direnin. (t.y.). Erişim: 01.12.2015 http://www.Milliyet.Com.Tr/parkinsona-hareketle-direnin--pembenar-detay-genelsaglik-1864976/
3. Spuch, C.,Navarro, C. (2011). Liposomes for targeted delivery of active agents against neurodegenerative diseases (alzheimer's disease and parkinson's disease). *Journal of Drug Delivery*, 1-12.
4. Modi, G., Pillay, V.,Choonara, Y. E. (2010). Advances in the treatment of neurodegenerative disorders employing nanotechnology. *Annals of the New York Academy of Sciences,* 1184, 154-172.
5. Modi, G., Pillay, V., Choonara, Y. E., Ndesendo, V. M., du Toit, L. C.,Naidoo, D. (2009). Nanotechnological applications for the treatment of neurodegenerative disorders. *Progress in Neurobiology*, 88 (4), 272-285.
6. Clarke, C. E., Speller, J. M.,Clarke, J. A. (2000). Pramipexole for levodopa-induced complications in parkinson's disease. *Cochrane Database Systematic Reviews* (3), 1-23.
7. Piercey, M. F. (1998). Pharmacology of pramipexole, a dopamine d3-preferring agonist useful in treating parkinson's disease. *Clinical Neuropharmacology*, 21 (3), 141-151.
8. Park, K. (2007). Nanotechnology: What it can do for drug delivery. *Journal of Controlled Release*, 120, 1-3.
9. Mamalis, A. G. (2007) Recent advances in nanotechnology. *Journal of Materials Processing Technology*, 181, 52-58.
10. Garnett, M. C.,Kallinteri, P. (2006). Nanomedicines and nanotoxicology: Some physiological principles. *Occupational Medicine*, 56 (5), 307-311.
11. Chan, V. S. (2006). Nanomedicine: An unresolved regulatory issue. *Regulatory Toxicology and Pharmacology*, 46 (3), 218-224.
12. Samad, A., Sultana, Y., Aqil, M. (2007). Liposomal drug delivery systems: An update review. *Current Drug Delivery*, 4 (4), 297-305.

13. Vyas, S. P., Sihorkar, V. (2001). S. P. Vyas, Dixit, V. (Ed.). *Advances in liposomal therapeutics* (s. 230). New Delhi: CBS Publishers
14. Rajera, R., Nagpal, K., Singh, S. K.,Mishra, D. N. (2011). Niosomes: A controlled and novel drug delivery system. *Biological and Pharmaceutical Bulletin*, 34 (7), 945-953.
15. Rose, J. S., Neal, J. M.,Kopacz, D. J. (2005). Extended-duration analgesia: Update on microspheres and liposomes. *Regional Anesthesia and Pain Medicine*, 30 (3), 275-285.
16. Illum, L., Davis, S. S. (1984). The organ uptake of intravenously administered colloidal particles can be altered using a non-ionic surfactant (poloxamer 338). *FEBS Letters*, 167 (1), 79-82.
17. Gabizon, A., Goren, D., Horowitz, A.T., Tzemach, D., Lossos, A., Siegal, T. (1997). Long-circulating liposomes for drug delivery in cancer therapy: A review of biodistribution studies in tumor-bearing animals. *Advanced Drug Delivery Reviews*, 24, 337-344.
18. Allen, T. M., Hansen, C.B., Lopez de Menzes, D.E. (1995). Pharmacokinetics of long-ciculating liposomes. *Advanced Drug Delivery Reviews*, 16, 267-284.
19. Haselbarth, VF., Justus-Obenauer, H., Peil, H.,et al (1994). Pharmacokinectics and bioavailability of pramipexole: Comparison of plasma levels after intravenous and oral administration in healthy volunteers (M/2730/0029). (Rapor No: Upjohn Technical Report 7215-94-016).
20. Holloway, RG., Shoulson, I., Fahn. S., Kieburtz, K., Lang, A., Marek, K., et al. (2004). Pramipexole vs levodopa as initial treatment for parkinson disease: A 4-year randomized controlled trial. *Archives of Neurology,* 61, 1044-1053.
21. Boehringer ingelheim (2009) boehringer ingelheim receives approval from european commission for mirapexin®/sifrol® prolonged-release, once daily tablet for treatment of parkinson's disease. (t.y.) Erişim: 13 Apr 2014 http://multivu.Prnewswire.Com/mnr/boehringeringelheim/39700/
22. Mizuno, Y., Yamamoto, M., Kuno, S., Hasegawa, K., Hattori, N., Kagimura, T. et al. (2012). Efficacy and safety of extended-versus immediate-release pramipexole in japanese patients with advanced and l-dopa under treated

parkinson disease: A double-blind, randomized trial. *Clinical Neuropharmacology,* 35, 174-181.

23. Jain, N. K., Rana, A. C., Jain, S. K. (1998). Brain drug delivery system bearing dopamine hydrochloride for effective management of parkinsonism. *Drug Development and Industrial Pharmacy*, 24 (7), 671-675.
24. Amicarelli, F., Gasbarri, A., Masciocco, L., Pompili, A., Pacitti, C., Carlucci, G. et al. (1999). The effect of intrastriatal injection of liposome-entrapped tyrosinase on the dopamine levels in the rat brain. *Cellular and Molecular Biology*, 45 (7), 1093-1097.
25. Shimoji, M., Zhang, L., Mandir, A. S., Dawson, V. L., Dawson, T. M. (2005). Absence of inclusion body formation in the mptp mouse model of parkinson's disease. *Molecular Brain Research*, 134 (1), 103-108.
26. Dick, F. D., De Palma, G., Ahmadi, A., Scott, N. W., Prescott, G. J., Bennett, J. et al. (2007). Environmental risk factors for parkinson's disease and parkinsonism: The geoparkinson study. *Occupational Environmental Medicine*, 64 (10), 666-672.
27. Kurosinski, P. G. M., Götz, J. (2000). Alzheimer's and parkinson's disease-overlapping or synergistic pathologies? *TRENDS in Molecular Medicine*, 8, 41-43.
28. Shafique, H. B. A., Chung, A., Logendrarajah, R. (2011). Causes of parkinson's disease: Literature review. *Journal of Parkinsonism & Restless Legs Syndrome*, 1, 5-7.
29. Yamaguchi, H., Kajitani, K., Dan, Y., Furuichi, M., Ohno, M., Sakumi, K. et al. (2006). Mth1, an oxidized purine nucleoside triphosphatase, protects the dopamine neurons from oxidative damage in nucleic acids caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Cell Death and Differentiation*, 13 (4), 551-563.
30. Speed, N. K. (2010). The role of insulin signaling on dopamine transporter trafficking. PhD Thesis, Faculty of the Graduate School of Vanderbilt University, Tennessee.
31. Tyrosine Erişim: 04 April 2015
http://www.Smartnootropics.Co.Uk/tyrosine/

32. Standaert, D.G., Roberson E.D.(2011). Treatment of central nervous system degenerative disorders. L.L. Brunton, B.A. Chabner , B.C. Knolman, (Ed.). *Goodman and gilman's the pharmacological basis of therapeutics* (Chapter 22). New York: McGraw-Hill
33. Cnsforum.Com. (t.y.). Erişim: 10 May 2015 https://www.cnsforum.com/
34. Weiner, R. I., Ganong, W. F. (1978). Role of brain monoamines and histamine in regulation of anterior pituitary secretion. *Physiological Reviews*, 58(4), 905-976.
35. Barbeau, A. (1962). The pathogenesis of parkinson's disease: A new hypothesis. *Canadian Medical Association Journal*, 87, 802-807.
36. Volkow, N. D., Wang, G. J. et al. (2002). Nonhedonic food motivation in humans involves dopamine in the dorsal striatum and methylphenidate amplifies this effect. *Synapse,* 44(3), 175-180.
37. New York Times Health Guides Parkinsons disease. (t.y.). Erişim: 12 November 2015 http://www.Nytimes.Com/health/guides/disease/parkinsons-disease/print.Html
38. Giros, B., Caron, M. G. (1993). Molecular characterization of the dopamine transporter. *Trends in Pharmacological Sciences,* 14(2), 43-49.
39. Singhal V. Parkinson's disease 2016. Erişim: 10 May 2015 http://www.Healthiply.In/mental-health/stress-and-depression/parkinsons-disease/
40. Pollock, M., Hornabrook, R. W. (1966). The prevalence, natural history and dementia of parkinson's disease. *Brain*, 89 (3), 429-448.
41. Lemke, M. R. (2008). Depressive symptoms in parkinson's disease. *European Journal of Neurology*, Suppl 1, 21-25.
42. Starkstein, S. E. (2012). Apathy in parkinson's disease: Diagnostic and etiological dilemmas. *Movement Disorders*, 27 (2), 174-178.
43. Rana, MA., Hafez, K. (2012). Depression in parkinson's disease. *Journal of Parkinsonism & Restless Legs Syndrome*, 2, 34-35.

44. Hely, M.A., Morris, J., Reid W.G., Trafficante, R. (2005). Sydney multicentre disease of pd: Non doparesponsive problems dominate dominate at least 15 years. *Movement Disorders,* 20, 1990-1995.
45. Ansari, A. Z., Joshi. D., Mishra, V. N., Chaurasia, R. N., Gupta, S., Kumar, B., et al. (2012). Clinical & neuropsychological profile in patients with parkinson's disease and parkinson's plus syndromes: Study from a tertiary care referral centre in a developing country. *Journal of Parkinsonism & Restless Legs Syndrome*, 2, 11-14.
46. Rana, M. A., Al-Shehri, M. (2012). Postural hypotension in parkinson's disease. *Journal of Parkinsonism & Restless Legs Syndrome*, 2, 32-33.
47. Bejjani, B.P., Damier, P., Arnulf, I., Papadopoulos, S., Bonnet, A.M., Vidailhet, M., et al. (1998). Deep brain stimulation in parkinson's disease: Opposite effects of stimulation in the pallidum. *Movement Disorders*, 13, 969-970.
48. Pouratian, N., Thakkar, S., Kim, W., Bronstein, J. M. (2012). Deep brain stimulation for the treatment of parkinson's disease: Efficacy and safety. *Degenerative Neurological and Neuromuscular Disiease*, 2012 (2), 107-117
49. Poewe, W., Antonini, A., Zijlmans, J. C., Burkhard, P. R., Vingerhoets, F. (2010). Levodopa in the treatment of parkinson's disease: An old drug still going strong. *Clinical Interventions in Aging*, 5, 229-238.
50. Aviles-Olmos, I., Martínez-Fernández, R., Foltynie, T. (2010). L-dopa-induced dyskinesias in parkinson's disease. *European Journal of Neurology,* 2, 91-100.
51. Learn Pharmacia's Blog. (t.y.). Erişim:14 December 2015 http://english-learnpharmacia.Blogspot.Fr/2011/09/medication-choice-in-parkinsons-disease.Html
52. Smith, K., Riche, D. M., Henyan N. (2010). Clinical drug data 11th edition, : McGraw Hill Professional.
53. Greenacre, J.K., Coxon, A., Petrie, A., Reid, J.L. (1976). Comparison of levodopa with carbidopa or benserazide in parkinsonism. *Lancet*, 7982, 381-384.

54. Hauser R.A. Parkinson's disease. (t.y.). Erişim: 20 June 2011 http://emedicine.Medscape.Com/article/1831191-overview
55. Zigmond, M.J., Burke, R.E. (2002). Pathophysiology Of Parkinson's Disease. Davis, K.L., Charney, D., Coyle J.T., Nemeroff, C. (Ed.). *Neuropsychopharmacology: The fifth generation of progress* (s. 1781-1793) Philadelphia, Lippincott.
56. Mirapex®, *PDR (2005)*, (s. 1434). Montvale: Thomson PDR,
57. Silindir, M., Ozer, A. Y. (2014). The benefits of pramipexole selection in the treatment of parkinson's disease. *Neurological Sci*ences, 35 (10), 1505-1511.
58. Ema science medicines health, ema/816841/2011, chmp, london. Erişim: 13 April 2014 http://www.Ema.Europa.Eu/docs/en_gb/document_library/epar_-_public_assessment_report/human/002291/wc500116760.Pdf
59. Bennett, J.P., Piercey, M.F. (1999). Pramipexole-a new dopamine agonist for treatment of parkinson's disease. *Journal of Neurological Sciences,* 163, 25-31.
60. Mirapex. (t.y.). Erişim: 7 November 2015 http://americareoncall.Com/yahoo_site_admin/assets/docs/mirapex.101155820.Pdf
61. Pelvig, D. P., Pakkenberg, H., Stark, A. K., Pakkenberg, B. (2008). Neocortical glial cell numbers in human brains. *Neurobiology of Aging*, 29 (11), 1754-1762.
62. WebMD Brain & Nervous System Health Center. (t.y.). Erişim: 13 December 2015 http://www.Webmd.Com/brain/picture-of-the-brain
63. Brightman, M. W., Reese, T.S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *The Journal of Cell Biology*, 40, 648-677.
64. Pardridge, W. M. (1997). Drug delivery to the brain. *Journal of Cerebral Blood Flow Metabolism*, 17, 713-731.

65. Deeken, J. F., Loscher, W. (2007) The blood-brain barrier and cancer: Transporters, treatment, and trojan horses. *Clinical Cancer Research*, 13 (6), 1663-1674.
66. Denora, N., Trapani, A., Laquintana, V., Lopedota, A., Trapani, G. (2009). Recent advances in medicinal chemistry and pharmaceutical technology-strategies for drug delivery to the brain. *Current Topics in Medicinal Chemistry*, 9 (2), 182-196.
67. Provenzale, J. M., Mukundan, S., Dewhirst, M. (2005). The role of blood-brain barrier permeability in brain tumor imaging and therapeutics. *American Journal of Roentgenology*, 185 (3), 763-767.
68. Pardridge, W. M. (1998). Introduction to the Blood-Brain Barrier. W. M. Pardridge (Ed.). *Introduction to the Blood-Brain Barrier Methodology, biology and pathology*. (s. 1-8), Cambridge: University Press
69. Mayhan, W. G. (2001). Regulation of blood-brain barrier permeability. *Microcirculation*, 8 (2), 89-104.
70. Begley, D. J. (1996). The blood-brain barrier: Principles for targeting peptides and drugs to the central nervous system. *Journal of Pharmacy and Pharmacology*, 48 (2), 136-146.
71. Tamai, I., Tsuji, A. (2000). Transporter-mediated permeation of drugs across the blood-brain barrier. *Journal of Pharmaceutical Sciences*, 89 (11), 1371-1388.
72. Fromm, M. F. (2000). P-glycoprotein: A defense mechanism limiting oral bioavailability and cns accumulation of drugs. *International Journal of Clinical Pharmacology and Therapeutics*, 38 (2), 69-74.
73. Kabanov, A. V., Batrakova, E. V. (2004). New technologies for drug delivery across the blood brain barrier. *Current Pharmaceutical Design*, 10 (12), 1355-1363.
74. Pardridge, W. M. (2007). Blood-brain barrier delivery. *Drug Discovery Today*, 12, 54-61.
75. Alam, M. I., Beg, S., Samad, A., Baboota, S., Kohli, K., Ali, J., et al. (2010). Strategy for effective brain drug delivery. *European Journal of Pharmaceutical Sciences*, 40, 385-403.

76. Pasha, S., Gupta, K. (2010). Various drug delivery approaches to the central nervous system. *Expert Opinion Drug Delivery*, 7, 113-135.
77. Pardridge, W. M. (1993). Brain drug delivery and blood-brain barrier transport. *Drug Delivery*, 83-101.
78. Gabathuler, R. (2009). Blood-brain barrier transport of drugs for the treatment of brain diseases. *CNS &Neurological Disorders-Drug Targets*, 8, 195-204.
79. Abbott, N.J., Rönnbäck, L., Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews Neurosciences,* 7, 41-53.
80. Zlokovic B.V. (1990). In vivo approaches for studying peptide interactions at the blood-brain barrier. *Journal of Controlled Release,* 13, 185-202.
81. Illum, L. (2003). Nasal drug delivery-possibilities, problems and solutions. *Journal of Controlled Release*, 87, 187-198.
82. Sherry Chow, H. H., Chen, Z., Matsuura, G. T. (1999). Direct transport of cocaine from the nasal cavity to the brain following intranasal cocaine administration in rats. *Journal of. Pharmaceutical Sciences*, 88, 754-758.
83. Sakane, T. Akizuki, M. Yoshida, M., Yamashita, S., Nadai, T., Hashida, M., et al. (1991). Transport of cephalexin to the cerebrospinal fluid directly from the nasal cavity. *Journal of Pharmacy and Pharmacology* , 43, 449-451.
84. Kern, W., Born, J., Schreiber, H., Fehm, H.L. (1999). Central nervous system effects of intranasally administered insulin during euglycemia in men. *Diabetes*, 48, 557-563.
85. Bickel, U., Yoshikawa, T., Pardridge, W. M. (2001). Delivery of peptides and proteins through the blood-brain barrier. *Advanced Drug Delivery Reviews*, 46, 247-279.
86. Olesen, S.P., Crone, C. (1986). Substances that rapidly augment ionic conductance of endothelium in cerebral venules. *Acta Physiologica*, 127 233-241.
87. Sanovich, E., Bartus, R.T., Friden, P.M., Dean, R. L., Le, H.Q., Brightman, M. W. (1995). Pathway across blood-brain barrier opened by the bradykinin agonist, rmp-7. *Brain Research*, 705, 125-135.

88. Erdlenbruch, B., Alipour, M., Fricker, G., Miller, D.S., Kugler, W., Eibl, H., et al. (2003). Alkylglycerol opening of the blood-brain barrier to small and large fluorescence markers in normal and c6 glioma-bearing rats and isolated rat brain capillaries. *British Journal of Pharmacology*, 140, 1201-1210.
89. Caban, S. (2010). Beyne hedeflendirilmiş kaspaz inhibitörleri ve bazik fibroblast büyüme faktörü (bfgf) taşıyan nanopartiküllerin hazırlanması, beyne geçişinin ve etkinliğinin değerlendirilmesi.Yüksek Lisans Tezi, Hacettepe Üniversitesi, Ankara.
90. Pardridge, W. M. (2003). Blood-brain barrier drug targeting: The future of brain drug development. *Molecular Interventions*, 3, 90-105.
91. Pardridge, W. M. (2001). Bbb-genomics: Creating new openings for brain-drug targeting. *Drug Discovery*, 6, 381-383.
92. Freitas, R. A. (2005). Current status of nanomedicine and medical nanorobotics. *Journal of Computational and Theoretical Nanoscience*, 2, 1-25.
93. Kaur, I. P., Bhandari, R., Bhandari, S.,Kakkar, V. (2008). Potential of solid lipid nanoparticles in brain targeting. *Journal of Controlled Release,* 127(2), 97-109.
94. Di Stefano, A., Sozio, P. Iannitelli, A., Cerasa, L.S. (2009). New drug delivery strategies for improved parkinson's disease therapy. *Expert Opinion Drug Delivery,* 6(4), 389-404.
95. Garbayo, E., Ansorena, E., Blanco-Prieto, M. J. (2012). Brain drug delivery systems for neurodegenerative disorders. *Current Pharmaceutical Biotechnology*, 13, 2388-2402.
96. Kura, A. U., Hussein Al Ali, S. H., Hussein, M. Z., Fakurazi, S., Arulselvan, P. (2013). Development of a controlled-release anti-parkinsonian nanodelivery system using levodopa as the active agent. *International Journal of Nanomedicine*, 8, 1103-1110.
97. Li, F., Jin, L., Han, J., Wei, M., Li, C. (2009). Synthesis and controlled release properties of prednisone intercalated mg-al layered double hydroxide composite. *Industrial& Engineering Chemistry Research.*, 48, 5590-5597.

98. Buse, J., El-Aneed, A. (2010). Properties, engineering and applications of lipid-based nanoparticle drug-delivery systems: Current research and advances. *Nanomedicine*, 5, 1237-1260.
99. Bangham, A. D., Standish, M.M., Watkins, J.C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, 13, 238-252.
100. Mitra, A., Nan, A., Line, B. R., Ghandehari, H. (2006). Nanocarriers for nuclear imaging and radiotherapy of cancer. *Current Pharmaceutical Design*, 12, 4729-4749.
101. Huynh, N. T., Roger, E., Lautram, N., Benoît, J. P., Passirani, C. (2010). The rise and rise of stealth nanocarriers for cancer therapy: Passive versus active targeting. *Nanomedicine*, 5, 1415-1433.
102. Goins, B., Klipper, R, Rudolph, A S, Phillips, W T. (1994). Use of technetium-99m-liposomes in tumor imaging. *Journal of Nuclear Medicine*, 35, 1491-1498.
103. Biotechnology Applications Including The Characterisation And Use Of Liposomes With Zeta Potential and Photon Correlation Spectroscopy Equipment By Malvern Instruments. (t.y.). Erişim: 16 January 2015 http://www.Azonano.Com/article.Aspx?Articleid=1222
104. Silindir M, Ozer. A. Y., Erdogan S. (2012). The use and importance of liposomes in positron emission tomography. *Drug Delivery*, 19(1), 68-80.
105. Betageri, G. V., Jenkins, S.A., Parsons D.L. (1993). Liposome drug delivery systems. USA: Technomics Publishing.
106. Hamoudeh, M., Kamleh, M. A., Diab, R., Fessi, H. (2008). Radionuclides delivery systems for nuclear imaging and radiotherapy of cancer. *Advanced Drug Delivery Reviews,* 60, 1329-1346.
107. Gregoriadis, G., Florence, A. T. (1999). Liposomes in drug delivery. Clinical, diagnostic and ophthalmic potential. *Drugs*, 45 (1), 15-28.
108. Volkmar, W. (2010). *Liposomes-methods and protocols.* USA: Humana Press.

109.Goyal, P., Goyal, K., Vijaya Kumar, S.G., Singh, A., Katare, O.P., Mishra, D.N. (2005). Liposomal drug delivery systems-clinical applications. *Acta Pharmaceut*ica 55, 1-25.

110.Alkan, H. (1983) Lipozomlar I. Özellikleri ve hazırlama yöntemleri. *FABAD Journal of Pharmaceutical Sciences*, 8, 181-196.

111.Mirafzali Z. (2011). How many liposome based drugs are in the market? Erişim: 11 December 2015 http://www.Quora.Com/how-many-liposome-baseddrugs-arein-the-market

112.Turner, A.F., Presant, C.A., Proffitt, R.T., Williams, L.E., Winsor, D.W., Werner, J.L. (1988). In-111-labeled liposomes: Dosimetry and tumor depiction. *Radiology*, 166, 761-765.

113.Kubo, A., Nakamura, K., Sammiya, T., Katayama, M., Hashimoto, T., Hashimoto, S., et al. (1993). Indium-111-labelled liposomes: Dosimetry and tumour detection in patients with cancer. *European Journal of Nuclear Medicine*, 20, 107-113.

114.Mujoriya, R., Bodla, R.B., Dhamande, K., Singh, D., Patle, L. (2011). Niosomal drug delivery system: The magic bullet. *Journal of Applied Pharmaceutical Science*, 1, 20-23.

115.Ozer, A.Y., Hincal, A. A., Bouwstra, J.A. (1991). A novel drug delivery systems: Non-ionic surfactant vesicles. *European Journal of Pharmacy*, 37, 75-79.

116.Baillie, A. J., Florence, A.T., Hume, L.R., Muirhead, G.T., Rogerson, A. (1985). The prepararti on and properties of niosomes-non-ionic surfactant vesicles. *Journal of Pharmacy and Pharmacology* , 37, 863-868.

117.Uchegbu, I. F., Vyas, S.P. (1998). Non-ionic surfactant vesicles (niosomes) in drug delivery. *International Journal of Pharmacy* , 172, 33-70.

118.Huwyler, J., Wu, D., Pardridge ,W.M. (1996). Brain drug delivery of small molecules using immunoliposomes. *Proceeding of the National Academy of Sciences of the United States of America Neurobiology*, 93, 14164-14169.

119.Weiner, N., Martin, F., Riaz, M. (1989). Liposomes as a drug delivery system. *Drug Development and Industrial Pharmacy*, 15 (10), 1523-1554.

120.Gregoriadis, G. (1995). Engineering of liposomes for drug delivery: Progress and problems, *Trends in Biotechnology*, 13 (12) 527-537.

121.Crommelin, D. J. A., Storm, G. (1987). Liposomes as drug delivery systems in therapy: Their potential and limitations. *International Pharmacy Journal*, 1 (5) 179-181.

122.Allen, T. M. (1997). Liposomes: Opportunities in drug delivery. *Drugs*, 54 (4), 8-14.

123.Storm, G., Crommelin, D. J. A. (1998). Liposomes:Qua vadis? *Primary Science Teaching Trust*, 1 (1), 19-31.

124.Gregoriadis, G. (1989). Liposomes as carriers of drugs. Observations on vesicle fate after injection and its control. *Subcellular Biochemistry*, 14, 363-378.

125.Bakker-Woudenberg, I. A., Storm, G., Woodle, M. C. (1994). Liposomes in the treatment of infections. *Journal of Drug Targeting*, 2 (5), 363-371.

126.Daemen, T., Hofstede, G., Ten Kate, M. T., Bakker-Woudenberg, I. A., Scherphof, G. L. (1995). Liposomal doxorubicin-induced toxicity: Depletion and impairment of phagocytic activity of liver macrophages. *International Journal of Cancer*, 61 (5), 716-721.

127.Storm, G., Ten Kate, M., Working, P., Bakker-Woudenberg, I. (1998). Doxorubicin entrapped in sterically stabilized liposomes: Effects on bacterial blood clearance capacity of the mononuclear phagocyte system. *Clinical Cancer Research*, 4 (1), 111-115.

128.Lasic, D., Papahadjopoulos, D. (1995). Liposomes revisited. *Science* 267 (5202) 1275-1276.

129.Allen, T. M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A. (1991). Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1066 (1), 29-36.

130.Torchilin, V. P., Lukyanov, A.N. (2003). Peptide and protein drug delivery to and into tumors: Challenges and solutions. *Drug Discovery Today,* , 8, 259-266.

131.Park, Y. S. (2002). Tumor-directed targeting of liposomes. *Bioscience Reports*, 22 (2), 267-281.

132.Kontermann, R. E. (2006). Immunoliposomes for cancer therapy. *Current Opinion in Molecular Therapeutics*, 8 (1), 39-45.

133.Torchilin, V. P. (2006). Multifunctional nanocarriers. *Advanced Drug Delivery Reviews*, 58 (14) 1532-1555.

134.Dandamudi, S., Patil, V., Fowle, W., Khaw, B. A., Campbell, R. B. (2009). External magnet improves antitumor effect of vinblastine and the suppression of metastasis. *Cancer Science*, 100 (8) 1537-1543.

135.Hu, Y. Z., Zhu, J. A., Jiang, Y.G., Hu, B. (2009) Ultrasound microbubble contrast agents: Application to therapy for peripheral vascular disease. *Advances in Therapy*, 26 (4), 425-434.

136.Ren, J. L., Wang, Z. G., Zhang, Y., Zheng, Y. Y., Li, X. S., Zhang, Q. X., Wang, Z. X., Xu, C. S. (2008). Transfection efficiency of tdl compound in huvec enhanced by ultrasound-targeted microbubble destruction. *Ultrasound in Medicine & Biology*, 34 (11) 1857-1867.

137.Schnyder, A., Huwyler, J. (2005). Drug transport to brain with targeted liposomes. *NeuroRx* 2 (1), 99-107.

138.Pardridge, W. M. (2001). Brain drug targeting and gene technologies.*The Japanese Journal of Pharmacology*, 87, 93-103.

139.Kurihara, A., Pardridge, W. M. (1999b). Imaging brain tumors by targeting peptide radiopharmaceuticals through the blood-brain barrier. *Cancer Research*, 59, 6159-6163.

140.Immordino, M. L., Dosio, F., Cattel, L. (2006). Stealth liposomes: Review of the basic science, rationale, and clinical applications, existing and potential. *International Journal of Nanomedicine*, 1(3), 297-315.

141.Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K. (2000). Tumor vascular permeability and the epr effect in macromolecular therapeutics: A review. *Journal of Controlled Release*, 65 (1-2), 271-284.

142.Kwon, G. S., Suwa, S., Yokoyama, M., Okano, T., Sakurai, Y., Kataoka, K. (1994). Enhanced tumor accumulation and prolonged circulation times of

micelle-forming poly(ethylene oxideaspartate) block copolymers-andriamycin conjugates. *Journal of Controlled Release*, 29, 17-23.

143.Weissig, V., Whiteman, K. R., Torchilin, V.P. (1998). Accumulation of protein-loaded long-circulating micelles and liposomes in subcutaneous lewis lung carcinoma in mice. *Pharmaceutical Research*, 15, 1552-1556.

144.Torchilin, V. P. (2007). Micellar nanocarriers: Pharmaceutical perspectives. *Pharmaceutical Research*, 24 (1), 1-16.

145.Papahadjopoulos, D., Gabizon, A. (1990). Liposomes designed to avoid the reticuloendothelial system. *Progress in Clinical and Biological Research*, 343, 85-93.

146.Gabizon, A., Papahadjopoulos, D. (1988). Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proceedings of the National Academy of Sciences of the United States of America*, 85 (18), 6949-6953.

147.Wu, N. Z., Da, D., Rudoll, T. L., Needham, D., Whorton, A. R., Dewhirst, M. W. (1993). Increased microvascular permeability contributes to preferential accumulation of stealth liposomes in tumor tissue. *Cancer Research*, 53 (16), 3765-3770.

148.La, S. B., Okano, T., Kataoka, K. (1996). Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated poly(ethylene oxide)-poly(betabenzyl l-aspartate) block copolymer micelles. *Journal of Pharmaceutical Sciences*, 85 (1), 85-90.

149.Lukyanov, A. N., Torchilin, V. P. (2004). Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. *Advanced Drug Delivery Reviews*, 56 (9), 1273-1289.

150.Afergan, E., Epstein, H., Dahan, R., Koroukhov, N., Rohekar, K., Danenberg, H. D., et al. (2008). Delivery of serotonin to the brain by monocytes following phagocytosis of liposomes. *Journal of Controlled Release* 132, 84-90.

151.Sharma, U. S., Sharma, A., Chau, R. I., Straubinger, R. M. (1997). Liposome-mediated therapy of intracranial brain tumors in a rat model. *Pharmaceutical Research,* 14, 992-998.

152.Siegal, T., Horowitz, A., Gabizon, A. (1995). Doxorubicin encapsulated in sterically stabilized liposomes for the treatment of a brain tumor model: Biodistribution and therapeutic efficacy. *Journal of Neurosurgery*, 83, 1029-1037.

153.Soni, V., Kohli, D. V., Jain, S. K. (2008). Transferrin-conjugated liposomal system for improved delivery of 5-fluorouracil to brain. *Journal of Drug Targeting*, 16(1), 73-78.

154.Pichandy, M., Mishra M., Kanaiyan, S., Srinivasa, R. T., Anbu, J. (2010). Formulation and psychopharmacological evaluation of surfactant modified liposome for parkinsonism disease. *Asian Journal of Pharmaceutical and Clinical Research*, 3, 46-54.

155.Di Stefano, A., Carafa, M., Sozio, P., Pinnen, F., Braghiroli, D., Orlando, G., et al. (2004). Evaluation of rat striatal l-dopa and da concentration after intraperitoneal administration of l-dopa prodrugs in liposomal formulations. *Journal of Controlled Release*, 99, 293-300.

156.During, M. J., Freese, A., Deutch, A. Y., Kibat, P. G., Sabel, B. A., Langer, R., et al. (1992). Biochemical and behavioral recovery in a rodent model of parkinson's disease following stereotactic implantation of dopamine-containing liposomes. *Experimental Neurology*, 115(2), 193-199.

157.Yurasov, V. V., Kucheryanu, V. G., Kudrin, V. S., Zhigal'tsev , I. V., Nikushkin, E. V., Sandalov, Y. G., et al. (1997). Effect of long-term parenteral administration of empty and l-dopa liposomes on the turnover of dopamine and its metabolites in the striatum of mice with experimental parkinson's syndrome. *Bulletion of Experimental Biology and Medicine,* 123 126-129.

158.Ismail, M. F., ElMeshad, A. N., Abdel-Hameed Salem, N. (2013) Potential therapeutic effect of nanobased formulation of rivastigmine on rat model of alzheimer's disease. *International Journal of Nanomedicine* 8,393-406.

159.Kizelsztein, P., Ovadia, H., Garbuzenko, O., Sigal, A., Barenholz, Y. (2009). Pegylated nanoliposomes remote-loaded with the antioxidant tempamine ameliorate experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* 213, 20-25.

160.Schmidt, J., Metselaar J., Wauben, M. H. M., Toyka, K. V., Storm, G., Gold, R.(2003). Drug targeting by long-circulating liposomal glucocorticosteroids increases therapeutic efficacy in a model of multiple sclerosis. *Brain,* 126, 1895-1904.

161.Awasthi, V., Yee, S. H., Jerabek, P., Goins, B., Phillips, W. T. (2007). Cerebral oxygen delivery by liposome-encapsulated hemoglobin: A positron emission tomographic evaluation in a rat model of hemorrhagic shock. *Journal of Applied Physiology,* 103, 28-38.

162.Shazeeb, M. S., Feula, G., Bogdanov, A. Jr. (2014). Liposome-encapsulated superoxide dismutase mimetic: Theranostic potential of an mr detectable and neuroprotective agent. *Contrast Media and Molecular Imaging*, 9 221-228.

163.Caraglia, M., Luongo L., Salzano, G., Zappavigna, S., Marra, M., Guida, F., Lusa, S., et al. (2013). Stealth Liposomes encapsulating zoledronic acid: A new opportunity to treat neuropathic *Pain. Molecular Pharmaceutics,* 10, 1111−1118.

164.Langer, R. (1990). New methods of drug delivery. *Science*, 249, 1527-1533.

165.Fu, Y., Kao, W. J. (2010). Drug release kinetics and transport mechanisms of nondegradable and degradable polymeric delivery systems. *Expert Opinion Drug Delivery,* 7(4), 429-444.

166.Noyes, A. A., Whitney, W. R. (1897). The Rate Of Solution Of Solid Substances In Their Own Solutions. *Journal of American Chemical Society*, 19, 930-934.

167.Dash, S., Murthy, P., Nath, L., Chowdhury, P. (2010). Kinetic modeling on drug release from controlled drug delivery systems. *Acta Poloniae Pharmaceutica ñ Drug Research*, 67, 217-223.

168.Ozturk, S. S., Palsson. B. O., Donohoe, B., Dressman, J. B. (1988). Kinetics of Release from Enteric-Coated Tablets. *Pharmaceutical Research* , 5, 550-565.

169.Mauger J. W., Chilko, D., Howard, S. (1986). On the analysis of the dissolution data. *Drug Development and Industrial Pharmacy*, 12, 969-992.

170.Polli, J. E., Rekhi, G. S., Augsburger, L. L., Shah, V.P. (1997). Methods to compare dissolution profiles and a rationale for wide dissolution

specifications for metoprolol tartrate tablets. *Journal of Pharmaceutical Sciences*, 86(6), 690-700.

171.Costa, P., Lobo, J. M. S. (2001). Modeling and comparison of dissolution profiles. *European Journal of Pharmaceutical Sciences,* (13), 123-133.

172.Shah, V. P., Lesko, L. J., Fan, J., Fleischer, N., Handerson, J., Malinowski, H., et al. (1997). Fda guidance for industry: Dissolution testing of immediate release solid oral dosage forms. *Dissolution Technology*, 4, 15-22.

173.Crank, J., (1975). *The mathematics of diffusion*. Oxford: Clarendon Press,.

174.Costa, P. (2001). An alternative method to the evaluation of similarity factor in dissolution testing. *International Journal of Pharmaceutics*, 220, 77-83.

175.Moore, J. W., Flanner, H. H. (1996). Mathematical comparison of dissolution profiles. *Pharmaceutical Technology*, 20, 64-74.

176. US Department of Health and Human Services, Food and Drug Administration. 1995. Guideline For Industry Immediate Release Solid Oral Dosage Forms Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation. Maryland: US Department of Health and Human Services, Food and Drug Administration

177.Arhewoh, I. M., Okhamafe, A. O. (2004). An overview of site-specific delivery of orally administered protein/peptides and modelling considerations. *Journal of Medical and Biomed*ical Sciences, 3, 7-20.

178.Narashimhan B., Mallapragada, S. K., Peppas, N. A. (1999). Release kinetics, data interpretation. E. Mathiowist (Ed.). *Encyclopedia of controlled drug delivery* (s. 921). New York John Wiley and Sons Inc.

179.Hadjiioannou, T. P., Christian, G. D., Koupparis, M. A., Macheras, P. A. (1993). *Quantitative calculations in pharmaceutical practice and research* . New York: VCH Publishers Inc.

180.Libo Y., Reza. F. (1996). Kinetic modeling on drug release from controlled drug delivery systems. *Journal of Pharmaceutical Sciences* , 85, 170.

181.Freitas, M. N., Marchetti, J. M. (2005). Nimesulide pla microspheres as a potential sustained release system for the treatment of inflammatory diseases. *International Journal of Pharmaceutics,* 295(1-2), 201-211.

182.Bourne, D. W. A. (2002). Pharmacokinetics. G. S. Banker, C. T. Rhodes (Ed.). *Modern pharmaceutics* (s.77-102). New York: Marcel Dekker Inc

183.Higuchi, T. (1963). Mechanism of sustained-action medication. Theoretical analysis of rate of release of solid drugs dispersed in solid matrices. *Journal of Pharmaceutical Sciences,* 52, 1145-1149.

184.Cell culture basics handbook, invitrogen gibco. Erişim: 02 March 2015 www.Invitrogen.Com/cellculturebasics.

185.Whonamedit - ringer's solution. (t.y.). Erişim: 05 March 2015 http://www.whonamedit.com/

186.Zurlo, J., Rudacille, D., Goldberg, A. M. (2001). *Animals and alternatives in testing: History, Science, and Ethics*.Erişim:31 May 2015 http://caat.jhsph.edu/publications/animal_alternatives/

187.Schiff, J. A. *An unsung hero of medical research*. Retrieved 2006-04-19. Yale alumni magazine, february 2002. Erişim: 30April 2015 http://archives.yalealumnimagazine.com/issues/02_02/old_yale.html

188.Coriell Institute For Medical Research.(t.y.). Erişim:10 April 2015 https://www.Coriell.Org/research-services/cell-culture/what-is-cell-culture

189.Weksler, B., Romero, I. A., Couraud, P. O. (2013). The hcmec/d3 cell line as a model of the human blood brain barrier. *Fluids and Barriers of the CNS*, 10 16-26.

190.Gumbleton, M., Audus, K. L. (2001). Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. *Journal of Pharmaceutical Sciences,* 90, 1681-1698.

191.Reichel, A. , Begley, D. J., Abbott, N. J. (2003). An overview of in vitro techniques for blood-brain barrier studies. *Methods Molecular Medicine,* 89, 307-324.

192.Raghnaill, M. N., Bramini, M., Ye, D., Couraud, P. O., Romero, I. A., Weksler, B., et al. (2014). Paracrine signalling of inflammatory cytokines

from an in vitro blood brain barrier model upon exposure to polymeric nanoparticles. *Analyst*, 139, 923-930.

193. Weksler, B. B., Subileau, E. A., Perrière, N., Charneau, P., Holloway, K., Leveque, M., et al. (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *The Faseb Journal*, 19(13), 1872-1874.

194. Hatherell, K., Couraud, P. O., Romero, I. A., Weksler, B., Pilkington, G. J. (2011). Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation transwell models. *Journal of Neurosciences Methods*, 199(2), 223-229.

195. Dos Santos, T., Varela, J., Lynch, I., Salvati, A., Dawson, K. A. (2011). Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines. *PLoS ONE*, 6, 1-10.

196. Blandini, F., Armentero, M. T. (2012). Animal models of parkinson's disease. *FEBS Journal,* 279, 1156-1166.

197. Senoh, S., Witkop, B. (1959). Nonenzymatic conversions of dopamine to norepinephrine and trihydroxyphenethylamine. *Journal of American Chemical Society,* 81, 6222-6231.

198. Senoh, S., Creveling, C. R., Udenfriend, S., Witkop, B. (1959). Chemical, enzymatic and metabolic studies on the mechanism of oxidation of dopamine. *Journal of American Chemical Society,* 81, 6236-6240.

199. Simola, N., Morelli, M., Carta, A. R. (2007). The 6-hydroxydopamine model of parkinson's disease. *Neurotoxicity Research*, 11, 151-167.

200. Tieu, K. (2011). A guide to neurotoxic animal models of parkinson's disease. *Cold Spring Harbor Perspectives Medicine,*1-20.

201. Porter, C. C., Totaro, J. A., Stone, C. A. (1963). Effect of 6-hydroxydopamine and some other compounds on the concentration of norepinephrine in the hearts of mice. *Journal of Pharmacology Experimental Therapy,* 140, 308-316.

202. Porter, C. C., Torato, J. A., Burcin, A. (1965). The relationship between radioactivity and norepinephrine concentrations in the brains and hearts of

mice following administration of labeled methyldopa or 6-hydroxydopamine. *Journal of Pharmacology Experimental Therapy,*150, 17-22.

203.Tranzer, J. P., Thoenen, H. (1968). An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia*, 24, 155-156.

204.Jonsson, G. (1980). Chemical neurotoxins as denervation tools in neurobiology. *Annual Reviews Neurosciences*, 3, 169-187.

205.Ungerstedt, U. (1968). 6-hydroxydopamine induced degeneration of central monoamine neurons. *European Journal of Pharmacology,* 5,107-110.

206.Saner, A., Thoenen, H. (1971). Model experiments on the molecular mechanism of action of 6-hydroxydopamine. *Molecular Pharmacology,* 7, 147-154.

207.Cohen G (1994). Free radicals, oxidative stress, and neurodegeneration. D.B. Calne (Ed.). *Neurodegenerative diseases* (s. 139-161). Philadelphia: W.B. Saunders

208.Joonsson, G. (1983). Chemical lesioning techniques: Monoamine neurotoxins. A. Björklund, T. Hökfelt (Ed.). *Handbook of chemical neuroanatomy vol 1: Methods in chemical neuroanatomy* (s. 463-507). Amsterdam: Elsevier

209.Bezard, E., Imbert, C., Gross, C. E. (1998). Experimental models of parkinson's disease: From the static to the dynamic. *Reviews Neurosciences,* 9,71-90.

210.Bezard, E., Przedborski, S. (2011). A tale on animal models of parkinson's disease. *Movement Disorders*, 26, 993-1002.

211.Agid, Y., Javoy, F., Glowinski, J., Bouvet, D., Sotelo, C. (1973). Injection of 6-hydroxydopamine into the substantia nigra of the rat. II. Diffusion and specificity. *Brain Research,* 58, 291-301.

212.Przedborski, S., Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donaldson, D., et al. (1995). Dosedependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. *Neuroscience,* 67, 631-647.

213.Przedborski, S., Tieu, K. (2006). Toxic animal models. M. F. Beal, A. E. Lang, A. C. Ludolph (Ed.). *Neurodegenerative diseases* (s. 196-221). Cambridge: Cambridge University Press.

214.Faull, R. L., Laverty, R. (1969). Changes in dopamine levels in the corpus striatum following lesions in the substantia nigra. *Experimental Neurology,* 23, 332-340.

215.Jeon, B.S., Jackson-Lewis, V., Burke, R. E. (1995). 6-hydroxydopamine lesion of the rat substantia nigra: Time course and morphology of cell death. *Neurodegeneration*, 4, 131-137.

216.Sauer, H., Oertel, W. H. (1994). Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: A combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience*, 59, 401-405.

217.Branchi, I., D'Andrea, I., Armida, M., Cassano, T., Pezzola, A., Potenza, et al. (2008). Nonmotor symptoms in parkinson's disease: Investigating early-phase onset of behavioral dysfunction in the 6-hydroxydopamine-lesioned rat model. *Journal of Neuroscience Research*, 86, 2050-2061.

218.Tadaiesky, M. T., Dombrowski, P. A., Figueiredo, C.P., Cargnin-Ferreira, E., Da Cunha, C., Takahashi, R.N. (2008). Emotional, cognitive and neurochemical alterations in a premotor stage model of parkinson's disease. *Neuroscience* 156, 830-840.

219.Cannon, J. R., Greenamyre, J. T. (2010). Neurotoxic in vivo models of parkinson's disease recent advances. *Progress in Brain Research*, 184, 17-33.

220.Ungerstedt, U., Arbuthnott, G. (1970). Quantitative recording of rotational behaviour in rats after 6-hydroxydopamine lesions of the nigrostriatal dopamine system. *Brain Research*, 24, 485-493.

221.Heft, M.W., Gracely, R. H., Dubner, R., McGrath, P. A. (1980). A validation model for verbal description scaling of human clinical pain. *Pain*, 9(3), 363-373.

222.Jiang, H., Jackson-Lewis, V., Muthane, U., Dollison, A., Ferreira, M., Espinosa, A., et al. (1993). Adenosine receptor antagonists potentiate

dopamine receptor agonist-induced rotational behavior in 6-hydroxydopamine-lesioned rats. *Brain Research*, 613, 347-351.

223.Chan, H., Paur, H., Vernon, A. C., Zabarsky, V., Datla, K. P., Croucher, M. J., et al. (2010). Neuroprotection and functional recovery associated with decreased microglial activation following selective activation of mglur2/3 receptors in a rodent model of parkinson's disease. *Parkinsons Disease*, vol 2010, 1-12.

224.Ilijic E, Guzman, J. N., Surmeier, D. J. (2011) The l-type channel antagonist isradipine is neuroprotective in a mouse model of parkinson's disease *Neurobiology of Disease*, 43(2), 364-371.

225.Bjorklund, L. M., Sánchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., McNaught, K. S., et al. (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a parkinson rat model. *Proceedings of the National Academy of Sciences,* 99, 2344-2349.

226.Kirik, D., Georgievska, B., Burger, C., Winkler, C., Muzyczka, N., Mandel, R. J., et al. (2002). Reversal of motor impairments in parkinsonian rats by continuous intrastriatal delivery of l-dopa using raav-mediated gene transfer. *Proceedings of the National Academy of Sciences,* 99, 4708-4713.

227.Roy, N.S., Cleren, C., Singh, S. K., Yang, L., Beal, M. F., Goldman, S.A. (2006). Functional engraftment of human es cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nature Medicine*, 12, 1259-1268.

228.Davis, G. C., Williams, A. C., Markey, S. P., Ebert, M. H., Caine, E. D., Reichert, C. M., et al. (1979). Chronic parkinsonism secondary to intravenous injection of meperidine analogs. *Psychiatry Research*, 1, 249-254.

229.Langston, J. W., Ballard, P., Tetrud, J. W., Irwin, I. (1983). Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, 219, 979-980.

230.Fox, S. H, Brotchie, J. (2010). The mptp-lesioned nonhuman primate models of parkinson's disease. Past, present, and future. *Progress in Brain Research,* 184, 133-157.

231.Dauer W, Przedborski, S. (2003). Parkinson's disease: Mechanisms and models. *Neuron*, 39, 889-909.

232.Rappold, P. M. , Tieu, K. (2010). Astrocytes and therapeutics for parkinson's disease. *Neurotherapeutics*, 7, 413-423.

233.Nelson, J. A. (2001). *Treatment for schizophrenia and other dopamine system dysfunctions*, U.S. Patent No. US6232326 B1.Washington, DC: U.S. Patent and Trademark Office.

234.Cui, M., Aras, R., Christian, W. V., Rappold, P. M., Hatwar, M., Panza, J., et al. (2009). The organic cation transporter-3 is a pivotal modulator of neurodegeneration in the nigrostriatal dopaminergic pathway. *Proceedings of the National Academy of Sciences,*106, 8043-8048.

235.Nicklas, W. J., Vyas. I., Heikkila, R. E. (1985). Inhibition ofnadhlinked oxidation in brain mitochondria by mppþ, a metabolite of the neurotoxin mptp. *Life Science*, 36, 2503-2508.

236.Mizuno, Y., Sone, N., Saitoh, T. (1987). Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenylpyridinium ion on activities of the enzymes in the electron transport system in mouse brain. *Journal of Neurochemistry*, 48, 1787-1793.

237.Przedborski, S., Jackson-Lewis, V., Naini, A., Jakowec, M., Petzinger, G., Miller, R., et al. (2001). The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (mptp): Atechnical reviewof its utility and safety. *Journal of Neurochemistry*,76, 1265-1274.

238.Porras, G., Li Q., Bezard E. (2012). Modeling parkinson's disease in primates: The mptp model. *Cold Spring Harbor Perspectives in Medicine*, 2(3), 1-10.

239.Giovanni, A., Sieber, B. A., Heikkila, R. E., Sonsalla, P. K. (1994). Studies on species sensitivity to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Part 1: Systemic administration. *Journal of Pharmacology Experimental Therapeutics,* 270, 1000-1007.

240.Forno, L. S., DeLanney, L., Irwin, I., Langston, J. W. (1993). Similarities and differences between mptp-induced parkinsonism and parkinson's disease: Neuropathologic considerations. *Advanced Neurology,* 60, 600-608.

241.Ogawa, N., Hirose, Y., Ohara, S., Ono, T., Watanabe, Y. (1985). A simple quantitative bradykinesia test in mptp-treated mice. *Research Communication of Chemical Pathoogy andl Pharmacology,* 50, 435-441.

242.Anderson, G., Noorian A. R., Taylor, G., Anitha, M., Bernhard, D., Srinivasan, S., et al. (2007). Loss of enteric dopaminergic neurons and associated changes in colon motility in an mptp mouse model of parkinson's disease. *Experimental Neurology*, 207, 4-12.

243.European society of molecular imaging (esmi) study group on "image-guided drug delivery- igdd". (t.y.). Erişim: 7 January 2015 http://www.E-smi.Eu/index.Php?Id=2527

244.Wang, Y. X., Deng, M. (2010). Medical imaging in new drug clinical development. *Journal of Thoracic Disease,* 2,245-252.

245.Kääriäinen, T. M., Käenmäki, M., Forsberg, M. M., Oinas, N., Tammimäki, A., Männistö, P. T. (2011). Unpredictable rotational responses to l-dopa in the rat model of parkinson's disease: The role of l-dopa pharmacokinetics and striatal dopamine depletion. *Basic & Clinical Pharmacology & Toxicology*, 110, 162-170.

246.Hefti, F., Melamed, E., Sahakian, B. J., Wurtman, R. J. (1980). Circling behavior in rats with partial, unilateral nigro-striatal lesions: Effect of amphetamine, apomorphine, and dopa. *Pharmacology Biochemistry and Behavior*, 12, 185-188.

247.Frey, H., Lahtinen, A., Heinonen, T., Dastidar, P. (1999). Clinical application of MRI image processing in neurology. *International Journal of Business and Emerging Markets,* 1, 1, 47-53.

248.Small, G. W. (2002). Structural and functional brain imaging of alzheimer disease. K. L. Davis, D. Charney, J. T. Coyle, C. Nemeroff (Ed.). *Neuropsychopharmacology: The fifth generation of progress* (s. 1232-1242). Philadelphia, Pennsylvania: Lippincott Williams & Wilkins.

249.Acer, N., Tuncay, A., Özsunar, Y., Turgut, M. (2011). Quantification of volumetric changes of brain in neurodegenerative diseases using magnetic resonance imaging and stereology. R. Chuen-Chung Chang (Ed.).

Neurodegenerative diseases - processes, prevention, protection and monitoring (s. 453-476). Crotia: Intech

250.Barthe, N., Coulon, P., Hennion, C., Ducassou, D., Basse-Cathalinat, B., Charpak, G. (1999). Optimization of a new scintillation gas detector used to localize electrons emitted by 99mtc. *Journal of Nuclear Medicine*, 40 (5), 868-875.

251.Roger A. W. (1979). Techniques of autoradiography. North Holland: Elsevier.

252.Grounds M. Autoradiography presenter. (t.y.). Erişim:11 March 2015 http://www.Lab.Anhb.Uwa.Edu.Au/hb313/main_pages/timetable/lectures/autoradiography.Htm

253.Autoradiograph Erişim:11 March 2015 http://en.Wikipedia.Org/wiki/autoradiograph

254.Lattes, J., Lacassagne A. (1924). Repartition du polonium (injecte sous la peau) dans l'organisme de rats de grees cancereuses. *Comptes Rendus Hebdomadaires des Séances et Mémoires de la Société de Biologie et des ses Filiales*, 90, 352-353.

255.Stumpf, W. E. (2003). *Drug localization in tissues and cells*. North Carolina: Chapel Hill.

256.Velasco, J. C. (2009). *High throughput digital β autoradiography imaging.* U.K: Centre for Vision, Speech and Signal Processing School of Electronics and Physical Sciences University of Surrey Guildford.

257.The neuroscience reseach program, the physchiatric institute, department of psychiatry, The University of Illinois at Chicago. (t.y.). Erişim: 08 March 2015 https://www.Uic.Edu/orgs/psych/

258.Autoradiography Erişim. (t.y.). 09 April 2015 https://www.Nationaldiagnostics.Com/electrophoresis/article/autoradiography

259.Autoradiography. (t.y.). Erişim:11 January 2015 http://en.Wikipedia.Org/wiki/autoradiograph

260.Rupassara, S. I., Larson, R. A., Sims, G. K., Marley, K. A. (2002). Degradation of atrazine by hornwort in aquatic systems. *Bioremediation Journal,* 6(3), 217-224.

261.Kuhar, M., Yamamura, H. I. (1976). Localization of cholinergic muscarinic receptors in rat brain by light microscopic radioautography. *Brain Research*, 110 (2), 229-243.

262.Young, W. S., Kuhar, M. (1979). A new method for receptor autoradiography: [3h]opioid receptors in rat brain. *Brain Research*, 179 (2), 255-270.

263.Jin, L., Lloyd, R. (1997). In situ hybridization: Methods and applications. *Journal of Clinical Laboratory Analysis*, 11 (1), 2-9.

264.Donnard, J. (2008). *Étude et conception d'un imageur β à très haute résolution spatiale*.THÈSE DE DOCTORAT, Université de Nantes, Nantes

265.Mirapex® *PDR 2005* (s. 1002-1007). Montvale: Thomson PDR

266.Bunnel, R. (1997). Using computer simulated results of a bulk drug substance assay to determine acceptance criteria for method validation. *Pharmaceutical Research*, 14, 156-163.

267.New, R. R. C. (1990). Preparation of liposomes-characterization of liposomes. R. R. C. New (Ed.). *Liposomes a practical approach* (s. 33-103 105-160). New York: Oxford University Press.

268.Griffin, W. C. (1949). Classification of surface-active agents by hlb. *Journal of the Society of Cosmetic Chemists,* 1 (5), 311-326.

269.Griffin, W. C. (1954). Calculation of hlb values of non-ionic surfactants. *Journal of the Society of Cosmetic Chemists,* 5 (4), 249-256.

270.Speck, U. (1993). Contrast media-overview. U. Speck (Ed.). *Use and pharmaceutical aspects* (s. 27-40). Berlin:Springer-Verlag

271.Erdoğan, S. (1996). *Radyokontrast Etken Madde Taşıyan Lipozom Ve Nisv Taşıyıcı Sistemler Üzerinde Çalışmalar*.Yükseklisans Tezi, Hacettepe Üniversitesi, Ankara.

272.Hattori, Y., Maitani, Y. (2004). Enhanced in vitro DNA transfection efficiency by novel folate-linked nanoparticles in human prostate cancer and oral cancer. *Journal of Controlled Release*, 97 (1), 173-183.

273.Grant, C. W., Stephen, K., Florio, E. (1989). A liposomal mri contrast agent: Phosphatid ylethanolamine-dtpa. *Magnetic Resonance in Medicine*, 11, 236-243.

274.Elbayoumi, T, Torchilin, V. P. (2006). Enhanced accumulation of long-circulating liposomes modified with the nucleosome-specific monoclonal antibody 2c5 in various tumours in mice: Gamma-imaging studies. *European Journal of Nuclear Medicine Molecular Imaging*, 33, 1196-1205.

275.Silindir, M. (2009). *Spect/Bt Yöntemi İle Tümör Teşhis Ve Görüntülenmesinde Kullanilacak Nanoboyutlu Lipozom Formülasyonlarinin Geliştirilmesi*.Yüksek Lisans Tezi, Hacettepe Üniv, Ankara.

276.Oku, N., Yamashita, M., Katayama, Y., Urakami, T., Hatanaka, K., Shimizu, K., et al. (2011). Pet imaging of brain cancer with positron emitter-labeled liposomes. *International Journal of Pharmaceutics*, 403, 170-177.

277.Rouser, G., Fleischer, S.,Yamamoto, A. (1970). Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*, 5 (5), 494-496.

278.Hoiuchi, A., Satou. T., Akao, N., Koike, K., Fujita, K., Nikaido, T. (2005). The effect of free and polyethylene glycol-liposome-entrapped albendazole on larval mobility and number in toxocano canis infected mice. *Veterinary Parasitology*, 129, 83-87.

279.Bicker, J., Alves, G., Fortuna, A., Falcão, A. (2014). Blood-brain barrier models and their relevance for a successful development of cns drug delivery systems: A review. *European Journal of Pharmaceutics and Biopharmaceutics*, 87, 409-432.

280.Henry, B., Crossman, A. R., Brotchie, J. M. (1998). Characterization of a rodent model in which to investigate the molecular and cellular mechanisms underlying the pathophysiology of l-dopa-induced dyskinesia. *Advanced Neurology*, 78, 53-61.

281.Thiele, S. L., Ware, R., Nash, J. E. (2012). Development of a unilaterally-lesioned 6-ohda mouse model of parkinson's disease. *Journal of visualized experiments*, 60, 3234.

282.Bjorklund, A., Rosenblad, C., Winkler, C., Kirik, D. (1997). Studies on neuroprotective and regenerative effects of gdnf in a partial lesion model of parkinson’s disease. *Neurobiology Disease,* 4,186-200.

283.Kirik, D, Rosenblad, C., Bjorklund, A. (1998). Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Experimental Neurology*, 152, 259-277.

284.Paxinos, G., Watson, C. (1986). The rat brain in stereotaxic coordinates. New York: Academic Press.

285.Maia, S., Arlicot, N., Vierron, E., Bodard, S., Vergote, J., Guilloteau, D., et al. (2012). Longitudinal and parallel monitoring of neuroinflammation and neurodegeneration in a 6-hydroxydopamine rat model of parkinson's disease. *Synapse*, 66, 573-583.

286.Pycock, C. M. (1980). Turning behavior in animals. *Neuroscience*, 5, 461-514.

287.Ungerstedt, U. (1971). Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behavior. *Acta physiologica scandinavica,* 82, 49-68.

288.Papa, S. M., Engber, T., Kask, A. M., Chase, T. N. (1994). Motor fluctuations in levodopa treated parkinsonian rats: Relation to lesion extent and treatment duration. *Brain Research,* 662, 69-74.

289.Emond, P., Guilloteau, D., Chalon, S. (2007). PE2I: A radiopharmaceutical for in vivo exploration of the dopamine transporter. *CNS Neuroscience & Therapeutics*, 14, 47-64.

290.Chalon, S., Garreau, L., Emond, P., Zimmer, L., Vilar, M. P., Besnard, J. C., et al. (1999). Pharmacological characterization of (e)-n-(3- iodoprop-2-enyl)-2beta-carbomethoxy-3beta-(40-methylphenyl)n ortropane as a selective and potent inhibitor of the neuronal dopamine transporter. *Journal of Pharmacology Experimental Therapeutics,* 291, 648-654.

291.Stepanov, V., Schou, M., Jarv, J., Halldin, C. (2007). Synthesis of 3h-labeled n-(3-iodoprop-2e-enyl)-2β-carbomethoxy-3β-(4-methylphenyl)nortropane (pe2i) and its interaction with mice striatal membrane fragments. *Applied Radiatian and Isotopes,* 65, 293-300.

292.Guilloteau, D, Emond, P., Baulieu, J. L., Garreau, L., Frangin, Y., Pourcelot, L., Mauclaire, L., Besnard, J. C., Chalon, S. (1998). Exploration of the

dopamine transporter: In vitro and in vivo characterization of a high-affinity and high-specificity iodinated tropane derivative (e)-n-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4 -methylphenyl)nortropane (pe2i). *Nuclear Medicine and Biology,* 25, 331-337.

293.Berthommier, E., Loc'h, C., Chalon, S., Olivier, C., Emond, P., Dao Boulanger, H., et al. (2002). New preparation of [^{123}I]PE2I: Investigation of the oxidation and purification steps. *Journal of Labelled Compounds Radiopharmaceuticals*, 45, 1019-1102.

294.Arıca, B. (1992). *Primakin Difosfat Lipozomlarının Formülasyonu, İn Vitro Salıverilmesi Ve İn Vivo Dağılımı Üzerinde Çalışmalar*.Yükseklisans Tezi, Hacettepe Üniversitesi, Ankara.

295.Mazda, F. (1993). K*ozmetik Amaçlı Üre Lipozom Ve Niozomlarının Formülasyonları, Hazırlanması Ve İn Vitro, İn Vivo Değerlendirilmeleri*.Yükseklisans Tezi, Hacettepe Üniversitesi, Ankara.

296.Taravella, B. C., Lesieur, S., Chopineau, J., Lesieur, P., Ollivon, M. (2002). Phase behavior of mixed aqueous dispersions of dipalmitoylphosphatidylcholine and dodecyl glycosides: A differential scanning calorimetry and x-ray diffraction investigation. *Langmuir*, 18(2), 325-335.

297.Skoog, D. A., Holler, F. J., Nieman, T. A. (1998). *Principles Of İnstrumental Analysis* ABD: Sonders College Publishing.

298.Korkmaz, M. (1996). *Dtpa İçeren Lipozom Ve Niozom Taşıyıcı Sistemleri Ve Tc-99m-Dtpa Kitlerinin Görüntülemede Kullanımları Ve Kalite Kontrolleri Üzerine Çalışmalar.* Bilim Uzmanlığı Tezi, Hacettepe Üniversitesi, Ankara.

299.Türker, S. (2004). *Nükleer Görüntüleme Teknikleri Kullanılarak Romatoid Artrit Tedavisinde Diklofenak Sodyum İçeren İlaç Taşıyıcı Sistemlerin, Konvansiyonel Dozaj Şekilleriyle Etkinliklerinin Karşılaştırılması*. Bilim Uzmanlığı Tezi, Hacettepe Üniversitesi, Ankara.

300.Gent, W. L.1969). Thermal properties of cholesterol and estimation of impurity by differential scanning calorimetry. *Journal of Scientific Instruments*, 2 (2), 69-72.

301.Erdoğan, S. (2001). *Derin Ven Trombuslarının Teşhisi Ve Sintigrafik Görüntülenmesi Amacıyla Geliştirilen İlaç Taşıyıcı Sistemler Üzerinde İn Vitro Ve İn Vivo Çalışmalar.* Doktora Tezi, Hacettepe Üniversitesi,, Ankara.

302.Wan, S. C. L., Lee, P. F. S. (1974). Cmc of polysorbates. *Journal of Pharmacutical Sciences*, 63, 136.

303.Erdoğan, S., Özer, A. Y., Ercan, M. T., Aydın, K., Hıncal, A. A. (1998). Biodistribution and computed tomography studies on iopromide liposomes. *STP Pharma Sciences*, 8, 133-137.

304.Arıca, B., Ozer, A. Y., Ercan, M. T., Hıncal, A. A. (1995). Characterization, in vitro and in vivo studies on primaquine diphosphate liposomes. *Journal of Microencapsulation*, 12, 469-485.

305.Mirapex dosage. (t.y.). Erişim: 17 March 2015 http://www.Drugs.Com/dosage/mirapex.Html

306.Parkyn, İlaç prospektüsü available from. (t.y.). Erişim: 24 March 2015 http://www.Ilacprospektusu.Com/ilac/2/parkyn-0-250-mg-100-tablet

307.Abib, E., Duarte L. F., Pereira, R. (2012). Comparative bioavailability: Two pramipexole formulations in healthy volunteers after a single dose administration under fasting conditions. *Journal of Bioequivalance and bioavailability*, 4, 56-59.

308.VonVoigtlander, P. F., Fici, G., Althaus, J. S. (1998). Pharmacological approaches to counter toxicity of dopa. *Amino Acids*, 14, 189-196.

309.Hall, E. D., Andrus, P., Oostveen, J., Althaus, J. S., VonVoigtlander, P. F. (1996). Neuroprotective effects of dopamine d2/d3 agonist pramipexole against postischemic or methamphetamine-induced degeneration of nigrostriatal neurons. *Brain Research,* 742, 80-88.

310.Kitamura, Y., Kohno. Y., Nakaazawa, M., Nomura Y. (1997). Inhibitory effect of talipexole and pramipexole on mptp-induced dopamine reduction in striatum of c57bl/6n mice. *The Japanese Journal of Pharmacology*, 74, 51-57.

311.Zou, L., Xu, J., Jankovic, J. He, Y., Appel, S. H., Le, W. (2000). Pramipexole inhibits lipid peroxidation and reduces injury in substantia nigra

induced by dopaminergic neurotoxin mptp in C57BL/6 mice. *Neuroscience Letters,* 281, 167-170.

312.Ema science medicines health, ema/816841/2011, chmp, london.Erişim: 22 January 2015 Http://www.Ema.Europa.Eu/docs/en_gb/document_library/epar__public_assessment_report/human/002291/wc500116760.Pdf

313.Ferger, B., Buck, K., Shimasaki, M., Koros, E., Voehringer, P., Buerger, E. (2010). Continuous dopaminergic stimulation by pramipexole is effective to treat early morning akinesia in animal models of parkinson's disease: A pharmacokinetic-pharmacodynamic study using in vivo microdialysis in rats. *Synapse*, 64, 533-541.

314.Wakode, R.R., Bajaj, A. N. (2008) *Formulation and characterization of pramipexole loaded microspheres*. Erişim: 13 May 2015 http://priory.Com/pharmacy/pramipexole.Htm

315.Metselaar, J. M., Mastrobattista, E., Storm, G. (2002). Liposomes for intravenous drug targeting: Design and pplications. *Mini Reviews in Medicinal Chemistry*, 2, 319-329.

316.Torchilin, V. P. (2000). Drug targeting. *European Journal of Pharmaceutical Sciences*, 11(2), 81-91.

317.Naveen, K., Jain, A. C. R., Sanjay, K. (1998). Jainbrain drug delivery system bearing dopamine hydrochloride for effective management of parkinsonism. *Drug Development and Industrial Pharmacy*, 24(7), 671475.

318.Bragagni, M., Mennini, N., Ghelardini, C., Mura, P. (2012). Development and characterization of niosomal formulations of doxorubicin aimed at brain targeting. *Journal of Pharmaceutica Pharmaceutical Sciences*, 15(1), 184 - 196.

319.Maruyama, K. (2002). Peg-immunoliposomes. *Bioscience Reports*, 22 (2), 251-266.

320.Yoss, N. L., Mahfouz, M. M., Diao, H. S., Kummerow, F. A. (1992). Sphingomyelin Favors Precipitation of Negatively Charged Liposomes in 1 mM ca^{+2}. *The Journal of Lipid Research*, 2, 237-256.

321.Vemuri, S., Rhodes, C. T. (1995). Prepararti̇on and chararcterization of liposomes as therapeutic delivery systems: A review. *Pharmaceutica Acta Helvetica*, 70, 95-111.

322.Tilcock, C., Unger, E., Cullis, P., MacDougall, P. (1989). Liposomal gd-dtpa: Preparation and characterization of relaxivity. *Radiology*, 171(1), 77-80.

323.Phillips, W. T., Goins, B. (1995). Targeted delivery of imaging agents by liposomes. V. P. Torchilin (Ed.). *Handbook of targeted delivery of imaging agents* (s. 149-173). Boca Raton FL: CRC Press

324.Gregoriadis, G. (1993). *Liposome technology, 2nd edition*. Boca Raton, FL: CRC Press.

325.Schwendener, R. A., Wuthrich, R., Duewell, S., Wehrli, E., Von Schulthess, G. K. (1990). A pharmacokinetic and mri study of unilamellar gadolinium-, manganese-and iron-dtpa-stearate liposomes as organ-specific contrast agents. *Investigational Radiology*, 23, 922-932.

326.Harwood, L. M., Moody, C. J. (1989). *Experimental organic chemistry: Principles and practice*. United States: Blackwell Scientific Publications

327.Reich, E., Schibli, A. (2007). *High-performance thin-layer chromatography for the analysis of medicinal plants*. New York: Thieme.

328.Measurement of hydrophile - lipophile balance (hlb) values, intertek. (t.y.). Erişim: 13 June 2015 http://www.Intertek.Com/chemicals/hlb/

329.Dean, J. A. (1995). *The analytical chemistry handbook.* New York: McGraw Hill.

330.Pungor, E. (1995). *A practical guide to instrumental analysis.* Florida: Boca Raton.

331.O'Neill, M. J. (1964). The analysis of a temperature-controlled scanning calorimeter. *Analytical Chemistry*, 36 (7), 1238-1245.

332.Reusch, W. Infrared spectroscopy. Virtual textbook of organic chemistry: Michigan State University. (t.y.). Erişim: 18April 2015 https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/Spectrpy/InfraRed/infrared.htm#ir1

333.Atkins, P., Paulo, J. D. (2009). *Elements of physical chemistry.* Oxford: Oxford U.P.

334.Li, H., Hu, H., Zhao, Y., Chen, X., Li, W., Qiang, W., et al. (2015) Multifunctional aptamer-silver conjugates as theragnostic agents for specific cancer cell therapy and fluorescence-enhanced cell imaging. *Analytical Chemistry*, 87 (7), 3736-3745.

335.Koi L, Bergman, R., Brüchner, K., Pietzsch, J., Pietzsch, H. J., Krause, M. et al. (2014) Radiolabeled anti-EGFR-antibody improves local tumor control after external beam radiotherapy and offers theragnostic potential. *Journal of the European Society for Radiotherapy and Oncology*, 110(2), 362-369.

336.Sivakumar, B., Aswathy, R., Nagaoka, Y., Suzuki, M., Fukuda, T., Yoshida, Y., et al. (2013) Multifunctional carboxymethyl cellulose-based magnetic nanovector as a theragnostic system for folate receptor targeted chemotherapy, imaging, and hyperthermia against cancer. *Langmuir*, 29(10), 3453-3466.

337.Ryu, J. H., Koo, H., Sun, I. C., Yuk, S. H., Choi, K., Kim, K., et al. (2012) Tumor-targeting multi-functional nanoparticles for theragnosis: New paradigm for cancer therapy. *Advanced Drug Delivery Reviews*, 64(13), 1447-1458.

338.Talsma, H. (1991). *Preparation, characterization and stabilization of liposomes*. PhD Thesis, Utrecht University, Utrecht.

339.Mutlu, N. B. (2007). *Rivastigminin lipozom formülasyonunun geliştirilmesi ve hücre kültürlerinden geçişinin incelenmesi*. Master of Science, Gazi University, Ankara.

340.Flonja, L. (2011). Tümör teşhis ve tedavisi İçin geliştirilen nanoboyutta lipozomal İlaç taşıyıcı sistemler üzerinde in vitro çalışmalar. Master of Science, Hacettepe University, Ankara.

341.Gabizon, A. A., Barenholz, Y., Bialer, M. (1993). Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing polyethylene glycol-derivatized phospholipid: Pharmacokinetic studies in rodents and dogs. *Pharmaceutical Research*, 10, 703-708.

342.Bally, M. B., Nayar, R., Masin, D., Hope, M. J., Cullis, P. R., Mayer, L. D. (1990). Liposomes with entrapped doxorubicin exhibit extended blood residence times. *Biochimica et Biophysica Acta,* 1023, 133-139.

343.Al-Angary, A. A., Bayomi, M .A., Khidr, S. H., Al-Meshal, M. A., Al-Dardiri, M. (1995). Characterization, stability and in vivo targeting of liposomal formulations containing cyclosporin. *International Journal of Pharmaceutics*, 114(2) 221-225.

344.Acartürk F, (2007). Bölüm 10: Reaksiyon kinetiği ve stabilite. F. Acartürk, İ. Ağabeyoğlu, N. Çelebi, T. Değim, Z. Değim, S. Takka, F. Tırnaksız (Ed.). *Modern farmasötik teknoloji* (s. 141-182). Ankara:Türk Eczacıları Birliği Eczacılık Akademisi Yayını

345.Glavas-Dodov, M., Fredro-Kumbaradzi, E., Calıs, S., Gorasinova, K., Mladenovska, K., Simonovska, M. et al. (2004). Formulation and characterisation of 5-fluorouracil loaded liposomes. *Bulletin of the Chemists and Technologists of Macedonia,* 23, 13-18.

346.Tóth, A., Veszelka, S., Nakagawa, S., Niwa, M., Deli, M. A. (2011). Patented in vitro blood-brain barrier models in cns drug discovery. *Recent Patents Drug Discovery*, 6, 107-118.

347.Naik, P., Cucullo, L. (2012). In vitro blood-brain barrier models: Current and perspective technologies. *Journal of Pharmaceutical Sciences*, 101, 1337-1354.

348.Nielsen, P. A., Anderson, O., Hansen, S. H., Simonsen, K. B., Andersson, G. (2011). Models for predicting blood-brain barrier permeation. *Drug Discovery Today*, 16, 472-475.

349.Provenzale, J. M., Silva, G. A. (2009). Uses of nanoparticles for central nervous system imaging and therapy. *American Journal of Neuroradiology*, 30, 1293-1301.

350.Masserini, M. (2013). Nanoparticles for brain drug delivery. *ISRN Biochemistry*, 1-18.

351.Gabathuler, R. (2010). Approaches to transport therapeutic drugs across the blood-brain barrier to treat brain diseases. *Neurobiology of Disease,* , 37, 48-57.

352.Corrodi, H., Fuxe, K.,Ungerstedt, U. (1971). Evidence for a new type of dopamine receptor stimulating agent. *Journal of Pharmacy and Pharmacology*, 23, 989-991.

353.Glick, S. D., Jerussi, T. P., Fleisher, L. N. (1976). Turning in circles: The neuropharmacology of rotation. *Life Science*, 18, 88-96.

354.Moore, K. E., Kelly, P. H. (1978). Biochemical pharmacology of mesolimbic and mesocortical dopaminergic neurons. M. A. Lipton, A. DiMascio, K. F. Killam (Ed.). *Psychopharmacology: A generation of progress* (s. 221-234). New York: Raven Press

355.Amphetamine. (t.y.). Erişim: 29 June 2015 http://www.Drugs.Com/amphetamine.Html

356.Newman, T. Amphetamine: Uses, Side Effects and Contraindications. (t.y.). Erişim: 16 May 2015 http://www.Medicalnewstoday.Com/articles/221211.Php

357.Papathanou M, Rose, S., McCreary, A., Jenner, P. (2011). Induction and expression of abnormal involuntary movements is related to the duration of dopaminergic stimulation in 6-ohda-lesioned rats. *European Journal of Neuroscience*, 33(12), 2247-2254.

358.Holtman, L., Van Vliet, E. A., Appeldoorn, C., Gaillard, P. J., De Boer, M., Dorland, R., et al. (2014). Glutathione pegylated liposomalmethylprednisolone administration afterthe early phase of status epilepticus did notmodify epileptogenesis in the rat. *Epilepsy Research*, 108, 396-404.

359. Arica, B., Kas, H. S., Moghdam, A., Akalan, N., Hincal, A. A. (2005). Carbidopa/levodopa-loaded biodegradable microspheres: in vivo evaluation on experimental Parkinsonism in rats. *Journal of Controlled Release*, 102, 689-697.

360. Jeon, M. Y., Lee, W. Y., Kang, H. Y., Chung, E. J. (2007). The effects of l-3,4-dihydroxyphenylalanine and dopamine agonists on dopamine neurons in the progressive hemiparkinsonian rat models. *Neurology Research*, 29(3), 289-295.

SUPPLEMENTS AND PUBLICATIONS

Supplement 1.

CV

She was borned at Ankara in 1984. She was graduated from Hacettepe University in 2006 and she was won a scholarship from Erasmus Student Exchange Programme to resume her undergraduate programme at Ljubljana University Faculty of Pharmacy in Ljubljana/Slovenia for 5 months in 2005. She was compeleted her master thesis in 2009 at Hacettepe University Faculty of Pharmacy Department of Radiopharmacy. She began her doctoral thesis at the same year and she was won a scholarship from Campus France in 2011 to proceed her cotutelle doctoral thesis in a collaboration between Hacettepe University and François Rabelais de Tours University. She has been working as a research assistant at Hacettepe University Faculty of Pharmacy Department of Radiopharmacy since 2006 and she is the treasurer of Association of Radiopharmacy since 2007.

Supplement 2.

Publications Performed within the Concept of PhD Thesis

- **Articles**
- Silindir Gunay M, Ozer AY, Erdogan S, Bodard S, Baysal I, Gulhan Z, Guilloteau D, Chalon S. Development of Nanosized, Pramipexole-Encapsulated Liposomes and Niosomes for the Treatment of Parkinson's Disease. Journal of Nanoscience and Nanotechnology, (Submitted).

- Silindir Gunay M, Ozer AY, Chalon S. Drug Delivery Systems For Imaging and Therapy of Parkinson's Disease. Current Neuropharmacology, 2016, 14, 376-91.

- Silindir, M, Ozer, A Y. The benefits of pramipexole selection in the treatment of Parkinson's Disease. Neurological Sciences, 2014, 35(10), 1505-1511.

- Silindir, M, Erdogan, S, Ozer, A Y, Maia, S. Liposomes and their application in molecular imaging. Journal of Drug Targeting, 2012, 20(5), 401-415.

- **Oral Presentations**
- Silindir M, Ozer AY. Radiopharmaceuticals and their use for Parkinson's Diseases, International Multidisciplinary Symposium on Drug Research and Development- DRD 2015, Proceedings Book, 69, Anadolu University Faculty of Pharmacy, Eskisehir, 15-17 October 2015.
- Silindir Gunay M. Radyofarmasötikler ve Yeni Gelişmeler. 5. Biyomedikal Mühendisliği Öğrenci Sempozyumu. Tüyap Fuarı, 24-25 March 2016.

- **Poster Presentations**
- Silindir, M, Erdoğan, S, Özer A Y. Characterization of Nanosized Theragnostic Liposomes for the Diagnosis and Therapy of Parkinson's Disease, 16th International Pharmaceutical Technology Symposium (IPTS-

16), Proceedings Book, Lara The Marmara Hotel, Antalya-Turkey, September 10-12 2012. (pp. 121-123).

- Silindir M, Erdogan S, Ozer A Y. Design of Novel Nanosized Theragnostic Niosomes for Diagnosis and Therapy of Parkinson's Disease. 40th Annual Meeting and Exposition of the Controlled Release Society (CRS), Hawaii Convention Center, Honolulu, Hawaii, U.S.A, 21-24 July 2013.

- Silindir M, Erdogan S, Ozer A Y, Guilloteau D, Chalon S. Comperative Evaluation of Pramipexole Encapsulated Theranostic Liposomes And Niosomes For Parkinson's Disease. International Conference on Clinical PET-CT and Molecular Imaging (IPET 2015): PET-CT in the era of multimodality imaging and image-guided therapy, Proceedings Book: IAEA-CN-232/85, IAEA, Vienna, Austria, 5-9 October 2015.

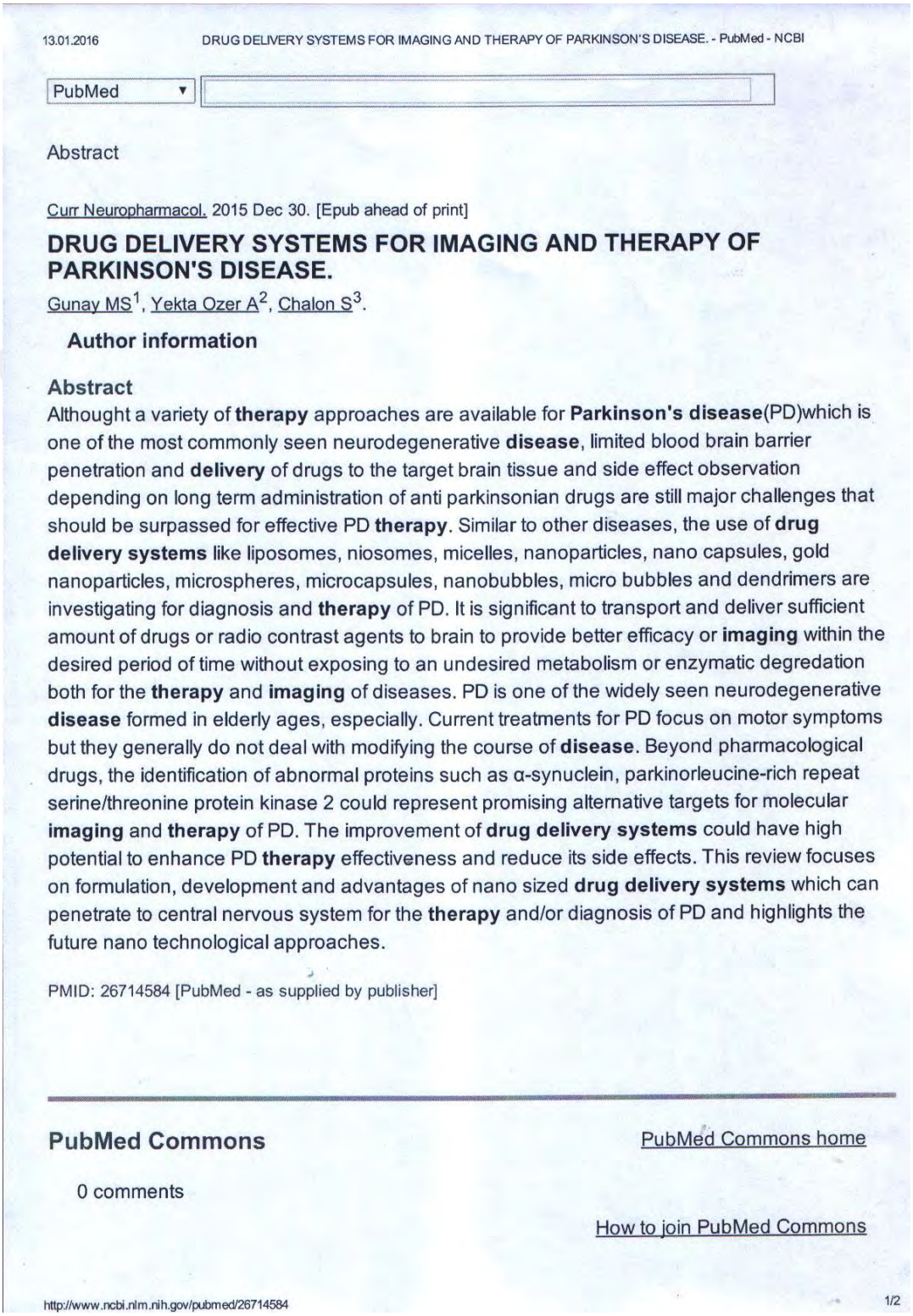
- Silindir Gunay M, Ozer AY, Erdogan S, Baysal I, Guilloteau D, Chalon S. In Vitro Studies on BBB Penetration of Pramipexole Encapsulated Theranostic Liposomes for the Therapy of Parkinson's Disease. 18th European Symposium on Radiopharmacy and Radiopharmaceuticals, 07-10. April 2016, pp. 79, Salzburg/Austria.

- **Project**
- Preparation of Theragnostic Immunoliposomes for the Diagnosis and Therapy of Parkinson's Disease, TUBITAK, Project No: 112S244, December 2012-2013 (As Scholarship Student) (budget: 29.600 TL).

- **Scholarship**
- French Embassy Coutotelle Doctorate Thesis Scholarship supported by Campus France which was supervised by François Rabelais Université de Tours, Faculty of Medicine, Equipe 3, Molecular Imaging and Brain, Inserm U930 and Hacettepe University, Faculty of Pharmacy, Department of Radiopharmacy (2011-2015).

Supplement 3.

Articles



13.01.2016 DRUG DELIVERY SYSTEMS FOR IMAGING AND THERAPY OF PARKINSON'S DISEASE. - PubMed - NCBI

PubMed

Abstract

Curr Neuropharmacol. 2015 Dec 30. [Epub ahead of print]

DRUG DELIVERY SYSTEMS FOR IMAGING AND THERAPY OF PARKINSON'S DISEASE.

Gunay MS[1], Yekta Ozer A[2], Chalon S[3].

Author information

Abstract

Althought a variety of **therapy** approaches are available for **Parkinson's disease**(PD)which is one of the most commonly seen neurodegenerative **disease**, limited blood brain barrier penetration and **delivery** of drugs to the target brain tissue and side effect observation depending on long term administration of anti parkinsonian drugs are still major challenges that should be surpassed for effective PD **therapy**. Similar to other diseases, the use of **drug delivery systems** like liposomes, niosomes, micelles, nanoparticles, nano capsules, gold nanoparticles, microspheres, microcapsules, nanobubbles, micro bubbles and dendrimers are investigating for diagnosis and **therapy** of PD. It is significant to transport and deliver sufficient amount of drugs or radio contrast agents to brain to provide better efficacy or **imaging** within the desired period of time without exposing to an undesired metabolism or enzymatic degredation both for the **therapy** and **imaging** of diseases. PD is one of the widely seen neurodegenerative **disease** formed in elderly ages, especially. Current treatments for PD focus on motor symptoms but they generally do not deal with modifying the course of **disease**. Beyond pharmacological drugs, the identification of abnormal proteins such as α-synuclein, parkinorleucine-rich repeat serine/threonine protein kinase 2 could represent promising alternative targets for molecular **imaging** and **therapy** of PD. The improvement of **drug delivery systems** could have high potential to enhance PD **therapy** effectiveness and reduce its side effects. This review focuses on formulation, development and advantages of nano sized **drug delivery systems** which can penetrate to central nervous system for the **therapy** and/or diagnosis of PD and highlights the future nano technological approaches.

PMID: 26714584 [PubMed - as supplied by publisher]

PubMed Commons PubMed Commons home

0 comments

How to join PubMed Commons

http://www.ncbi.nlm.nih.gov/pubmed/26714584 1/2

Neurol Sci
DOI 10.1007/s10072-014-1891-5

REVIEW ARTICLE

The benefits of pramipexole selection in the treatment of Parkinson's disease

Mine Silindir · A. Yekta Ozer

Received: 27 May 2014 / Accepted: 14 July 2014

Abstract Levodopa administration as a gold standard in Parkinson's disease (PD) treatment is very valuable, however, long-term administration may cause some motor complications such as abnormal unintended movements and shortening response to each dose (wearing off phenomenon). Dopamine agonists were developed to reduce duration of immobile off periods and dependence to levodopa for improving motor impairments (Clarke et al., Cochrane Libr 1:1–23, 2000). Pramipexole is one of these nonergot dopamine agonists with high relative in vitro specificity and full intrinsic activity at D2 subfamily of dopamine receptors, with a higher binding affinity to D3 than to D4 or D2 receptor subtypes (Piercey, Clin Neuropharmacol 21:141–151, 1998). It can be advantageously administered as monotherapy or adjunctive therapy to levodopa to decrease side effects and increase effectiveness in both early and advanced PD treatment.

Keywords Advantages of pramipexole · Parkinson's disease

Introduction

Due to increase in lifetime, an increase is observed in the incidence of geriatric diseases, parallelly in research in this field across the world. One of the most recently observed neurodegenerative diseases is PD diagnosed over age 50 generally; however, it can also be seen in young people about 5 % among all patients and is called juvenile PD. While disease incidence is 0.2–0.3 % in the community, it is 1 % among people over the age of 55. PD took its name from an English doctor James Parkinson in 1817, who published about the disease first in An Essay on Shaking Palsy. Its motor symptoms took its source from degeneration or death of dopamine-generating cells in substantia nigra of midbrain [3]. While movement-related symptoms are observed in earlier PD, cognitive, behavioral problems are generally observed in progressive levels of disease. In some cases of subsequent advanced stages, dementia may be occurred [4].

Antiparkinsonian drugs comprise dopaminergics and antimuscarinics. While dopaminergics are used for potentiating actions of dopamine, antimuscarinics are used for reducing excessive central cholinergic effects. Antimuscarinics are grouped as tertiary amines. Dopaminergics are grouped as levodopa, peripheral dopa-decarboxylase inhibitors, apomorphine, adamantamine and amantamine, ergot derivatives, various other nonergot dopamine agonists including pramipexole, specific monoamine oxidase type B inhibitors, and catechol-*O*-methyltransferase inhibitors [5]. Pramipexole is developed as either adjunctive therapy to levodopa or monotherapy in PD treatment.

Physicochemical properties

Pramipexole dihydrochloride monohydrate ((*S*)-2-amino-4,5,6,7-tetrahydro-6-propylamine-benzothiazole dihydrochloride) is a white to off-white crystalline powder (302,27 g mol^{-1}) and stable under ordinary conditions. Its solubility is more than 20 % in water, about 8 % in methanol, 0.5 % in ethanol and practically insoluble in dichloromethane [6]. Due to high solubility and high permeability

M. Silindir · A. Y. Ozer (✉)
Department of Radiopharmacy, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey
e-mail: yktzer@yahoo.com; ayozer@hacettepe.edu.tr

M. Silindir
e-mail: mines@hacettepe.edu.tr; msilindir@yahoo.com

Published online: 20 July 2014

Journal of Drug Targeting, 2012; 20(5): 401–415
© 2012 Informa UK, Ltd.
ISSN 1061-186X print/ISSN 1029-2330 online
DOI: 10.3109/1061186X.2012.685477

informa
healthcare

REVIEW ARTICLE

Liposomes and their applications in molecular imaging

Mine Silindir[1], Suna Erdoğan[1], A. Yekta Özer[1], and Serge Maia[2]

[1]*Department of Radiopharmacy, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey and* [2]*Service de Pharmacie-Radiopharmacie, Hôpital Bretonneau, CHRU de Tours, Tours, France*

Abstract
Molecular imaging is a relatively new discipline with a crucial role in diagnosis and treatment tracing of diseases through characterization and quantification of biological processes at cellular and sub-cellular levels of living organisms. These molecular targeted systems can be conjugated with contrast agents or radioligands to obtain specific molecular probes for the purpose of diagnosis of diseases more accurately by different imaging modalities. Nowadays, an interesting new approach to molecular imaging is the use of stealth nanosized drug delivery systems such as liposomes having convenient properties such as biodegradability, biocompatibility and non-toxicity and they can specifically be targeted to desired disease tissues by combining with specific targeting ligands and probes. The targeted liposomes as molecular probes in molecular imaging have been evaluated in this review. Therefore, the essential point is detection of molecular target of the disease which is different from normal conditions such as increase or decrease of a receptor, transporter, hormone, enzyme etc. or formation of a novel target. Transport of the diagnostic probe specifically to targeted cellular, sub-cellular or even to molecular entities can be performed by molecular imaging probes. This may lead to produce personalized medicine for imaging and/or therapy of diseases at earlier stages.

Keywords: Molecular imaging, molecular targeting, drug delivery systems, targeted liposomes, targeted delivery systems, liposomes for imaging

Introduction

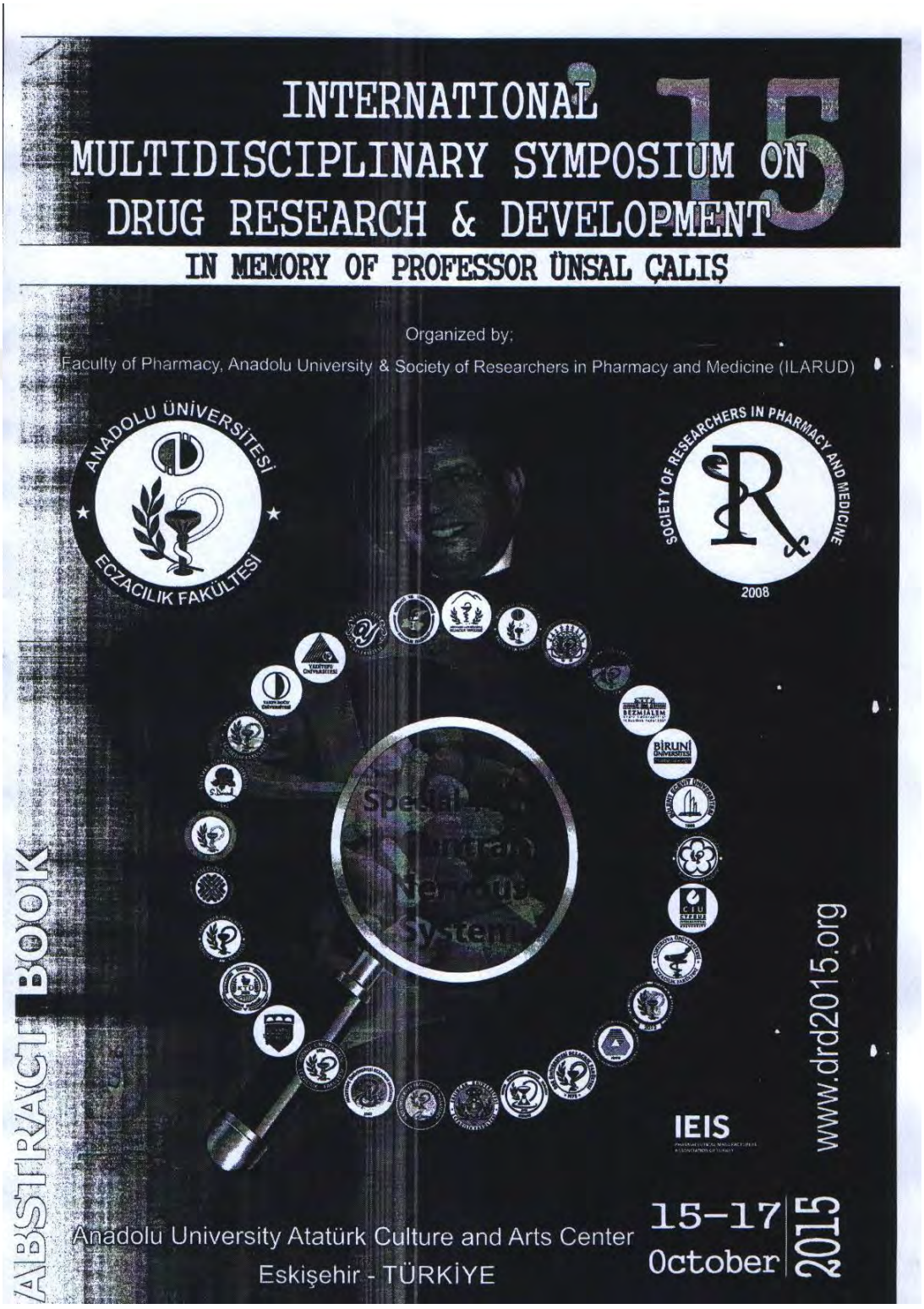
Considerable progress has been achieved in the field of molecular imaging due to recent advances in imaging modalities, molecular biology and chemistry (Joshi and Wang, 2010). Molecular imaging is defined as the measurement, characterization and visualization of biological processes in cellular and molecular level in human and other living systems (Blasberg, 2003; Cai and Chen, 2008). Imaging of multimolecular processes performing in the same time interval, gene expressions or protein–protein interactions, monitoring of cell targeting, drug and gene therapy optimization, determination of the progress of molecular pathologic diseases, imaging of drug effect at molecular and cellular level, supplying rapid, reproducible and three dimensional computerized images with quantitative results of therapeutic effects of gene products on animals or patients, therapy tracing are some significant application fields of molecular imaging (Blasberg, 2003; Massoud and Gambhir, 2003; Saha, 2004). Many branches like molecular biology, cellular biology and imaging technology are related with molecular imaging (Massoud and Gambhir, 2003). Table 1 represents significant properties of some imaging modalities and molecular probes (Massoud and Gambhir, 2003). The basic principle of molecular imaging depends on obtaining significantly high signal intensity by the use of minimal amount of molecular probe. Studies on the development of special reagents, ligands, protocols and devices for molecular imaging have been carried out over the past two decades. Better biocompatible probes/ligands for selecting appropriate cellular and sub-cellular targets for imaging were developed. The delivery of these probes by overcoming biological barriers and image amplification for detection of trace amount of target concentrations such as picomolar–nanomolar (pM–nM) and development of imaging systems to a level of higher spatial/temporal

Address for Correspondence: Prof.Dr.A.Yekta Özer, Department of Radiopharmacy, Faculty of Pharmacy, Hacettepe University, 06100, Sihhiye, Ankara, Turkey. Tel: +90(312)3052196. Fax: +90(312)3114777. E-mail: ayozer@hacettepe.edu.tr, yktzer@yahoo.com

(Received 01 December 2011; revised 26 March 2012; accepted 12 April 2012)

Supplement 4.

Oral Presentations



INTERNATIONAL MULTIDISCIPLINARY SYMPOSIUM ON DRUG RESEARCH & DEVELOPMENT 15

OP13 **RADIOPHARMACEUTICALS and THEIR USE for PARKINSON'S DISEASES**

Mine Silindir, A. Yekta Özer

Hacettepe University, Faculty of Pharmacy, Department of Radiopharmacy, 06100, Sıhhiye, Ankara, Turkey

(mines@hacettepe.edu.tr, msilindir@yahoo.com)

Radiopharmaceuticals are radioactive drugs composed of a radionuclide and a pharmaceutical for the diagnosis and/or therapy of many diseases comprising neurodegenerative diseases. While about 95% of the radiopharmaceuticals are used for the diagnostic purposes, the rest can be used for therapeutic purposes of the diseases (1). Although they are used in very minimal amounts, they should be sterile and pyrogen free, and they have to be undergone all the quality control measurements for drugs and also some special tests for the radioactive part (1). A variety of different applications were performed for the diagnosis of PD at Nuclear Medicine clinics with the use of specific radiopharmaceuticals. The precise differential diagnosis can be performed with the assessment of the accumulation of pathologic proteins and dopaminergic system besides brain perfusion and glucose metabolism (2).

Parkinson's disease (PD) is one of the widely seen neurodegenerative disease formed in elderly ages. Although PD is diagnosed over the age of 50 generally, it is not the disease only seen in geriatrics and can be seen as 5% in young people that is called as Juvenile PD. While about 10 million people suffer from PD worldwide, about 100-130 thousand people were diagnosed with PD in Turkey. It is expected that this number will be duplicated in 2030 in Turkey in which about 10 thousand people is diagnosed with PD every year (3, 4). Current treatments for PD generally focuses on curing the motor symptoms however these treatments generally do not deal with modifying the course of disease.

Although different diagnosis and therapy approaches are available for PD, there are still major challenges that should be surpassed for effective PD therapy such as limited blood brain barier (BBB) penetration, effective drug delivery to the target brain tissue and enhanced side effect observation depending on long term administration of antiparkinsonian drugs. It is significant to transport and deliver sufficient amount of drugs or radiocontrast agents to brain by obtaining the efficacy or imaging in the desired period of time without exposing to an undesired methabolism or enzymatic degredation both for the therapy and imaging of diseases. Similar to other diseases, the use of nanosized drug delivery systems like liposomes, niosomes, micelles, nanoparticles, nanocapsules, gold nanoparticles, microspheres, microcapsules, nanobubbles, microbubbles and dendrimers are investigating for diagnosis and therapy of PD for highlighting the future nanotechnological approaches.

References:

[1] Saha GB. Radiopharmaceuticals and Methods of Radiolabeling, In: Saha GB (editor), Fundamentals of Nuclear Pharmacy, 6th ed. USA: Springer, 2010, p. 83-114.

[2] Akdemir UO, Atay Kapucu LO. Parkinsonizmin görüntülenmesinde son gelişmeler: Nükleer tıp yaklaşımı, Parkinson Hastalığı ve Hareket Bozuklukları Dergisi 17: 13-24, 2014.

[3] Parkinson Hastalığı Derneği, 2015 Mar. Available from: URL: http://www.parkinsondernegi.com/

[4] Milliyet, Pembe Nar, Parkinsona Hareketle Direnin. 2015 Mar. Available from: URL: http://www.milliyet.com.tr/parkinsona-hareketle-direnin--pembenar-detay-genelsaglik-1864976/.

2006 NAMIK KEMAL ÜNİVERSİTESİ

Biyomedtek Ar-Ge Topluluğu NKÜ

5. Biyomedikal Mühendisliği Öğrenci Sempozyumu

24-25 Mart 2016

Biyomedikal Mühendisliği

cmf-bmm.web.nku.edu.tr
www.biyomedtekarge.org
5biyosemp.biyomedtekarge.org

25 Mart 2016 CUMA

10:30 - 11:15 Açılış
- **Doç. Dr. Betül Taşdelen**
(Namık Kemal Üniversitesi, Biyomedikal Mühendisliği Bölüm Başkanı)
- **Yrd. Doç. Dr. Hale Pınar Zengingönül**
(Namık Kemal Üniversitesi, Biyomedikal Mühendisliği Bölümü, Sempozyum Başkanı)

10:45 - 11:15
- **Prof. Dr. Musa Hakan Asyalı**
(Yıldız Teknik Üniversitesi, Biyomedikal Mühendisliği Bölüm Başkanı)
- **Doç. Dr. Kamuran A. Kadıpaşaoğlu**
(Yıldız Teknik Üniversitesi, Biyomedikal Mühendisliği Bölümü)

Biyomedikal Mühendisliğinde Son Gelişmeler

11:15 - 11:45 Şermin Bilginer
(İstem Tıbbi Cihazlar, Biyomedikal Mühendisi)
İstem Tıbbi Cihazlar ve Medikal Sektörün Gidişatı

11:45 - 12:30 Osman Fikret Küçükdeveci
(Kardiosis A. Ş., Şirket Ortağı ve Proje Yöneticisi)
Kardiosis Tanıtım ve Ar-Ge Çalışmaları

12:30 - 14:00 ARA

14:00 - 14:30 Ufuk Karanfil
(İstanbul Çekmece Kamu Hastaneler Birliği Genel Sekreterliği Birim Uzmanı, Tıbbi Hizmetler Başkanlığı Koordinatörü)
Kamu Hastaneler Birliği Klinik Mühendislik Hizmetleri ve Biyomedikal Mühendisliğinin Gelişimi

14:30 - 15:00 Kerem DÜNDAR
(Nöro Sağlık, Beyin Araştırmaları Eğitim Uygulama Merkezi Kurucusu)
Dijital Dünyada Beynin Kullanımı

15:00 - 15:30 Kuntay AKTAŞ
(BTECH İnnovation A. Ş., Genel Müdür)
3 Boyutlu Teknolojilerin Sağlık Sektöründe Kullanımı

15:30 - 16:00 Uzm. Ecz. Mine Silindir Günay
(Hacettepe Üniversitesi)
Radyofarmasötikler ve Yeni Gelişmeler

16:00 - 16:15 Muhammed Elmaoğlu
(Medical Imaging Academy Kurucusu)
Medikal Görüntüleme

16:15 - 17:00 MEZUNLAR BULUŞUYOR
- **Azat Achilov**
(Ekol Tıbbi Cihazlar, Biyomedikal Mühendisi)
- **Cemile Altun**
(Türkiye Kamu Hastaneler Kurumu, Klinik Mühendislik Hizmetler Birimi, Biyomedikal Mühendisi)
- **Ceren Yıldız**
(Meditel Medikal Teknik Elektronik A.Ş.,Biyomedikal Mühendisi)
- **Yağmur Birgi**
(Türkiye Kamu Hastaneler Kurumu, Klinik Mühendislik Hizmetler Birimi, Biyomedikal Mühendisi)

17:00 Kapanış

YER: EXPOMED Tüyap Fuar ve Kongre Merkezi
Karadeniz Salonu

RADYOFARMASÖTİKLER VE YENİ GELİŞMELER

Mine Silindir Gunay, A. Yekta Özer

Hacettepe Üniversitesi, Eczacılık Fakültesi, Radyofarmasi ABD., 06100, Sıhhiye, Ankara, Türkiye.

Radyasyonun sağlık için kullanılması günlük yaşantımızda büyük yararlar sağlamaktadır. Radyofarmasötikler, tıpta teşhis ve tedavi amacıyla kullanılan radyoaktif maddelerdir. Nükleer tıp'ta radyofarmasötiklerin %95'i teşhis, ancak % 5'i tedavi amacıyla kullanılmaktadır. Radyofarmasötikleri diğer ilaçlardan ayıran husus herhangi bir farmakolojik etkileri olmamasıdır. Çünkü eser miktarlarda kullanılmaktadırlar [1]. Radyofarmasötiklerin çoğu kullanılacakları nükleer tıp laboratuarlarında uygun korunma sağlanarak sıcak lab.'larda hazırlanırlar. Radyofarmasötiklerin hazırlanması, kalite kontrolü, dağıtımı, kayıtlarının tutulması, saklanması vb. işlerle uğraşan bilim dalına da, "Radyofarmasi (Nükleer Eczacılık)" adı verilir.

Radyofarmasötiklere ait gelişmeler ilk radyofarmasötiğin 1948'de Abbott Laboratuvar'ları ile piyasaya sürmelerinden bugüne kadar geçen sürede büyük gelişme ve büyüme göstermiştir. Radyofarmasi alanındaki bu gelişmeler kısa sürede gelişme ve yaygınlaşma sayesinde Amerikan Eczacıları Birliği 1978'de Eczacılık Uzmanlık Kurulu'nda "Radyofarmasi"yi ayrı bir uzmanlık dalı olarak tanımlamasını da içermektedir. Radyofarmasi'nin Eczacılık eğitiminin lisans ve yüksek lisans programına girerek özel bir uzmanlık alanı olarak ortaya çıkması önemli bir gelişme olmuştur [2].

Nükleer tıp ve Radyoloji alanında kullanılan görüntüleme cihazlarındaki gelişmelere bağlı olarak, hedefe spesifik olarak gidebilen ve hastalığın olduğu bölgeyi etrafındaki normal doku ve organlardan ayır edebilen, yüksek duyarlılık ve özgünlükteki radyofarmasötiklere ihtiyaç duyulmaktadır. Bu amaçla pek çok hastalığa spesifik olarak hedeflendirilebilen modifiye edilmiş multifonksiyonel nanopartiküler taşıyıcı sistemlerin hazırlanması ile teşhis ve/veya tedavi sağlanabilmektedir. Dünyada olduğu gibi ülkemizde de son yıllarda kanser, nörodejeneratif hastalıklar, kardiyoloji ve pek çok alanda yüzel modifikasyonu, polimer kimyası ve hedeflendirme ile hedefe spesifik radyofarmasötik ve teragnostikler üzerinde umut vaad eden çalışmalar yürütülmektedir.

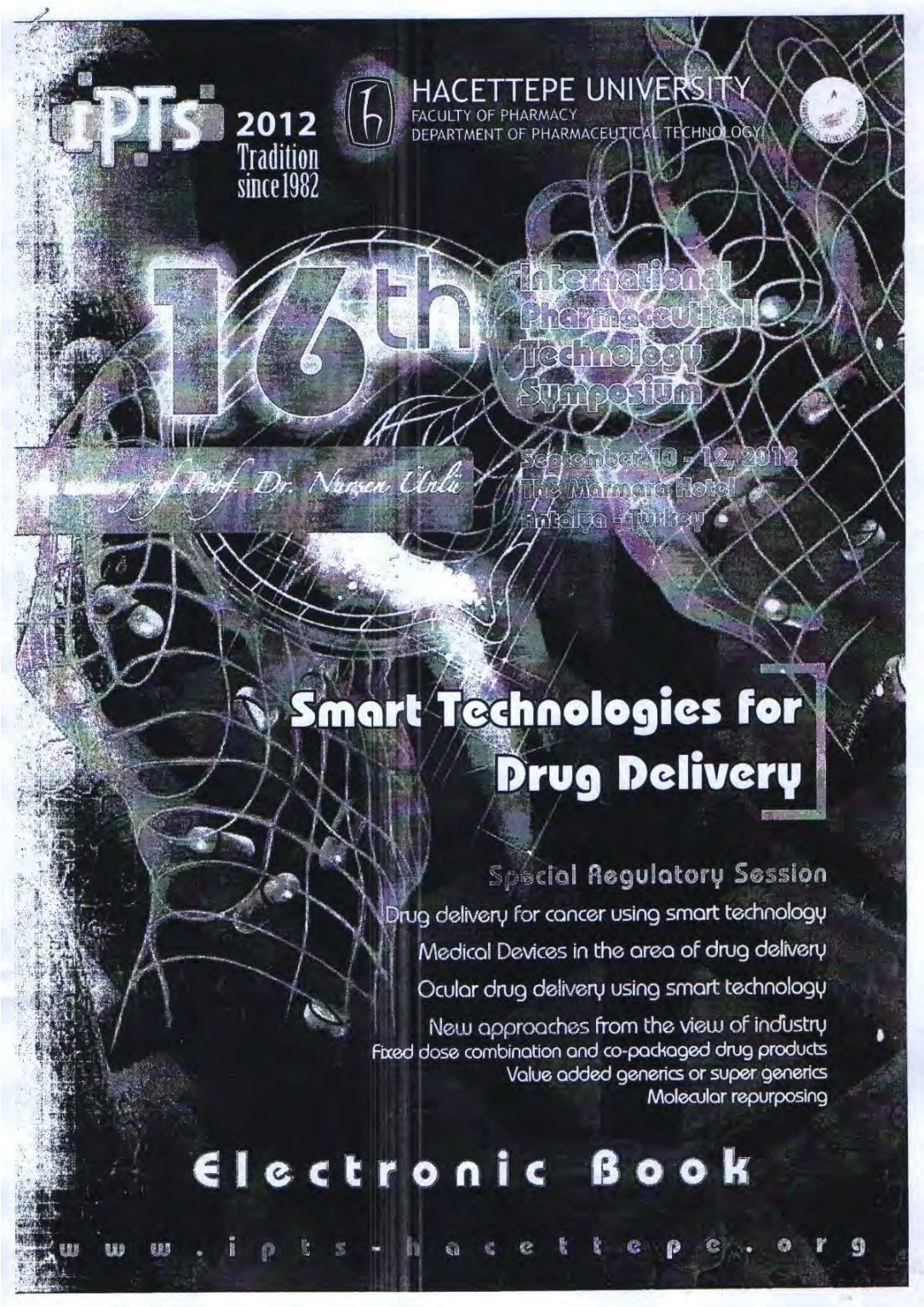
Anaktar kelimeler: Radyofarmasötikler, radyofarmasist, hedefe spesifik multifonksiyonel nanopartiküler sistemler.

References:

1. M.T.Ercan, Radyofarmasötikler, www.google.com.tr/search
2. Özer AY. Radyofarmasötik İlaçlar, İlaç Endüstrisinde Fikri Mülkiyet Hakları. Fersa Ofset, Ed: Goknur Aktay, Ankara: 2009, pp: 1-68.

Supplement 5.

Poster Presentations



P 14

Characterization of Nanosized Theragnostic Liposomes for the Diagnosis and Therapy of Parkinson's Disease

M. Silindir, S. Erdoğan, A.Y. Özer

Hacettepe University, Faculty of Pharmacy, Department of Radiopharmacy, Ankara, Turkey.

Introduction

One of the most recently observed neurodegenerative disease among geriatric diseases is the Parkinson's Disease (PD) which is diagnosed generally over age 50 generally in Turkey and the world. It can also be seen in 5% of young people. PD is hard to diagnose. By using the imaging modalities such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT), the decline in the accumulation of the radiotracer in substantia nigra of the brain can be detected. Therefore, some molecular targets such as Dopamine (DA) and Dopaminergic Transporter (DAT) should be specifically chosen. Drug therapy is one of the mostly used methods for the therapy of PD. One of the most crucial points in drugs that are used for the therapy is the limited amount of the drug penetrated into brain. However, any increase in the drug concentration also increases the risk of side effect observation as proportional. Therefore, it is needed to increase the drug concentration by not causing any rise in the side effects. Nanosized drug delivery systems such as liposomes have many advantages in brain delivery for the purpose of either therapy or diagnosis which can be targeted passively or actively. The studies performed in recent years generally depend on the development of drug delivery systems for the purpose of both the diagnosis and therapy which are called theragnostics. Diagnosis can be managed at the same time of the therapy and the effectiveness of the therapy can also be evaluated.

In this research, it is aimed to develop PolyEthylene Glycole (PEG) coated, nanosized, radiolabeled with ^{99m}Tc for molecular imaging with SPECT and antiparkinson drug pramipexole dihydrochloride monohydrate (pramipexole) encapsulated liposomes for the diagnosis and therapy of PD. Following the preparation of liposomal dispersions, the characterization studies were also performed.

Keywords:

Brain Targeting, Molecular Imaging, Parkinson's Disease, Theragnostic Liposomes, Pramipexole.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC) (Avanti Polar Lipids Inc.) is used as phospholipid, cholesterol (Chol) (Sigma) is used as bilayer condenser and DSPE-PEG_{2000} (Lipoid) for PEG-coating. Pramipexole (Abdi İbrahim) is used as antiparkinson agent.

Synthesis of DTPA-PE to prepare DTPA-PE for radiolabeling of liposomes, 4 mL of chloroform was added on 0,2 mmol Egg PC and stirred. 30µL triethylamine was added on the mixture. 1 mmol DTPA (Diethylene triamine penta acetate) anhydride was dissolved in 20 mL of DMSO (dimethysulfoxide) in another place and this mixture was added dropwise on the other mixture in the existence of argon gas and incubated for 3 hours. Precipitation was obtained after adding distilled water on the mixture and filtered and lyophilized (1, 2). The synthesis of DTPA-PE was observed by IR spectra with pressed potassium bromide tablets at 4000-400 cm^{-1} bands. TLC determination was also performed for the formation of the complex by observing the spot at UV lamp with 254 nm.

Preparation of Theragnostic Liposomes

Pramipexole encapsulated DPPC:Chol:PEG_{2000}-PE:DTPA-PE (10:5:1:0.16 mole ratios %) and positive charged DPPC:Chol:PEG_{2000}-PE:SA:DTPA-PE (10:4:1:1:0.16 mole ratios %) liposomes were prepared according to the film method (3). Briefly, phospholipid and other ingredients were dissolved in chloroform and afterwards chloroform was evaporated in a rotavapor under vacuum system. The film layer was hydrated with Tris buffer (20 mM pH: 7.4) containing pramipexole (5 $mg.mL^{-1}$) for 30 min at 70°C. Liposomes were

CRS 2013 Program

40th Annual Meeting & Exposition of the Controlled Release Society

July 21–24, 2013
Hawaii Convention Center
Honolulu, Hawaii, U.S.A.

CRS
Leading Delivery Science and Technology

Emerging Challenges for Global Delivery

Download the CRS Annual Meeting Mobile App

201 Bifunctional Solid Lipid Nanoparticle System as a Potential Tool for Tumor Detection Using Optical and MR Imaging. Presenter: Xiao Yu Wu, Departmen of Pharmaceutical Sciences, University of Toronto, Canada. Author(s): P. Prasad, A. Abbasi, X. Wu. VIEW ABSTRACT

202 Biomimetic Nanoarchitectures of Peptide Dendrimers for Drug/Gene Delivery. Presenter: Zhongwei Gu, National Engineering Research Center for Biomaterials, Sichuan University, Prc Peoples Rep of China. Author(s): Z. Gu, K. Luo, X. Xu, B. He. VIEW ABSTRACT

203 Bioreducible Nanoparticles for Intracellular Hydrogen Peroxide Generation. Presenter: Fwu-Long Mi, Department of Biochemistry, School of Medicine, Taipei Medical University, Taipei, Taiwan, Taiwan. Author(s): F. Mi, Y. Su, S. Yu, D. Tang, Y. Ho, A. Chao. VIEW ABSTRACT

204 Bladder cancer targeting nanomicelle. Presenter: Tzuyin Lin, UCDavis, U.S.A. Author(s): T. Lin. VIEW ABSTRACT

206 Chitosan oligosaccharide-arachidic acid-based nanoprobes for magnetic resonance imaging of cancer. Presenter: Hyun-Jong Cho, Kangwon National University, Korea. Author(s): H. Cho, U. Termsarasab, D. Kim. VIEW ABSTRACT

207 Comparing the Acoustic Release of Doxorubicin from Targeted and non-Targeted Polymeric Micelles. Presenter: Ghaleb Husseini, American University of Sharjah, Uae. Author(s): G. A. Husseini, L. Kherbeck, W. G. Pitt, J. A. Hubbell. VIEW ABSTRACT

209 DDS Evaluation Using Intravital Real-Time Laser Scanning Confocal Microscopy. Presenter: Yu Matsumoto, The University of Tokyo, Japan. Author(s): Y. Matsumoto, T. Nomoto, K. Toh, N. Nishiyama, T. Yamasoba, K. Kataoka. VIEW ABSTRACT

210 Design of Novel Nanosized Theragnostic Niosomes for Diagnosis and Therapy of Parkinson's Disease. Presenter: A. Yekta özer, Hacettepe University Faculty of Pharmacy Department of Radiopharmacy, Turkey. Author(s): M. Silindir, S. Erdogan, A. özer. VIEW ABSTRACT

212 Development of Targeted [64Cu]CuS Nanoparticles for PET/CT Imaging and Photothermal Ablation Therapy. Presenter: Lili Cui, university of Houston, U.S.A. Author(s): L. Cui, M. Zhou, C. Xiong, C. Li, D. Chow. VIEW ABSTRACT

213 Development of Three-Dimensional Lung Multicellular Spheroids for the Evaluation of Anti-Cancer Therapeutics. Presenter: Samantha Meenach, University of Kentucky, U.S.A. Author(s): S. A. Meenach, A. N. Tsoras, R. C. McGarry, H. M. Mansour, J. Z. Hilt, K. W. Anderson. VIEW ABSTRACT

214 Differentiation of drug release from geopolymer formulations by a new bench-top method. Presenter: Bing Cai, Uppsala University, Sweden. Author(s): B. Cai, H. Engqvist, S. Bredenberg. VIEW ABSTRACT

215 Dual Temperature- and pH-Responsive Fluorescence Molecular Probe for Cellular Imaging utilizing a-Functional Polymer. Presenter: Minami Matsuura, Keio University, Japan. Author(s): M. Matsuura, T. Funatsu, Y. Maekawa, Y. Hiruta, T. Okano, H. Kanazawa. VIEW ABSTRACT

216 Dual-targeting paclitaxel-loaded nanoparticles for the treatment of glioma in mice. Presenter: Zhiqing Pang, Department of Pharmaceutics, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, Prc Peoples Rep of China. Author(s): Z. Pang, B. Zhang, Y. Hu. VIEW ABSTRACT

217 Dynamic contrast enhanced computed tomography imaging of perfusion predicts for the heterogeneous spatial distribution of liposomes. Presenter: Shawn Stapleton, University of Toronto, Canada. Author(s): S. Stapleton, M. Pintilie, C. Allen, D. A. Jaffray. VIEW ABSTRACT

218 Effect of Polymers on API Precipitation Determined by Polarized Light Microscopy. Presenter: Anette Müllertz, Department of Pharmacy, University of Copenhagen, Denmark. Author(s): L. G. Jensen, J. X. Wu, T. Rades, J. Rantanen, B. Abrahamsson, A. Müllertz. VIEW ABSTRACT

219 EPR effect in metastatic and autochthonous tumor and antimetastatic effect of SMA-pirarubicin (THP). Presenter: Hiroshi Maeda, Sojo University, Japan. Author(s): H. Maeda, H. Nakamura, K. Tsukigawa, L. Liao, J. Fang. VIEW ABSTRACT

220 Facile Preparation of Calcium Phosphate Nanoparticles for Noninvasive Cancer Diagnosis. Presenter: Peng Mi, The University of Tokyo, Japan. Author(s): P. Mi, D. Kokuryo, H. Cabral, M. Kumagai, I. Aoki, N. Nishiyama, K. Kataoka. VIEW ABSTRACT

222 Fluorescence Real-time Imaging of Cell Dynamics in Tissue of a Living Mouse using Tissue Fixing Suction Device. Presenter: Yuriko Higuchi, Graduate School of

International Conference on Clinical PET-CT and Molecular Imaging (IPET 2015): PET-CT in the Era of Multimodality Imaging and Image-Guided Therapy

5 – 9 October 2015
IAEA Headquarters, Vienna, Austria

Organized by the IAEA

In cooperation with:

- ACNM — American College of Nuclear Medicine (ACNM)
- ARSNM — Arab Society of Nuclear Medicine (ARSNM)
- AOFNMB — Asia Oceania Federation of Nuclear Medicine and Biology (AOFNMB)
- Asian School of Nuclear Medicine (ASNM)
- ARCCNM — Asian Regional Cooperative Council for Nuclear Medicine (ARCCNM)
- Australian and New Zealand Society of Nuclear Medicine (ANZSNM)
- BNMS — British Nuclear Medicine Society (BNMS)
- CANM ACMN — Canadian Association of Nuclear Medicine (CANM)
- EANM — European Association of Nuclear Medicine (EANM)
- EFOMP — European Federation of Organisations in Medical Physics (EFOMP)
- ESR — European Society of Radiology (ESR)
- IOMP — International Organization for Medical Physics (IOMP)
- ISORBE — International Society of Radiolabeled Blood Elements (ISORBE)
- MDACC — MD Anderson Cancer Center
- Singapore General Hospital — Singapore General Hospital (SGH)
- SNMMI — Society of Nuclear Medicine and Molecular Imaging (SNMMI)
- Society of Radiopharmaceutical Sciences — Society of Radiopharmaceutical Sciences (SRS)
- World Association of Radiopharmaceutical and Molecular Therapy (WARMTH)
- World Federation of Nuclear Medicine and Biology (WFNMB)
- WMIS — World Molecular Imaging Society (WMIS)

International Conference on Clinical PET-CT and Molecular Imaging (IPET 2015): PET-CT in the Era of Multimodality Imaging and Image-Guided Therapy

BOOK OF ABSTRACTS

IAEA-CN-232

International Conference on Clinical PET-CT and Molecular Imaging (IPET 2015): PET-CT in the Era of Multimodality Imaging and Image-Guided Therapy

IAEA-CN-232/85

Comparative Evaluation of Pramipexole Encapsulated Theranostic Liposomes and Niosomes for Parkinson's Disease

M. Silindir[1], A.Y. Ozer[2], S. Erdogan[2], D. Guilloteau[3], S. Chalon[4]

[1] Department of Radiopharmacy, Faculty of Pharmacy, Hacettepe University, 06100,Sihhiye, Ankara, Turkey and UMR INSERM U930, Faculty of Medicine, Université François Rabelais de Tours, Tours, France
[2] Department of Radiopharmacy, Faculty of Pharmacy, Hacettepe University, 06100, Sihhiye, Ankara, Turkey
[3] UMR INSERM U930, CNRS ERL 3106, Université François Rabelais de Tours, Tours, France
[4] UMR INSERM U930, Faculty of Medicine, Université François Rabelais de Tours, Tours, France

Corresponding Author: msilindir@yahoo.com

Background: Parkinson's disease (PD) is defined as a degenerative disorder of CNS. It is a chronic and progressive movement disorder. Its motor symptoms result from the death of dopamine generating cells in the substantia nigra of midbrain. PD is assumed as one of the most recently observed neurodegenerative disease among geriatric diseases diagnosed generally over age 50 all around the world. However, it can also be seen in 5% of young people. PD is hard to diagnose. By using the imaging modalities such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), the decline in the accumulation of the radiotracer in substantia nigra can be detected.
For PD treatment, drug therapy is one of the mostly used methods. Limited amount of drug penetrating into the brain is one of the most crucial points. It is needed to increase the drug concentration by not causing any rise in side effects. Passively or actively targeted nanosized drug delivery systems such as liposomes and niosomes have many advantages in brain delivery for the purpose of either therapy or diagnosis. Recent studies performed around this issue generally depend on development of new drug delivery systems in which diagnosis can be managed together with therapy, which is called theranostics, and therapy effectiveness can also be evaluated.

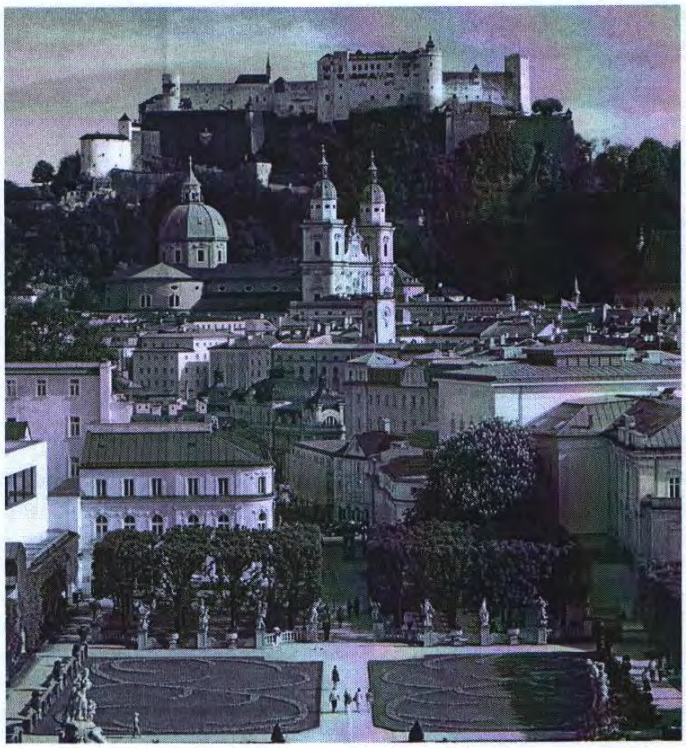
Methodology: In this research, liposomes and niosomes were formulated for the diagnosis and therapy of PD through passive brain targeting; they were coated in polyethylene glycol (PEG), nanosized, neutral or positively charged, 99mTc labeled for SPECT imaging, encapsulated with pramipexole dihydrochloride monohydrate (pramipexole). Following preparation of liposomal and niosomal dispersions, their characterization and release kinetics were evaluated and compared.

Results: In both formulations nanosized particles and proper zeta potentials and about 10% pramipexole encapsulation efficiency was observed.

Conclusion: Promising characterization and release profiles for both diagnosis and therapy of PD were obtained with these theranostic systems. To obtain better results it is needed to perform further in vivo animal studies and our studies are continuing.

(The authors would like to thank for generous gifts of Abdi Ibrahim Ilaç for Pramipexole, to Lipoid for PEG-PE. This study was supported by the grant of TUBITAK (Project No: 112S244)).

18th European Symposium on Radiopharmacy and Radiopharmaceuticals



FINAL PROGRAMME

April 07-10, 2016 • Salzburg, Austria

18th European Symposium
on Radiopharmacy and Radiopharmaceuticals
April 07-10, 2016 • Salzburg, Austria

PP21

In Vitro Studies on BBB Penetration of Pramipexole Encapsulated Theranostic Liposomes for the Therapy of Parkinson's Disease

Mine Silindir Gunay1, A. Yekta Ozer1, Suna Erdogan1, Ipek Baysal3, Denis Guilloteau2, Sylvie Chalon2
1Department of Radiopharmacy, Faculty of Pharmacy, Hacettepe University, 06100, Ankara, Turkey.
2UMR INSERM U930,Université François Rabelais de Tours, Tours, France.
3Department of Biochemistry, Faculty of Pharmacy, Hacettepe university, 06100, Ankara, Turkey.

Introduction: Brain penetration and targeting is hard due to complex structure with different barriers such as blood–brain barrier (BBB) with blood–cerebrospinal fluid (CSF) interface and CSF–blood interface (1, 2). Although these tight and rigid barriers protect brain, they also prevent penetration of molecules, drugs and radiopharmaceuticals for diagnosis and therapy of many neurodegenerative diseases. Parkinson's disease (PD) is assumed to be one of the most frequently observed neurodegenerative disease among geriatric disorders. PD comprises motor symptoms resulting from the death of dopamine generating cells in the substantia nigra. By using the imaging modalities such as single photon emission computed tomography (SPECT), the decline in the accumulation of specific radiotracer in the striatum can be detected. For PD treatment, limited brain penetration of drug is a major concern. Passively or actively targeted, nanosized drug delivery systems such as liposomes have different interests for either therapy or diagnosis. Recent studies generally depend on the development of new delivery systems, theranostics, in which diagnosis can be managed together with therapy by evaluating therapeutic effect. **Materials and Methods:** Theranostic liposomes were formulated by polyethylene glycole (PEG) coated, nanosized, either neutral or positively charged, 99mTc labeled for SPECT imaging and pramipexole encapsulated for PD therapy. Their characterization and in vitro release kinetics were evaluated. In vitro penetration of both formulations was evaluated in a BBB cell co-culture model. **Results:** Both neutral and positively charged liposomes showed proper characterization with about 10% encapsulation efficiency and around 100 nm particle sizes. All formulations fitted to the first-order release kinetics (3). Both formulations were found BBB permeable in cell culture studies with fluorescent images and fluorospectroscopy. **Conclusion:** Promising characterization and release profiles were obtained with theranostic liposomes for both diagnosis and therapy of PD. Both neutral and positively charged formulations found BBB permeable in vitro. In vivo animal studies are continuing to obtain more accurate data. (The authors thank to Abdi Ibrahim Ilaç for Pramipexole. This study was supported by the grant of TUBITAK, Project No: 112S244).

References:

1. Choi YK, Kim KW, [2008], BMB Rep 41: 345-52. 2. Saunders NR, et al., [2008], Trends Neurosci 31: 279-86. 3. Silindir M, et al., IPET [2015], Proceed. Book, IAEA-CN-232/85, IAEA, Vienna.