1. INTRODUCTION

1.1. DEPRESSION: THE INVISIBLE DISEASE

Depression is a serious medical condition and is associated with decrease in functioning and well-being, high levels of disability, and high work absenteeism and health care costs. It is a serious condition that affects a person's mind and body. It impacts all aspects of everyday life including eating, sleeping, working, relationships, and how a person thinks about himself/herself. If they do not receive appropriate treatment their symptoms can continue for weeks, months, or years.

1.1.1. Burden of Depression

Depression is associated with increased mortality due to suicide and its interactions with other medical illness. Depression affects approximately 19 million Americans, or 9.5% of the population in any given one-year period. At some point in their lives, 10%-25% of women and 5%-12% of men will likely become clinically depressed. In fact, it affects so many people that it is often referred to as the "common cold" of mental illness. The risk of suicide ranges from 43 to 224 per 100,000 person-years for those receiving treatment in primary care and in inpatient psychiatry settings, respectively (Simon and Von Korff 1998). Over 60 percent of suicides are attributed to major depressive disorder. Detrimental effects on personal productivity, interpersonal relationships, and the ability to perform usual daily activities are pervasive. Studies examining the effects of depression on health-related quality of life demonstrate decrements that equal or exceed those of patients with chronic medical illnesses such as diabetes mellitus or ischemic heart disease (Hays et al., 1995; Kazis et al., 1998; Covinsky et al., 1997; Spitzer et al., 1995).

In addition to personal costs, depression extracts a high economic toll on society as 1 of the 10 most costly illnesses in the United States. Estimated annual costs total about $44 billion (1990 dollars), with $12 billion per year going toward direct treatment costs and $31 billion per year lost in indirect costs (Greenberg et al., 1993). The indirect costs are attributed to premature death ($8 billion) and lost productivity in the workplace ($23 billion). This estimate encompasses costs for major depression, bipolar disorder, and dysthymia, with major depression accounting for over 85 percent of the cost. This estimate, however, does not represent the true cost to society because it does not measure the adverse effects of pain and suffering and other quality-of-life issues.
Furthermore, these estimates are conservative; they fail to include other important costs such as additional out-of-pocket expenses incurred by families, excessive hospitalization for nonpsychiatric conditions due to depression, and unnecessary diagnostic tests for depressed patients who present with somatic symptoms. They also exclude costs associated with individuals who have symptoms which do not meet full diagnostic criteria for major depression (Simon and Von Korff 1998; Hirschfeld et al., 1997).

1.1.2. Reason of depression

Neurotransmitters are chemical messengers within the brain that facilitate communication between nerve cells. Figure 1.1 depicts the junction between two nerve cells. Packets of neurotransmitter e.g., serotonin molecules are released from the end of the presynaptic cell (the axon) into the space between the two nerve cells (the synapse). These molecules may then be taken up by serotonin receptors of the postsynaptic nerve cell (the dendrite) and thus pass along their chemical message. Excess molecules are taken back up by the presynaptic cell and reprocessed.

Figure 1.1: Junction between two neurons exhibiting release of neurotransmitter.
Several things might potentially go wrong with this process and lead to a serotonin deficit including the followings;

- Not enough serotonin is produced
- There are not enough receptor sites to receive serotonin
- Serotonin is being taken back up too quickly before it can reach receptor sites
- Chemical precursors to serotonin (molecules that serotonin is manufactured from) may be in short supply, or
- Molecules that facilitate the production of serotonin may be in too short supply.

As a consequence, if there is a breakdown anywhere along the path, neurotransmitter supplies may not be adequate for the brain's needs. Inadequate supplies lead to the symptoms that are known as depression.

1.1.3. Brain areas involved in depression

Because of the diversity of the symptoms in major depression it is likely that multiple brain regions are involved in this disease. This is supported by brain imaging studies that have reported changes in blood flow (Drevets 2001; Liotti and Mayberg 2001) and anatomical abnormalities (Miguel-Hidalgo and Rajkowska 2002; Rajkowska 2000; Rajkowska et al., 1999) in prefrontal and cingulated cortex, hippocampus, striatum, amygdala, and thalamus. Of course, these various brain regions would operate in a complex neural circuitry, together yielding the symptomatic picture seen in major depression. Even so, the large majority of studies on major depression point out frontal cortex and hippocampus as key structures involved in affective disorders. The hippocampus is of particular interest in depression as it is a key member of the limbic system controlling emotional behavior and in regulating the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.2) (De Kloet et al., 1998).

In depression, the hypothalamic-pituitary (HPA) axis is upregulated with a down regulation of its negative feedback controls. Corticotropin-releasing factor (CRF) is hyper secreted from the hypothalamus and induces the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH interacts with receptors on adrenocorticol cells and cortisol is released from the adrenal glands. Release of cortisol into the circulation has number of effects, including elevation of blood glucose. The negative feedback of cortisol to the hypothalamus, pituitary and immune
system is impaired. This leads to continuous activation of the HPA axis and excess cortisol release (Figure 1.2). Cortisol receptors become desensitized leading to increased activity of the pro-inflammatory immune mediators and disturbances in neurotransmitter transmission (Stuart and Cole, 1996).

**Research Findings**

Brain imaging research is revealing that in depression, neural circuits responsible for moods, thinking, sleep, appetite, and behavior fail to function properly, and that the regulation of critical neurotransmitters is impaired (Soares and Mann 1997).

Genetics research, including studies of twins, indicates that genes play a role in depression. Vulnerability to depression appears to result from the influence of multiple genes acting together with environmental factors (NIMH Genetics Workgroup 1998).

Other research has shown that stressful life events, particularly in the form of loss such as the death of a close family member, may trigger major depression in susceptible individuals (Mazure et al., 2000).
The hypothalamic-pituitary-adrenal (HPA) axis, the hormonal system that regulates the body's response to stress, is overactive in many people with depression. Research findings suggest that persistent overactivation of this system may lay the groundwork for depression (Arborelius et al., 1999).

Studies of brain chemistry, mechanisms of action of antidepressant medications, and the cognitive distortions and disturbed interpersonal relationships commonly associated with depression, continue to inform the development of new and better treatments.

1.1.4. Types of depression

There are three main types of depressive disorders:

1. Dysthymic disorder
2. Bipolar disorder (manic-depressive illness)
3. Major depressive disorder

1. Dysthymic disorder

Some people have a chronic but less severe form of depression, called dysthymic disorder, which is diagnosed when depressed mood persists for at least 2 years (1 year in children) and is accompanied by at least 2 other symptoms of depression. Many people with dysthymia develop major depressive episodes.

2. Bipolar disorder (manic-depressive illness)

Episodes of depression also occur in people with bipolar disorder. In this disorder, depression alternates with mania, which is characterized by abnormally and persistently elevated mood or irritability and symptoms including overly-inflated self-esteem, decreased need for sleep, increased talkativeness, racing thoughts, distractibility, physical agitation, and excessive risk taking. Because bipolar disorder requires different treatment than major depressive disorder or dysthymia, obtaining an accurate diagnosis is extremely important.

3. Major depressive disorder

Major depressive disorder is also known as major depression, clinical depression, or unipolar depression. The term unipolar refers to the presence of one pole, or one extreme of mood-
depressed mood. This may be compared with bipolar depression which has the two poles of depressed mood and mania (i.e., euphoria, heightened emotion and activity). Major depression is a highly familial disease and the heritability for depression is approximately 40% (Sullivan et al., 2000; Levinson 2006). Heritability seems to be more pronounced in women than in men (Marcus et al., 2005), but genes alone are not predictive for development of affective disorders. It is well acknowledged today that affective disorders result from an interaction between genetic liability and environmental risk factors (Kendler et al., 1995; Caspi et al., 2003; Farmer et al., 2005). An important environmental risk factor is stress and stressful life events often precipitate the onset of major depression (Caspi et al., 2003; Williamson et al., 1998).

Different people are affected in different ways by major depression. Some people have trouble sleeping, they lose weight, and they generally feel agitated and irritable. Others may sleep and eat too much and continuously feel worthless and guilty. Still others can function reasonably well at work and put on a "happy face" in front of others, while deep down they feel quite depressed and disinterested in life. There is no one way that people look and behave when they have major depression. However, most people will either have depressed mood or a general loss of interest in activities they once enjoyed, or a combination of both. In addition they will have other physical and mental symptoms that may include fatigue, difficulty with concentration and memory, feelings of hopelessness and helplessness, headaches, body aches, and thoughts of suicide.

Major depression is a serious neuropsychiatric illness, probably the western world’s largest public health burdens. It is predicted to be the second leading cause of premature death or disability by 2020 (World Health Organisation 2001; Murray and Lopez 1997). In adults, major depressive disorder affects twice as many women as men. For both genders it is most common in those who are 25-44 years of age, and least common for those over the age of 65. In children, clinical depression affects girls and boys at about the same rate. Within an entire lifetime, major depression can affect 10-25% of women and 5-12% of men. At any one point in time, 5-9% of women and 2-3% of men are likely to be clinically depressed. Although major depression can occur at any age, the average age for developing the illness seems to be in a person's mid-20. However, the average age of onset of the condition appears to be decreasing. Those with a parent or sibling who has had major depression may be 1.5 to 3 times more likely to develop the condition than those who do not.
It is estimated that 10-25% of those who develop major depressive disorder have previously had dysthymic disorder, and each year about 10% of those with dysthymic disorder will develop a first major depressive episode. There are some people who have had dysthymia prior to developing major depression. The presence of both conditions at the same time is sometimes called "double depression." Those who are in this situation may have a greater chance of developing additional depressive episodes and have more difficulty fully recovering between the episodes. They may also need to have a longer period of continued care in order to overcome their symptoms.

The development of major depressive disorder may be related to certain medical illnesses. As many as 20-25% of those who have illnesses such as cancer, stroke, diabetes, and myocardial infarction are likely to develop major depressive disorder sometime during the presence of their medical illness. Managing or treating a medical condition can be more difficulty if a person is also clinically depressed. The prognosis for the medical problem may also be less positive.

It has been shown that other mental health conditions may often co-exist with major depressive disorder. Some of these are alcohol/drug abuse, anxiety and panic disorders, obsessive-compulsive disorder, eating disorders, and borderline personality disorder.

Major depressive disorder should be taken very seriously since up to 15% of those with this condition die by suicide.

Diagnosis of Major Depressive Disorder

1. For a major depressive episode a person must have experienced at least five of the nine symptoms below for the same two weeks or more, for most of the time almost every day, and this is a change from his/her prior level of functioning. One of the symptoms must be either (a) depressed mood, or (b) loss of interest.
   a. Depressed mood. For children and adolescents, this may be irritable mood.
   b. A significantly reduced level of interest or pleasure in most or all activities.
   c. A considerable loss or gain of weight (e.g., 5% or more change of weight in a month when not dieting). There may also be an increase or decrease in appetite. For children, they may not gain an expected amount of weight.
   d. Difficulty falling or staying asleep (insomnia), or sleeping more than usual (hypersonnia).
e. Behavior that is agitated or slowed down. Others should he able to observe this.

f. Feeling fatigued, or diminished energy.

g. Thoughts of worthlessness or extreme guilt (not about being ill).

h. Ability to think, concentrate, or make decisions is reduced.

i. Frequent thoughts of death or suicide (with or without a specific plan), or attempt of suicide.

2. The persons' symptoms do not indicate a mixed episode.

3. The person's symptoms are a cause of great distress or difficulty in functioning at home, work, or other important areas.

4. The person's symptoms are not caused by substance use (e.g., alcohol, drugs, medication), or a medical disorder.

5. The person's symptoms are not due to normal grief or bereavement over the death of a loved one, they continue for more than two months, or they include great difficulty in functioning, frequent thoughts of worthlessness, thoughts of suicide, symptoms that are psychotic, or behavior that is slowed down (psychomotor retardation).

1.1.5. Treatments of depression

Three types of therapies have proved effective for depressive disorders: pharmacotherapy, psychotherapy, and electroconvulsive therapy (ECT).

1. Pharmacotherapy: Antidepressant medications are widely used for effective treatments of depression (Muldrow et al., 1998). A number of antidepressant medications are available to treat depression. Antidepressants are generally categorized by how they affect the neurotransmitters in the brain to change the mood. Types of antidepressants include:

- **Selective serotonin reuptake inhibitors (SSRIs).** These are safer and generally cause fewer side effects than other types of antidepressants. SSRIs include fluoxetine (Prozac), paroxetine (Paxil), sertraline (Zoloft), citalopram (Celexa) and escitalopram (Lexapro). The most common side effects include decreased sexual desire and delayed orgasm. Other side effects may include digestive problems, jitteriness, restlessness, headache and insomnia.

- **Serotonin and norepinephrine reuptake inhibitors (SNRIs).** These include duloxetine (Cymbalta), venlafaxine (Effexor) and desvenlafaxine (Pristiq). Side effects are similar to those caused by SSRIs. In high doses these medications can cause increased sweating and dizziness.
Norepinephrine and dopamine reuptake inhibitors (NDRIs). Bupropion (Wellbutrin) falls into this category. It's one of the few antidepressants that doesn't cause sexual side effects. At high doses, bupropion may increase the risk of having seizures.

Atypical antidepressants. These are called atypical because they don't fit neatly into another antidepressant category. They include trazodone (Desyrel) and mirtazapine (Remeron). Both of these antidepressants are sedating and are usually taken in the evening. In some cases, one of these medications is added to other antidepressants to help with sleep.

Tricyclic antidepressants. These antidepressants have been used for years and are generally as effective as newer medications. Because they tend to have more numerous and more severe side effects, a tricyclic antidepressant generally is not prescribed unless an SSRI first tried without an improvement in the depression. Side effects can include low blood pressure, dry mouth, blurred vision, constipation, urinary retention, fast heartbeat and confusion. Older adults taking these medications are susceptible to memory problems, confusion and hallucinations. Tricyclic antidepressants are also known to cause weight gain.

Monoamine oxidase inhibitors (MAOIs). MAOIs include tranylcypromine (Parnate) and phenelzine (Nardil). These are usually prescribed as a last resort, when other medications haven't worked. That's because MAOIs can have serious harmful side effects. They require a strict diet because of dangerous (or even deadly) interactions with foods such as certain cheeses, pickles and wines and some medications including decongestants. Selegiline (Emsam) is a newer MAOI present in the form of transdermal patch. This may cause fewer side effects than other MAOIs.

Other medication strategies. In some cases, combination of two or more antidepressants or other medications are recommended for better effect. This strategy is known as augmentation. These may include stimulants, mood-stabilizing medications, anti-anxiety medications or antipsychotic medications.

2. Psychotherapy: Certain types of psychotherapy, specifically cognitive-behavioral therapy (CBT) and interpersonal therapy (IPT), have been found helpful for depression. Research indicates that mild to moderate depression often can be treated successfully with either therapy alone; however, severe depression appears more likely to respond to a combination of psychotherapy and medication (Hyman et al., 2000). More than 80 percent of people with
depressive disorders improve when they receive appropriate treatment (National Advisory Mental Health Council 1993).

3. Electroconvulsive therapy (ECT): In situations where medication, psychotherapy, and the combination of these interventions prove ineffective, or work too slowly to relieve severe symptoms such as psychosis (e.g., hallucinations, delusional thinking) or suicidality, electroconvulsive therapy (ECT) may be considered. ECT is a highly effective treatment for severe depressive episodes. The possibility of long-lasting memory problems, although a concern in the past, has been significantly reduced with modern ECT techniques. However, the potential benefits and risks of ECT, and of available alternative interventions, should be carefully reviewed and discussed with individuals considering this treatment and, where appropriate, with family or friends (National Institute of Mental Health 1999).

One herbal supplement, *Hypericum* (St. John's wort) appears to be more effective for the short-term treatment of mild to moderately severe depressive disorders. Adverse effects occur significantly less frequently with hypericum compared with first generation tricyclic antidepressants. It is not clear whether hypericum is more or less effective than standard antidepressive agents.
1.2. BRAIN TARGETING FOR DEPRESSION

1.2.1. Brain and BBB

The nervous system is our body's decision and communication center. The central nervous system (CNS) is made of the brain and the spinal cord while the peripheral nervous system (PNS) is made of nerves. Together they control every part of our daily life, from breathing to blinking. As a result, the nature has protected the CNS by tough barriers. Consequently, many neurotherapeutics are unsuccessful in treating CNS disorders because they cannot be effectively delivered to the brain. Drug delivery to the brain is a challenge even though there is relatively high blood flow. There are two physiological barriers separating the brain from its blood supply controlling the transport of compounds. One is the blood–brain barrier (BBB) and the other is the blood–cerebrospinal fluid barrier (BCSFB).

Internally brain is protected from foreign organisms and noxious chemicals by highly strengthened membrane system called as blood-brain barrier (BBB). The barrier is located at the brain blood capillaries, which are unusual in two ways. Firstly, the cells which make up the walls of these vessels (the endothelium) are sealed together at their edges by tight junctions (composed of smaller subunits, frequently dimers that are transmembrane proteins such as occludin, claudins, junctional adhesion molecule and others (Figure 1.3). Each of these transmembrane proteins is anchored into the endothelial cells by another protein complex that includes ZO-1 and associated proteins) that form a key component of the barrier. Secondly, these capillaries are enclosed by the flattened astrocyte cell projections called astrocytic feet (also known as "glia limitans") surrounding the endothelial cells of the BBB. Thus, BBB is a specialized system of capillary endothelial cells that protects the brain from harmful substances in the blood stream, while supplying the brain with the required nutrients for proper function. It is a semi permeable, selective barrier which was confirmed for the first time by Ehrlich who showed that cerebrospinal fluid (CSF) injection of tryptophan blue dye stained the entire brain parenchyma but could not enter into brain capillary microvasculature (Hawkins and Thomas, 2005). BBB is responsible for several functions like maintenance of neuronal microenvironment, tissue homeostasis, vasotonous regulation, fibrinolysis and coagulation, blood cell activation and migration during physiological and pathological processes, and also helps in vascularisation of normal neoplastic tissues (Risau, 1995). Physiologically BBB is made up of three layers such as inner endothelial cell layer which forms the wall of the capillary and contains tight junctions
followed by presence of basement membrane upon which pericytes and astrocytic feet processes lies (Egleton, 1997). Due to the presence of such tight junctions between endothelial cells a very high electrical resistance of around 1500-2000 $\Omega cm^2$ results (Crone and Olesen, 1982) as compared to 3.33 $\Omega cm^2$ in other body tissue proving the barrier function of BBB (Crone and Cristensen, 1981).

Figure 1.3: Structure of the BBB and tight junction.

(A) The BBB is formed in the central nervous system by capillary endothelial cells and surrounding perivascular elements (basal lamina, pericyte, astrocyte end-foot, and interneurons). (B) The tight junction is established by the interaction between the transmembrane proteins (claudins, occludin, and junction adhesion molecule) on adjacent endothelial cells. The C-terminal of these transmembrane proteins is linked to cytoskeletal actin through ZO-1. In response to pathological stimuli, $\delta$PKC may directly or indirectly increase phosphorylation of ZO-1, thus disrupting the association between ZO-1 and the actin cytoskeleton. The disorganization of proteins at the tight junction may result in the aberrant permeability of the BBB.
Astrocytes and pericytes help in differentiation as well as maintenance of BBB function. Astrocytes are the most abundant non-neuron cells and play many essential roles in the healthy central nervous system (CNS), including biochemical support of endothelial cells which form the blood-brain barrier, regulation of blood flow, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a principal role in the repair and scarring process of the brain and spinal cord following traumatic injuries (Sarafian et al., 2010). Pericytes are perivascular cells which are important for the maturation, remodeling and maintenance of the vascular system via the secretions of growth factors or modulation of the extracellular matrix. They are also involved in the transport across the BBB and the regulation of vascular permeability (Allt and Lawrenson, 2001).

The blood-cerebrospinal fluid barrier (BCSFB) is another barrier (after BBB) that a systemically administered drug encounters before entering the CNS. It functions together with the BBB and the meninges, to control the internal environment of the brain. It is sited at the choroid plexus epithelium, secretes CSF, which circulates through the ventricles and around the outside of the brain and spinal cord (Engelhardt et al., 2009). In the last twenty years the choroidal epithelium has emerged as a complex organ with many additional functions that include neuroendocrine signaling, neuroimmune and neuroinflammatory responses, drug and toxin metabolism and transport. The improved knowledge has established the role of the choroid plexus in brain function which has the potential to open new avenues for the treatment and prevention of neurological disorders. Choroid plexus function changes with disease and aging and knowledge of the processes involved is important for understanding CNS disease states, such as Alzheimer’s, Parkinson’s, HIV, and disorders of CSF circulation (Szmydynger et al., 2009).

In addition, some regions of the CNS called as circumventricular organs (CVO) are present adjacent to the ventricles of brain where BBB capillary endothelial tight junctions are absent. These brain sites are unique in terms that they are highly vascularised as compared to other brain regions and lacks BBB because the capillary system supplying the CVOs contains fenestrated endothelial cells instead of epithelial tight junction (Cottrell and Ferguson, 2004). Examples of such areas include choroid plexus, pineal gland, neurohypophysis, median eminence, organosum vasculosum of lamina terminalis, subfornical organ (SFO), area postrema of the chemoreceptor trigger zone (CTZ) and nucleus tractus solitarius (NTS). These sites require intimate contact to closely monitor the composition of the blood and to respond accordingly. Compared to the area
of tight BBB capillaries, the relative surface area of the capillaries of CVOs is very less (5000:1) which enables CVOs not to allow a significant diffusion of substances into the CNS (Begley, 2004; Johanson et al., 2005).

1.2.2. BBB transport system
Unlike peripheral capillaries that allow relatively free exchange of substance across cells, the BBB rigorously limits transport into the brain. BBB not only functions as a physical barrier, but also as a biochemical barrier that expresses certain enzymes like peptidases along with several cytosolic enzymes and efflux p-glycoprotein system that helps effluxing drugs from the endothelial cells back into the blood which helps in its further protecting action towards the brain microenvironment (Bernacki et al., 2008). Thus the BBB is often the rate-limiting factor in determining permeation of therapeutic drugs into the brain. BBB is physiologically guided by two types of membranes such as luminal membrane and abluminal membrane. Even so, BBB has been found to be permeable in transport of nutrients like blood glucose, proteins, peptides and related peptide drugs (Egleton and Davis, 2005). Various transport mechanisms at the BBB have been explained for the transport of these substances (Figure 1.4). These transport systems mainly operate in the luminal and abluminal membranes i.e. from both blood-to-brain and brain-to-blood directions. But the blood-to-brain transport system is of considerable interest in drug delivery for targeting of drug molecules into brain as compared to brain-to-blood transport system.

Different substances are basically transported through free diffusion mechanism either paracellularly or transcellularly (Pardridge, 2005). Paracellular diffusion is a non-saturable and non-competitive movement of compounds (e.g., sucrose) between cells. It occurs to a limited extent at the BBB, due to the "tight junctions". Transcellular diffusion (transcytosis) is a non-saturable and non-competitive movement across cells of lipophilic substances (e.g. ethanol). Facilitated diffusion is a form of carrier-mediated endocytosis in which solute molecules bind to specific membrane protein carriers that trigger a conformational change in the protein; which results in carrying through of the substance to the other side of the membrane, from high to low concentration (passive diffusion). This mechanism has contributed to transport of various substances including amino acids, nucleoside, small peptide, monocarboxyates and glutathione (Egleton et al., 2005; Zolkovic et al., 1993).
Endocytosis can be isolated into bulk-phase (fluid phase or pinocytosis) endocytosis and mediated endocytosis (receptor and absorptive mediated). Bulk-phase endocytosis is the non-competitive, non-saturable, temperature and energy dependent nonspecific uptake of extracellular fluids. It occurs to a very limited degree in the endothelial cells of the cerebral microvasculature (Pardridge, 1995).

Receptor-mediated endocytosis provides a means for selective uptake of high molecular weight molecules by specific type receptors present on the luminal membrane (Table 1.1) (Nicholson and Sykova, 1998). Cells have different receptors for the uptake of many different types of ligands, including hormones, growth factors, enzymes, and plasma proteins. It occurs at the brain for macromolecular substances, such as transferrin, insulin, leptin, and IGF-I & IGF-II, and is a highly specific type of energy dependent transport. In addition to this several other receptors are found in BBB e.g., receptor for leptin as LEPR, Fc fragment of IgG receptor transporter (FCGRT) (Duffy et al., 1988; Pardridge, 2007). Absorptive-mediated transport is triggered by an electrostatic interaction between a positively charged substance (charge moiety of a peptide) and the negatively charge plasma membrane surface (i.e. glycocalyx). It has a lower affinity and higher capacity than receptor-mediated endocytosis (Bickel et al., 2001).

Carrier mediated transporter (CMT) system is expressed on both the luminal and abluminal membranes of the brain capillary endothelium and operates in both directions i.e., from blood to brain and brain to blood directions (Table 1.2) (Frank and Pardridge, 1981; Duffy et al., 1988). The CMT systems can be exploited for brain drug-delivery after reformulating the drug in such a way that the drug assumes a molecular structure mimicking that of the endogenous ligand. For example, pseudonutrients are the polar small drug molecules which are made by mimicking the structure of nutrients.
Figure 1.4: A plan diagram of cerebral capillary that form BBB and their associations with the astrocytes and glia cells. The major paths for the transport of molecules across the BBB are shown. (1) Paracellular pathway: Usually, the tight junctions limit transport of hydrophilic compounds, including polar drugs. However, due to aberrant permeability water soluble molecules manage to cross the BBB. (2) Adsorptive transcytosis: Cationization increases the uptake of poorly transportable local plasma proteins such as albumin by adsorptive-mediated endocytosis and transcytosis. (3) Transport proteins: The endothelial cells contain carrier proteins for choline, amino acids, glucose, purine bases, nucleosides etc. The transport by some transport proteins is energy-dependent (e.g., P-glycoprotein) and act as efflux transporters which extrude lipophilic molecules (e.g., Azidothymidine, AZT). (4) Transeellular pathway: It is an effective diffusive route for lipid-soluble agents (most CNS drugs enter via this route). (5) Receptor mediated transcytosis: Specific receptor-mediated endocytosis and transcytosis are offered for the transport of certain proteins, such as insulin and transferrin.
Chapter 1

Introduction

Table 1.1: Description of receptors responsible for the transport of molecules through BBB explained under receptor-mediated transport system

<table>
<thead>
<tr>
<th>Transport system</th>
<th>Receptors</th>
<th>Molecules</th>
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</thead>
<tbody>
<tr>
<td>Receptor-mediated transport (RMT)</td>
<td>Insulin receptor (INSR)</td>
<td>Insulin</td>
</tr>
<tr>
<td></td>
<td>Transferrin receptor (TFR)</td>
<td>Transferrin</td>
</tr>
<tr>
<td></td>
<td>Insulin-like growth factor receptors (IGF1R &amp; IGF2R)</td>
<td>Insulin like growth factor 1 &amp; 2 (IGF1 &amp; IGF2), mannose-6-phosphate</td>
</tr>
<tr>
<td></td>
<td>Leptin receptor (LEPR)</td>
<td>Leptin</td>
</tr>
<tr>
<td></td>
<td>Fc like growth factor receptor (FCGRT)</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>Scavenger receptor type B1 (SCARB1)</td>
<td>Modified lipoproteins, like acetylated low density lipoprotein (LDL)</td>
</tr>
</tbody>
</table>

Gabapentin (a γ-amino acid) successfully cross the BBB because the structure does mimic that of an α-amino acid and is recognized by large neutral amino acid transporter (Uchiino et al., 2002). They have the tendency to penetrate BBB, as these are the camouflage of true transporters and hence they use the carrier mediated transporting systems (Gloor et al., 2001). Several other drugs which have been successfully transported into the brain include melphalan for brain cancer, levodopa (L-Dopa) for Parkinson’s disease and α-methyl-DOPA for treatment of high blood pressure and endogenous substances or nutrients utilizing different transporters of CMT system (Pardridge, 2007; Tersaki and Tsuji, 1994). The uptake of nutrients from blood into the brain is facilitated by the solute carrier (SLC) transporter families. These influx carriers are involved in the transport of a broad range of substrates including glucose, amino acids, nucleosides, fatty acids, minerals and vitamins in various human tissues, including brain. There are 43 families summarized based upon amino acid homology. Amongst these, SLCO/SLC21, the organic anion transporting superfamily (OATPs), and SLC22, the organic cation/anion/zwitterion transporter family, are heavily involved in the uptake of many diverse substrates. Of the three SLC families i.e., SLC15, SLC22, and SLCO are considered especially to have a role in xenobiotic drug uptake (Girardin, 2006; Tsuji, 2005).
The active efflux transport (AET) is involved in extruding drugs from the brain and is a major obstacle for many pharmacological agents, with the ABC (ATP binding cassette) transporter P-glycoprotein being the principle efflux mechanism of these agents (Urquhart et al., 2009). It requires energy in most instances and unlike CMT it is a unidirectional transport process (Loscher and Potschka, 2005). Most abundantly present component of this system is efflux P-glycoprotein, which is a product of ABCB1 gene. Several other multiple members of this gene family are available as ABCCs, ABCG2. AET is operated by broadly two groups of transporters, one is entirely energy dependent like ABC gene family found at luminal membrane (Pardridge, 2005) and others are energy independent found at abluminal membrane are organic anion

### Table 1.2: Description of carrier mediated transport system with different transporters and endogenous molecules to be transported

<table>
<thead>
<tr>
<th>Transport system</th>
<th>Transporters</th>
<th>Molecules</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1 (Glucose transporter 1)</td>
<td>Glucose, hexose, 2-deoxyglucose, fluorodeoxy glucose</td>
<td></td>
<td>Positron emission tomography (PET) scanning</td>
</tr>
<tr>
<td>LAT1 (Large neutral amino acid transporter)</td>
<td>Large and small neutral amino acids, L-dopa (Levodopa), α-methyl-dopa (Methyldopa), α-methyl-para-tyrosine or gabapentin</td>
<td></td>
<td>In parkinsonism, hypertension and in delivery of antiepileptic drugs</td>
</tr>
<tr>
<td>CAT1 (Cationic amino acid transporter 1)</td>
<td>Basic amino acids, like as arginine or lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT1 (Monocarboxylic acid transporter 1)</td>
<td>Lactate, pyruvate, ketone bodies and monocarboxylic acid drugs like probenecid</td>
<td></td>
<td>In treatment of gout &amp; urinary incontinence</td>
</tr>
<tr>
<td>CNT2 (concentrative nucleoside transporter 2)</td>
<td>Purine nucleosides, and certain pyrimidine nucleosides as uridine</td>
<td></td>
<td>In delivery of several anticancer and antiviral drugs</td>
</tr>
<tr>
<td>SLCs (Choline transporter) (Sodium dependent)</td>
<td>Choline</td>
<td></td>
<td>A cholinergic agent used in experimental techniques, not as a drug</td>
</tr>
</tbody>
</table>
transporter 1 (OAT), organic anion transporting polypeptide (OATP), glutamic acid amino acid transporter (EAAT) etc (Table 1.3). They carry substrates to brain due to their ion transporting mechanism without utilizing energy (Conford et al., 1994).

Table 1.3: Classification of active efflux transporter system (based on energy dependence) with transporters and drug molecules to be transported

<table>
<thead>
<tr>
<th>Transport system</th>
<th>Classification</th>
<th>Transporters</th>
<th>Drug molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active efflux transporter (AET)</td>
<td>Energy &amp; Na(^+) dependent transporters (Luminal membrane)</td>
<td>ABCB1 (Adenosine triphosphate-binding cassette transporter, subfamily B, member 1; [P-glycoprotein])</td>
<td>In targeting of antitumor drugs like doxorubicin, paclitaxel to brain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCC (Adenosine triphosphate-binding cassette transporter, subfamily C</td>
<td>Anticancer drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABCG2 (Adenosine triphosphate-binding cassette transporter, subfamily G, member 2;</td>
<td>Drugs used for cancer treatment</td>
</tr>
<tr>
<td></td>
<td>Energy independent &amp; Na(^+) independent transporters (Abluminal membrane of brain capillary endothelial cell)</td>
<td>OAT (Organic anion transporter)</td>
<td>Uremic toxins, (\rho)-Aminomhippuric acid (PAH), Valproic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OATP, OATP2 (Organic anion-transporting polypeptide 2)</td>
<td>Sulfated-conjugated steroid hormones</td>
</tr>
<tr>
<td>Na(^+) &amp; Cl(^-) dependent system</td>
<td>ATA2 (Acidic aminoacid transporter 2)</td>
<td></td>
<td>Small neutral amino acids like L-alanine, L-glycine, L-proline</td>
</tr>
<tr>
<td></td>
<td>TAUT (Taurine transporter)</td>
<td></td>
<td>Taurine</td>
</tr>
<tr>
<td>Na(^+) dependent low affinity system</td>
<td>No subtransporter is identified</td>
<td></td>
<td>Mainly allow stereoselective molecules</td>
</tr>
<tr>
<td>Na(^+) &amp; Cl(^-) dependent low affinity system</td>
<td>GAT1,2 (Gamma amino butyric acid transporter 2)</td>
<td></td>
<td>Suppressive neuro transmitters</td>
</tr>
<tr>
<td>Na(^+) dependent high affinity system</td>
<td>EAAT (Glutamic acid amino acid transporter)</td>
<td></td>
<td>Glutamic acid</td>
</tr>
</tbody>
</table>
1.2.3. Different strategies for brain targeting

The brain is a delicate organ with many vital functions and it is isolated and protected from the outside environment by several intriguing mechanisms. Unfortunately, these are the same mechanisms that prevent the CNS delivery of therapeutic agents. The tight junction of the BBB surrounding the brain is one such mechanism (Graff et al., 2005). It has been investigated that 100% of large molecule drugs and 98% of small molecule drugs do not cross BBB (Pardridge, 2007). For a small molecule drug to cross the BBB in significant amounts, the molecule must have two important characteristics like molecular mass must be under 400 Da and high lipid solubility (Pardridge, 2005). Due to these reasons the brain drug targeting becomes more difficult for the pharmaceutical industries. Several strategies have been investigated (Figure 1.5) for effective clinical outcome for different CNS conditions (Alam et al., 2010).

![Figure 1.5: Overview of different approaches of brain targeting. (ANS – autonomic nervous system; CNS – central nervous system; BBB – blood brain barrier; Mabs – monoclonal antibodies).](image-url)
1.3. INTRANASAL STRATEGY FOR BRAIN TARGETING

Now a day's delivery to the central nervous system (CNS) via nasal route is considered to be one of the most hot and challenging issues of research and development for pharmaceutical, nutraceutical and biotechnological candidates. Intranasal administration offers a non-invasive alternative route to the central nervous system (CNS) for drug delivery, effectively bypassing the BBB (Graff et al., 2005). This method allows drugs that do not cross the BBB to be delivered to the central nervous system (CNS) and eliminates the need for systemic delivery; thereby reducing unwanted systemic side effects (Figure 1.6).

![Diagram showing different methods of drug administration to the brain](image)

Figure 1.6: Different methods of drug administration to the brain.

The nasal route is one of the most permeable and highly vascularized site for drug administration ensuring rapid absorption and onset of therapeutic action. Various factors that synergistically enhance the permeation of nasally administered drugs are —

*High permeability of the nasal epithelium that allows for absorption of molecules with a molecular mass below 1000 Da (approximately).*
The nasal mucus membrane is folded which leads to a large air mucosa surface and thereby a large contact area for the drug formulation. Beneath the epithelium, a rich network of blood vessels ensures a fast and an efficient absorption.

There is a relatively low enzymatic activity in the nasal tract, which minimises primary metabolism of the active substance.

1.3.1. Anatomy & Physiology of the Nose

The nasal cavity is divided into two symmetrical halves by the nasal septum, a central partition of bone and cartilage; each side opens at the face via the nostrils and connects with the mouth at the nasopharynx. The nasal vestibule, the respiratory region and the olfactory region are the three main regions of the nasal cavity (Table 1.4). The lateral walls of the nasal cavity includes a folded structure which enlarges the surface area in the nose to about 150cm². This folded structure includes three turbinates: the superior, the median and the inferior. In the main nasal airway, the passages are narrow, normally only 1-3mm wide, and this narrows structure enables the nose to carry out its main functions. During inspiration, the air comes into close contact with the nasal mucosa and particles such as dust and bacteria are trapped in the mucous. Additionally, the inhaled air is warmed and moistened as it passes over the mucosa; and the high blood supply in the nasal epithelium. The submucosal zone of the nasal mucosa directly open to the systemic circulation, thus avoiding first pass metabolism. Another, perhaps more familiar, major function of the nose is olfactory region which is located on the roof of the nasal cavity.

The nasal cavity is covered with a mucous membrane which can be divided into nonolfactory and olfactory epithelium areas. The nonolfactory area includes the nasal vestibule, which is lined with skin-like cells, and respiratory region, which has a typical airways epithelium.

1.3.1.1. The Respiratory region

The nasal respiratory epithelium is generally described as a pseudo-stratified ciliated columnar epithelium. This region is considered to be the major site for drug absorption into the systemic circulation. The four main types of cells seen in the respiratory epithelium are ciliated columnar cells, non-ciliated columnar cells, goblet cells and basal cells. Although rare, neurosecretory cells...
may be seen but, like basal cells, these cells do not protrude into the airway lumen. The proportions of the different cell types vary in different regions of the nasal cavity. In the lower turbinate area, about 15-20% of the total numbers of cells are ciliated and 60-70% is non-ciliated epithelial cells. The numbers of ciliated cells increase towards the nasopharynx with a corresponding decrease in non-ciliated cells. The high number of nonciliated cells indicates their importance for absorption across the nasal epithelium. Both columnar cell types have numerous (about 300-400 per cell) microvilli. The large number of microvilli increases the surface area and this is one of the main reasons for the relatively high absorptive capacity of the nasal cavity. The role of the ciliated cells is to transport mucus towards the pharynx. Basal cells, which vary greatly in both number and shape, never reach the airway lumen. These cells are poorly differentiated and act as stem cells to replace other epithelial cells. About 5-15% of the mucosal cells in the turbinates are goblet cells, which contain numerous secretory granules filled with mucin. In conjunction with the nasal glands; the goblet cells produce secretions, which form the mucus layer (Schipper et al., 1991).
1.3.1.2 The olfactory region

In human, the olfactory region is located on the roof of the nasal cavities, just below the cribriform plate of the ethmoid bone, which separates the nasal cavities from the cranial cavity. Humans have relatively simple noses, since the primary function is breathing, while other mammals have more complex noses better adapted for the function of olfaction. The olfactory segment is lined with a specialized type of pseudostratified columnar epithelium, known as olfactory epithelium, which contains receptors for the sense of the smell. This segment is located along the dorsal roof of the nasal cavity. The olfactory tissue is often yellow in colour, in contrast to the surrounding pink tissue. Histological sections appear yellowish-brown due to the presence of lipofuscin pigments. Olfactory mucosal cell types include bipolar neurons, supporting (sustentacular) cells, basal cells, and Bowman's glands. The axons of the bipolar neurons form the olfactory nerve (cranial nerve I) which enters the brain through the cribiform plate. Bowman's glands are serous glands in the lamina propria, whose secretions trap and dissolve odoriferous substances. The primary challenge in achieving significant nose-to-brain drug delivery is depositing drug on the olfactory region of the nasal cavity. This region is the only part of the body where primary brain neurons are exposed to the outside environment (Figure 1.7). Due to the lack of a significant biological membrane barrier and the passive diffusion nature of the transport, studies have shown that both small molecules and biologics can be delivered along this pathway, leading up to 100 fold increases in brain concentration compared to IV dosing.

1.3.2. Nasal Drug Absorption Mechanism:

The first step in the absorption of drug from the nasal cavity is passage through the mucus. Small, unchanged particles easily pass through this layer. However, large or charged particles may find it more difficult to cross. Mucin, the principle protein in the mucus, has the potential to bind to solutes, hindering diffusion. Additionally, structural changes in the mucus layer are possible as a result of environmental changes (i.e. pH, temperature, etc.) (Illum et al., 1999).
Several mechanisms have been proposed but the following two mechanisms have been considered predominantly,

- The first mechanism involves an aqueous route of transport, which is also known as the paracellular route. This route is slow and passive. There is an inverse log-log correlation between intranasal absorption and the molecular weight of water-soluble compounds. Poor bioavailability was observed for drug with a molecular weight greater than 1000 Daltons (Aurora 2002).

- The second mechanism involves transport through a lipoidal route which is also known as the transcellular process and is responsible for the transport of lipophilic drugs that show a rate dependency on their lipophilicity. Drug also cross cell membranes by an active transport route via carrier-mediated means or transport through the opening of tight
junctions. For examples, chitosan, a natural biopolymer from shellfish, opens tight junctions between epithelial cells to facilitate drug transport (Dodane 1999).

1.3.3. Pathways to the CNS

The neural connections between the nasal mucosa and the brain provide a unique pathway for noninvasive delivery of therapeutic agents to the CNS (Thorne and Frey 2001; Illium 2000). It has been known for many years that pathogens and toxic metals could be transported from the nasal mucosa to the CNS along neural pathways (Mathison et al., 1998). However, it has only recently been appreciated that these same pathways can be used to deliver therapeutic agents to the CNS (Sippel et al., 1999; Thorne and Frey 2001).

The olfactory and trigeminal nerve components in the nasal epithelium provide pathways to deliver therapeutic agents to the olfactory bulb and brainstem (Figure 1.8 and Figure 1.9), respectively, where dispersion to other CNS areas may be possible via pulsatile flow within the perivascular spaces of cerebral blood vessels (Thorne et al., 2004; Thorne et al., 2008). The olfactory epithelium contains olfactory sensory neurons whose dendritic processes are exposed to the external environment, creating potential intracellular and extracellular pathways to the underlying submucosa and brain (Figure 1.10). The olfactory neural pathway provides both intraneuronal and extraneuronal pathways into the brain (Thorne and Frey 2001; Thorne et al., 1995; Balin et al., 1986; Shipley 1985). The intraneuronal pathway involves axonal transport and requires hours to days for drugs to reach different brain regions (Thorne and Frey 2001; Thorne et al., 1995; Baker and Spencer 1986). The extraneuronal pathway probably relies on bulk flow transport through perineural channels, which deliver drug directly to the brain parenchymal tissue, to the cerebrospinal fluid (CSF), or to both (Thorne and Frey 2001). This extraneuronal pathway allows therapeutic agents to reach the CNS within minutes (Thorne and Frey 2001; Thorne et al., 1995; Frey et al., 1995). Intranasal delivery of agents to the CSF is not surprising as CSF normally drains along the olfactory axon bundles as they traverse the cribriform plate of the skull and approach the olfactory submucosa in the roof of the nasal cavity where the CSF is then diverted into the nasal lymphatics (Kida et al., 1993; Thorne and Frey 2001).

The respiratory nasal epithelium contains free trigeminal nerve endings close to the epithelial surface, allowing possibilities for the transport of therapeutic agents. It was evidenced that the trigeminal neural pathway also may be involved in rapidly delivering protein therapeutic agents,
such as insulin-like growth factor-I to the brain and spinal cord following intranasal administration (Thorne et al., 2004). The trigeminal nerves innervating areas of the nasal cavity are responsible for most chemoperception other than olfaction and sense diverse stimuli, including hot spices, formaldehyde, and other chemicals (Feron et al., 2001). Intranasal application demonstrated the highest levels of therapeutic agent (cytokine interferon-β1b) within the olfactory bulbs and trigeminal nerves less than an hour after administration, along with significant targeting of the caudate, putamen, globus pallidus, substantia nigra and nucleus accumbens (Thorne et al., 2008).

Figure 1.8: Diagram of the olfactory area showing the olfactory epithelium, bulb and tract.
Figure 1.9: Representation of olfactory (red) and trigeminal (blue) pathways for entry of macromolecules into the brain and spinal cord following nasal application.

Figure 1.10: Theoretical mechanisms conveying substances from the submucosa (lamina propria) into the central nervous system following nasal application.
1.3.4. Advantages of nasal drug delivery

Delivery from the nose to the CNS occurs within minutes along both the olfactory and trigeminal neural pathways. Intranasal delivery occurs by an extracellular route and does not require that drugs bind to any receptor or undergo axonal transport. Intranasal delivery requires neither any modification of the therapeutic agents nor to be coupled with any carrier. It has been potentially explored as an alternative route for drugs with poor bioavailability (first-pass hepatic metabolism) and for the delivery of biosensitive (susceptible to enzymatic or acidic degradation) and high molecular weight (MW) compounds such as proteins, peptides and so on.

Intranasal insulin improved memory and mood in healthy adults (Benedict et al., 2004) and improved memory in patients with Alzheimer’s disease without altering blood levels of insulin or glucose (Reger et al., 2006). A large number of drugs ranging from proteins and peptides to hormones and vaccines have been delivered through the nasal cavity. Oxytocin, buserelin, desmopressin, calcitonin, insulin, luteinizing hormone releasing hormone, growth hormone and adrenocorticotropic hormone are some of the peptides that have been successfully administered through the nasal route (Jadhav et al., 2007). Apart from these, steroids (corticosteroids, estradiol, progesterone, testosterone, and so on), antihypertensives (nifedipine, nitroglycerine, propranolol, hydralazine, and so on), analgesics (buprenorphine), antibiotics and antivirals have been shown to produce considerable systemic effects when administered via the nasal cavity (Jadhav et al., 2007). The feasibility of the nasal route for administering vaccines against plague, diphtheria tetanus, influenza, cholera, and HIV has already been tested for inducing both mucosal and systemic immune response against the carried antigen (Arora et al., 2002).

Advantages

The advantages of intranasal delivery are considerable. This method is:

(1) Non-invasive, rapid and comfortable

(2) Bypasses the BBB and targets the CNS, reducing systemic exposure and thus systemic side effects

(3) Does not require any modification of the therapeutic agent being delivered

(4) Works for a wide range of drugs. It facilitates the treatment of many neurologic and psychiatric disorders
(5) Rich vasculature and highly permeable structure of the nasal mucosa greatly enhance drug absorption.

(6) Problem of degradation of peptide drugs is minimized up to a certain extent.

(7) Easy accessibility to blood capillaries.

(8) Avoids destruction in the gastrointestinal tract, hepatic “first pass” elimination and gut wall metabolism, allowing increased, reliable bioavailability.

1.3.5. Limitations of nasal drug delivery

Despite the advantages, the limitations of the nasal drug administration are:

1. Fast clearance from the nasal tract which leads to a very short retention time of the active substance due to the cilia assisted transport and continuous mucus production.

2. The mucus membranes increases the production of mucus dramatically if it is irritated which is very sensitive. Therefore, a nasal formulation normally has to have a pH of 4.0-6.5, a surfactant content less than 20 mg/ml and water as a solvent (Romeo et al., 1998). The problem of fast clearance of the nasal tract may be solved by the use mucoadhesive systems (polymers) that adhere to the mucus membrane by forming a gel when it comes into contact with the mucosa (e.g. chitosan, lectin), thus providing a prolonged retention time of the active substance.

3. The total amount of drugs accessing the brain has been reported to be low, especially for nasally applied biotech drugs such as peptides, proteins and DNA, which are poorly absorbed and highly susceptible to the harmful environment of the nasal cavity (Kim et al., 2000; Dufes et al., 2003).

4. Concentration achievable in different regions of the brain and spinal cord, varies with each agent.
5. Delivery is expected to decrease with increasing molecular weight of drug

6. Some therapeutic agents may be susceptible to partial degradation in the nasal mucosa or may cause irritation to the mucosa

7. Nasal congestion due to cold or allergies may interfere with this method of delivery

8. Frequent use of this route results in mucosal damage (e.g. infection, anosmia).

1.3.6. Factors affecting the intranasal drug delivery

When a drug is nasally administered to induce systemic effects or to act into CNS it needs to pass through the mucus layer and epithelial membrane before reaching the blood stream or pass directly to the CNS. The passage across the epithelium may occur by transcellular or paracellular mechanisms. Transcellular transport can be mediated by carriers that exist in the nasal mucosa, including organic cation transporters and amino acids transporters. In contrast, paracellular route is involved in the transport of small polar drugs and it takes place between adjacent epithelial cells through hydrophilic porous and tight junctions. Tight junctions are dynamic structures localized between the cells. Nevertheless, it is well known that their size is between 3.9-8.4 Å, avoiding the passage of bigger molecules, therefore this process of transport is highly dependent on the drug molecular weight (Pires et al., 2009; Ali et al., 2010).

I. Physicochemical properties of drugs

The influence of physicochemical characteristics of drug molecules on the rate and extent of gastrointestinal absorption is well understood. In same way, but with some differences, the physicochemical properties of drugs (molecular weight, lipophilicity, pKa, stability and solubility) can influence nasal absorption.

A. Molecular weight, lipophilicity and pKa

Lipophilic compounds having a molecular weight lower than 1 kDa can easily pass through nasal membrane whereas absorption of lipophilic drugs bigger than 1 kDa is significantly reduced. On the other hand, the rate and degree of nasal absorption of polar drugs is low and highly dependent on the molecular weight. The permeation of polar drugs with a molecular weight of
less than 300 Da is not considerably influenced by their physicochemical properties. By contrast, the rate of permeation is highly sensitive to molecular size if it is higher than 300 Da; i.e. an inverse relationship exists between rate of permeation and molecular weight. For some small polar molecules only a 10% bioavailability is suggested. The value goes down to 1% for large molecules such as proteins. The nasal membrane is predominantly lipophilic and hence, drug absorption is expected to diminish with a decrease in lipophilicity. However, if lipophilicity is too high, the drug does not dissolve easily in the aqueous environment of nasal cavity, hence, with accelerated mucociliary clearance the contact time with nasal membrane diminishes resulting in a reduced permeation through the wall.

B. Solubility
Due to the small size of nasal cavity, the allowable volume of drug solution is low for intranasal drug administration. Thereby, drugs poorly soluble in water and/or requiring high doses may constitute a problem. This can be overcome by enhancing the drug solubility.

C. pH
The extent of nasal absorption depends on the pKa of drug and pH at the absorption site, and also the pH of formulation. The pH of formulation can induce nasal mucosa irritation and, hence, it should be similar to that found on human nasal mucosa (5.0-6.5). It has been reported that if pH values were below 3 or above 10 damages were observed intracellularly and at membrane level (Pires at al., 2009).

II. Nasal physiological factors
A. Blood flow
The blood flow rate influences significantly the systemic nasal absorption of drugs, so that as it enhances more drug passes through the membrane, reaching the general circulation. It has been reported that vasoconstriction decreased nasal drug absorption by diminishing the blood flow (Pires at al., 2009).
Chapter 1

Introduction

B. Mucociliary clearance

Mucociliary clearance (MCC) is the self-clearing mechanism of the bronchi. MCC also influences significantly the nasal drug absorption. Briefly, all factors that increase mucus production, decrease mucus viscosity or increase ciliary beat frequency may increase the MCC. If MCC decreases, residence time of the drug product in nasal mucosa increase and, therefore, permeation is enhanced.

C. Enzymatic degradation

Drugs nasally administered circumvent gastrointestinal and hepatic first-pass effect. However, they may be significantly metabolized in lumen of nasal cavity or during the passage across the nasal epithelial barrier due to the presence of a broad range of metabolic enzymes in nasal tissues. Carboxyl esterases, aldehyde dehydrogenases, epoxide hydrolases and glutathione S-transferases have been found in nasal epithelial cells and are responsible for the degradation of drugs in nasal mucosa.

D. Transporters and efflux systems

Multidrug resistance transporters have been identified in human nasal respiratory and olfactory mucosa, which may be involved in the transport of a wide variety of hydrophobic and amphiphilic drugs. P-gp is an efflux transporter that exists in the apical area of ciliated epithelial cells and in the submucosal vessels of the human olfactory region. Several studies demonstrated that P-gp has an important role in preventing actively the influx of drugs from nasal membrane.

E. Absorption enhancers

Small and large hydrophilic drugs may be poorly permeable across nasal epithelium and may show an insufficient bioavailability. It is possible to greatly improve their absorption if they are administered in combination with absorption enhancers which induce reversible modifications on the structure of epithelial barrier. In intranasal drug delivery, mostly used absorption enhancers are surfactants (laureth-9), bile salts, fatty acids (taurodihydrofusidate) and polymeric enhancers (chitosan, cyclodextrins, poly-L-arginine and aminated gelatine).
Chapter 1

F. Mucoadhesive drug delivery systems

Mucociliary clearance is one of the most important limiting factors for nasal drug delivery, because it reduces the time allowed for drug absorption. Thus, improved nasal drug absorption can also be achieved by prolonging the contact time between drug and nasal mucosa. Mucoadhesion implies the attachment of the drug delivery system to the mucus, involving an interaction between mucin and a synthetic or natural polymer. Mucoadhesive polymers mostly used in intranasal drug delivery are chitosan, alginate and cellulose or its derivatives.

1.3.7. Strategies for improving drug availability in nasal administration

Various strategies used to improve the availability of the drug in the nasal mucosa, include:

1) Improving the nasal residence time
2) Enhancing the nasal absorption
3) Modifying drug structure to change physicochemical properties

1) Improving the nasal residence time

Mucociliary clearance acts to remove the foreign bodies and substances from nasal mucosa as quickly as possible. One way of delaying clearance is to apply the drug to the anterior part of the nasal cavity, an effect that is largely determined by the type of dosage form used. The preparation could also be formulated with polymers such as methylcellulose, hydroxy propyl methyl cellulose or polyacrylic acid, in which incorporation of polymer increases viscosity of the formulation and also acts as a bio adhesive with mucus. Increase in residence time does not necessarily lead to increase the absorption; this concept can be illustrated by considering insulin solution with similar viscosity containing carbopol and CMC. Here carbopol enhance the absorption whereas CMC solution doesn’t enhance the absorption of insulin. If we increase the viscosity, slow diffusion of drug from matrix causes retention in absorption with CMC. In case of carbopol causes enhancement of absorption due to opening the intracellular junctions. One more lucrative way to increase the nasal residence time is using biodegradable microspheres as a carrier for drug delivery. Biodegradable microspheres swell in presence of water thereby increasing the viscosity. This phenomenon leads to increase the nasal residential time.
2) Enhancing nasal absorption

The mechanism of action of absorption enhancer is increasing the rate at which drug passes through the nasal mucosa. Many enhancers act by altering the structure of epithelial cells in some way, but they should accomplish this while causing no damage or permanent change to nasal mucosa.

General requirement of an ideal penetration enhancer are as follows.

1. It should lead to an effective increase in the absorption of the drug
2. It should not cause permanent damage or alteration to the tissue
3. It should be non irritant and nontoxic.
4. It should be effective in small quantity
5. The enhancing effect should occur when absorption is required
6. The effect should be temporary and reversible
7. It should be compatible with other excipients.

Classification of penetration enhancer:

Chemical penetration enhancers are widely used in the nasal drug delivery. Classification of chemical penetration enhancer includes, following

1) Solvents 2) Alkyl methyl sulphoxides 3) Pyrrolidones 4) 1- Dodecyl azacycloleptan-2-one
5) Surfactants.

Mechanism of penetration enhancers is as follows,

• Increasing cell membrane permeability
• Opening tight junction and formation of intracellular aqueous channels
• Increasing lipophilicity of the charged drug by forming ion pair
• Inhibiting proteolytic activity.

3) Modifying drug structure

Modification of drug structure without altering pharmacological activity is one of the lucrative ways to improve the nasal absorption. Here modification of physiochemical properties such as molecular size, molecular weight, Pka and solubility, are favorable for nasal drug absorption.
1.3.8. Formulation Development Research in Nasal Drug Delivery

Most of the over the counter nasal preparation are formulated as solution, to treat the nasal symptoms of allergic rhinitis and common cold. A simple drug solution is adequate for this purpose as it produces better dispersion over greater surface area. The nasal residence time of such formulation is short (3-20 min) and exhibit high inter-individual variability. This route provides fast peak levels in circulation (Vitoria et al., 1999).

Large number of drugs has been evaluated for systemic bioavailability after transnasal administration in experimental animal models. Transnasal administration of drugs in diverse dosage forms such as sprays, powders, and microspheres has been attempted for improved residence and bioavailability. The nasal delivery is receiving attention for management of postoperative pain; mucosal administration requires only a 1.1-1.5 time higher dose of fentanyl than i.v. dose. The nasal delivery of vaccines is a very attractive route of administration in terms of efficacy (Vitoria et al., 1999).

1.3.9. Formulation design

1.3.9.1. Physiochemical Properties of Drugs

Chemical form:
The form of a drug can be important in determining absorption. For example, conversion of the drug into a salt or ester form can alter its absorption (Huang et al., 1985) studied the effect of structural modification of drug on absorption. It was observed that in –situ nasal absorption of carboxylic acid esters of L-Tyrosine a significantly greater than that of L-Tyrosine.

Polymorphism:
Polymorphism is known to affect the dissolution rate and solubility of drug and thus their absorption through biological membranes. It is therefore advisable to study the polymorphic stability and purity of drugs for nasal powders and/or suspensions.

Molecular Weight:
A linear inverse correlation has been reported between the absorption of drugs and molecular up to 300 Daltons. Absorption decreases significantly if the molecular weight is greater than 1000 Daltons except with the use of absorption enhancers.
**Particle Size:**

It has been reported that particle sizes greater than 10 μm are deposited in the nasal cavity. Particles that are 2 to 10 μm can be retained in the lungs, and particles of less than 1 μm are exhaled.

**Solubility and Dissolution Rate:**

Drug solubility and dissolution rates are important factors in determining nasal absorption from powders and suspensions. The particles deposited in the nasal cavity need to be dissolved prior to absorption. If drugs remain as particles or is cleared away, no absorption occurs.

**1.3.9.2. Delivery Systems**

The selection of delivery system depends upon the drug being used, proposed indication, patient population and last but not least, marketing preferences. Some of these delivery systems and their important features are summarized below:

**Nasal Drops:**

Nasal drops are one of the most simple and convenient systems developed for nasal delivery. The main disadvantage of this system is the lack of the dose precision and therefore nasal drops may not be suitable for prescription products. It has been reported that nasal drops deposit human serum albumin in the nostrils more efficiently than nasal sprays.

**Nasal sprays:**

Both solution and suspension formulations can be formulated into nasal sprays. Due to the availability of metered dose pumps and actuators, a nasal spray can deliver an exact dose from 25 to 200 μm. The particles size and morphology (for suspensions) of the drug and viscosity of the formulation determine the choice of pump and actuator assembly.

**Nasal Gels:**

Nasal gels are high-viscosity thickened solutions or suspensions. Until the recent development of precise dosing device, there was not much interest in this system. The advantages of a nasal gel includes the reduction of post-nasal drip due to high viscosity, reduction of taste impact due to reduced swallowing, reduction of anterior leakage of the formulation, reduction of irritation by using soothing/emollient excipients and target to mucosa for better absorption.
Chapter 1

Introduction

**Nasal Powder:**
This dosage form may be developed if solution and suspension dosage forms cannot be developed e.g., due to lack of drug stability. The advantages to the nasal powder dosage form are the absence of preservative and superior stability of the formulation. However, the suitability of the powder formulation is dependent on the solubility, particles size, aerodynamic properties and nasal irritancy of the active drug and/or excipients. Local application of drug is another advantage of this system.

1.3.10. Evaluation of Nasal Formulations
1.3.10.1. *In Vitro Testing Models for Nasal Drug Delivery*

The nasal mucosa provides a moist and highly vascularized membrane, crucial to rapid absorption into the blood stream and brain as well, thus facilitating faster transport to the site of action. Nasal drug delivery has been generating widespread interest in the drug delivery field because it could not only be used in local treatment and systematic administration but in brain delivery also. Therefore, evaluation of these novel formulations requires reliable *in vivo/in vitro* testing models. Compounds administrated via this route are absorbed directly into the circulation system, avoiding the first-pass hepatic metabolism and brain delivery, by-passing the BBB. This site of drug administration has been considered an ideal route for non-invasive delivery route. However, there are a number of factors that limits the widespread utility of this route including the limited maximum dose per spray, rapidity of clearance from the nasal cavity and enzymatic degradation in the mucus layer and nasal epithelium.

The physiology of the nasal cavity presents the most significant barrier to drug absorption. The primary physical barrier for intranasal delivery is the respiratory and olfactory epithelia of the nose. The permeability of the epithelial tight junctions in the body is variable (Maggio 2011). However, the problem associated with low bioavailability has been solved recently by developing novel drug delivery system to be delivered in solution of mucoadhesive substances (e.g., chitosan) (Wen 2011; Patel et al., 2011). The use of bioadhesive polymers has been shown to lengthen the residence time and enhance the bioavailability of drugs delivered to the nasal cavity.

Excised animal tissue models are frequently used for nasal drug absorption studies due to methodological and ethical limitations associated with the use of human nasal specimens,
although it is difficult enough to obtain human nasal tissue without these other issues (Lang et al., 1996; Schmidt et al., 2000). The porcine nasal mucosa (also sheep) has similarities with human nasal mucosa with regard to morphology (presence of ciliated and non-ciliated cells, basal cells, goblet cells, serous glands) and expression of P-glycoprotein. Thus, porcine nasal mucosa seems to be feasible for in vitro studies investigating the permeability of nasal tissue and prediction of absorption of drugs after nasal administration in vivo (Wadell et al., 2003). The fresh nasal tissue removed from the nasal cavity of sheep is also used for this purpose. The cumulative amounts of drug permeated within determination of the effective permeability coefficients across mucosal membrane are calculated. The histology of treated nasal mucosa membranes also has to be investigated after the completion of the experiments.

Mainly there are three in vitro testing models:

1. **Excised models**: Excised animal nasal mucosal tissue is obtained from various animals that are frequently used to study nasal transport and metabolism (rabbits, bovine, sheep tissue). It is crucial to select the right part of the nasal mucosa tissue for in vitro investigation studies. The different regions of ovine nasal mucosa cavity demonstrate quite different in vitro drug permeability properties. It has been established that the middle turbinate mucosa is the suitable model for in vitro studies because of its many advantages such as large surface area, highest drug permeability coefficient and high reproducibility.

2. **Cell line models**: RPMI 2650 human nasal epithelial cell line derived from a spontaneously formed tumor. These include primary and passaged cell culture, Liquid-Covered Culture method (LCC) and Air-Interfaced Culture method (AIC). Cultures of human nasal epithelial cell layers on Transwell inserted under AIC or LCC conditions are useful for in vitro drug transport studies. MatTek Corp. (200 Homer Avenue, Ashland, MA) offers EPI tissue models. However it must be noted that efforts to develop and characterize various nasal cell culture systems are still in their infancy.

3. **Artificial membranes**: Several artificial membranes have also been proven to be a good model for nasal drug delivery studies. For instance, the polydimethylsiloxan (PDMS) membranes have demonstrated good in vitro model properties during nasal mucosa drug permeation studies.
1.3.10.2. *In vitro* diffusion studies (Pisal et al., 2004a; Pisal et al., 2004b):

The nasal diffusion cell is fabricated in glass. The water-jacketed recipient chamber has total capacity of 60 ml and a flanged top of about 3mm; the lid has 3 openings, each for sampling, thermometer, and a donor tube chamber. The 10 cm long donor chamber, and a donor tube chamber has total capacity of 60 ml and a flanged top of about 3mm; the lid has 3 openings, each for sampling, thermometer, and a donor tube chamber the 10 cm long donor chamber tube has internal diameter of 1.13 cm. The nasal mucosa of sheep was separated from sub layer bony tissues and stoned in distilled water containing few drops at gentamycin injection. After the complete removal of blood from muscosal surface, is attached to donor chamber tube. The donor chamber tube is placed such a way that it just touches the diffusion medium in recipient chamber. At predetermined intervals, samples (0.5 ml) from recipient chamber are withdrawn and transferred to amber colored ampoules. The samples withdrawn are suitably replaced. The samples are estimated for drug content by suitable analytical technique. Throughout the experiment the temperature is maintained at 37 °C.

1.3.10.3. *In Vivo* Nasal Absorption studies (Chein et al., 1989; Romeo et al., 1998; Aulton et al., 2002):

**Animal models for nasal absorption studies**

The animal models employed for nasal absorption studies can be of two types, viz., whole animal or *in vivo* model and an isolated organ perfusion or *ex vivo* model. These models are discussed in detail below:

**Rat Model**

The surgical preparation of rat for *in vivo* nasal absorption study is carried out as follows: The rat is anaesthetized by intraperitoneal injection of sodium pentobarbital. An incision is made in the neck and the trachea is cannulated with a polyethylene tube. Another tube is inserted through the oesophagus towards the posterior region of the nasal cavity. The passage of the nasopalatine tract is sealed so that the drug solution is not drained from the nasal cavity through the mouth. The drug solution is delivered to the nasal cavity through the nostril or through the cannulation tubing. The blood samples are collected from the femoral vein. As all the probable outlets of
drainage are blocked, the drug can be only absorbed and transported into the systemic circulation by penetration and/or diffusion through nasal mucosa.

**Rabbit Model** (Corbo et al., 1998)

The rabbit offers several advantages as an animal model for nasal absorption studies:

1. It is relatively cheap, readily available and easily maintained in laboratory settings
2. It permits pharmacokinetic studies as with large animals (like monkey)
3. The blood volume is large enough (approx. 300ml)
4. To allow frequent blood sampling (1-2ml)

Thus it permits full characterization of the absorption and determination of the pharmacokinetic profile of a drug. Rabbits (approx. 3 kg) are either anaesthetized or maintained in the conscious state depending on the purpose of study. In the anaesthetized model, the rabbit is anaesthetized by an intramuscular injection of a combination of ketamine and xylazine. The rabbit's head is held in an upright position and the drug solution is administered by nasal spray into each nostril. During the experiment the body temperature of the rabbit is maintained at 37°C with the help of a heating pad. The blood samples are collected by an indwelling catheter in the marginal ear vein or artery.

**Dog Model** (Lee et al., 1991)

The dog is either anaesthetized or retained in the conscious condition depending on the drug characteristics and the purpose of experiment. The dog is anaesthetized by intravenous injection of sodium thiopental and the anesthesia is maintained with sodium Phenobarbital. A positive pressure pump through a cuffed endotracheal tube gives the ventilation. The body temperature is maintained at 37-38°C by a heating pad. The blood sampling is carried out from the jugular vein.

**Sheep Model** (Visor et al., 1987)

The sheep, rabbit and dog models are more practical and suitable for investigating nasal drug delivery from sophisticated formulations. They permit better evaluation of the parameters there involved. The *in vivo* sheep model for nasal delivery is essentially parallel to that for the dog model. Male in-house bred sheep are employed since they are free from nasal infections.
**Monkey Model**

Monkeys (approx. 8 kg) are anaesthetized, tranquillized or maintained in the conscious state as per the experimental purpose. The monkey is tranquillized by intramuscular injection of ketamine hydrochloride or anaesthetized by intravenous injection of sodium Phenobarbital. The head of the monkey is held in an upright position and the drug solution is administered into each nostril. Following the administration, the monkey is placed in a supine position in a metabolism chair for 5-10 min. throughout the course of study the monkey breaths normally through the nostrils. The blood samples are collected through an indwelling catheter in the vein.

1.3.10.4. *Ex Vivo* Nasal Perfusion Models (Chien et al., 1985)

Surgical preparation is the same as that is for *in vivo* rat model. During the perfusion studies, a funnel is placed between the nose and reservoir to minimize the loss of drug solution. The drug solution is placed in a reservoir maintained at 37°C and is circulated through the nasal cavity of the rat with a peristaltic pump. The perfusion solution passes out from the nostrils (through the funnel) and runs again into the reservoir. The drug solution in the reservoir is continuously stirred. The amount of drug absorbed is estimated by measuring the residual drug concentration in the perfusing solution. The drug activity due to stability problems may be lost during the course of experiment. This is especially true for peptide and protein drugs that may undergo proteolysis and aggregation.

Rabbit can also be used as the animal model for *ex vivo* nasal perfusion studies. The rabbit is anaesthetized with parenteral urethane-acepromazine. A midline incision is made in the neck and the trachea is cannulated with a polyethylene neonatal endotracheal tube. The oesophagus is isolated and ligated. The distal end of the oesophagus is closed with suture and flexible tygon tubing is inserted into the proximal end and advanced to the posterior part of the nasal cavity. The nasopalatine tract (that connects nasal cavity to the mouth) is closed with an adhesive to avoid drainage of drug solution from the nasal cavity. The drug in isotonic buffer solution is recirculated using aperistaltic pump.

1.3.11. *Estimation of drug-uptake*

The extent to which a substance gains access to the CNS needs to be determined for potential neuropharmaceuticals as well as for drug candidates with primary targets in the periphery. The
available experimental tools to study transport include in vivo techniques, in vitro models, and computational approaches (Table 1.5). Characteristics of the in vivo methods include classical pharmacokinetic techniques over brain perfusions, microdialysis and imaging techniques. In vivo measurements remain unmatched with respect to sensitivity and for the characterization of carrier-mediated uptake, receptor-mediated transport, and active efflux. Isolated microvessels are valuable tools for molecular characterization of transporters. Endothelial cell culture models of the blood brain barrier (BBB) are pursued as in vitro systems suitable for screening procedures. Recent applications of conditionally immortalized cell lines indicate that a particular weakness of culture models because of down regulation of BBB-specific transporter systems can be overcome. In silico approaches are being developed with the goal of predicting brain uptake from molecular structure at early stages of drug development. Currently, the predictive capability is limited to passive, diffusional uptake and predominantly relies on few molecular descriptors related to lipophilicity, hydrogen bonding capacity, charge, and molecular weight (Bickel 2001).

1.3.11.1. Gamma Scintigraphy

Animal study is performed for in vivo evaluation of the developed formulations with the help of gamma scintigraphy. The scintigraphy study involves the following steps.

1.3.11.1.1. Preparation of radiolabeled-drug formulation

The formulations are radiolabeled using suitable radioactive material (e.g., $^{99m}$Tc) (Babbar et al., 2000; Koziara et al., 2003). The radiochemical purity of $^{99m}$Tc-labeled formulations may be determined by thin layer chromatography (TLC) using silica gel-coated fiberglass sheets. The effects of incubation time, pH, and stannous chloride concentration on labeling are studied to achieve optimum reaction conditions (Kumar et al., 2008).

1.3.11.1.2. Biodistribution studies

Biodistribution studies are performed using suitable animal model (e.g., rat, rabbit). Radiolabeled drug formulations are administered through the tail vein and/or each nostril. Formulations are instilled into the nostrils with the help of micropipette (10–100µl). The rats are held from the back in slanted position during nasal administration. The rats are killed humanely at different time intervals and the blood is collected using cardiac puncture. Subsequently, brain and other tissues (liver, spleen, intestine, kidney and tail) are dissected, washed twice using normal saline,
made free from adhering tissue/fluid, and weighed. Radioactivity present in each tissue/organ is measured using shielded well-type gamma scintillation counter. Radio pharmaceutical uptake per gram in each tissue/organ is calculated as a fraction of administered dose using following equation (Saha, 1993).

\[
\text{% Radioactivity per gram of tissue} = \frac{\text{Counts in sample} \times 100}{\text{Weight of sample} \times \text{Total counts injected}}
\]

Pharmacokinetic parameters (Keck et al., 2002) for the formulations are calculated from drug concentration in blood vs time plot and blood/brain ratio. Brain targeting efficiency is calculated using two equations mentioned below (Vyas et al., 2005).

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

\[
\text{DTE %} = \left( \frac{\text{AUC}_{\text{brain}} / \text{AUC}_{\text{blood}} \text{i.n.}}{\text{AUC}_{\text{brain}} / \text{AUC}_{\text{blood}} \text{i.v.}} \right) \times 100
\]

Nose to brain direct transport percentage (DTP %) is calculated using equation;

\[
\text{DTP %} = \left( \frac{\text{B}_{\text{i.n.}} - \text{B}_x}{\text{B}_{\text{i.n.}}} \right) \times 100
\]

Where \( B_x = (\text{Bi.v.}/\text{Pi.v.}) \times \text{Pi.n.} \), \( B_x \) is the brain AUC fraction contributed by systemic circulation through the BBB following intranasal administration, \( \text{Bi.v.} \) is the \( \text{AUC}_{0-1} \) (brain) following intravenous administration, \( \text{Pi.v.} \) is the \( \text{AUC}_{0-1} \) (blood) following intravenous administration, \( \text{Bi.n.} \) is the \( \text{AUC}_{0-1} \) (brain) following intranasal administration, \( \text{Pi.n.} \) is the \( \text{AUC}_{0-1} \) (blood) following intranasal administration, AUC is the area under the curve.

1.3.11.1.3. Imaging

Radiolabeled drug formulations are injected through the tail vein of rats. Similarly, radiolabeled-drug formulations are administered in each nostril. The rats are anaesthetized and placed on the imaging board. Imaging is performed using Single Photon Emission Computerized Tomography (SPECT, LC 75-005, Diacam, Siemens AG, Erlanger, Germany) gamma camera (Koziara et al., 2003). The scintigraphy images following dosage administration are recorded.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Measured/ Estimated Parameter</th>
<th>Advantages</th>
<th>Disadvantages/caveat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous injection/ brain sampling</td>
<td>Influx; influx/efflux</td>
<td>Most physiological approach; highest sensitivity; low technical difficulty</td>
<td>May require good analytical tools to exclude metabolite uptake and careful pharmacokinetic analysis to discriminate unidirectional uptake versus bidirectional transfer</td>
</tr>
<tr>
<td>Brain uptake index</td>
<td>Influx</td>
<td>Fast procedure; moderate technical difficulty; permits wide range of modifications of injected composition; artifacts by metabolism largely excluded</td>
<td>Relatively insensitive (compared with intravenous injection and brain perfusion)</td>
</tr>
<tr>
<td>Brain perfusion</td>
<td>Influx</td>
<td>Higher sensitivity compared with BUI; permits modification of both perfusate composition and flow rates; artifacts by peripheral metabolism excluded</td>
<td>Technically more difficult than intravenous experiments and BUI</td>
</tr>
<tr>
<td>Quantitative autoradiography</td>
<td>Influx</td>
<td>Excellent spatial resolution</td>
<td>Time-consuming evaluation; no proof of integrity of tracer</td>
</tr>
<tr>
<td>External registration: MRI, SPECT, PET</td>
<td>Influx/efflux</td>
<td>Noninvasive and applicable in humans; allows time course measurements in individual subjects</td>
<td>Expensive equipment (MRI, PET) and tracers (PET); limited sensitivity (MRI) and availability of labeled tracers (MRI, PET); poor spatial resolution for small animals.</td>
</tr>
<tr>
<td>Microdialysis</td>
<td>Influx/efflux</td>
<td>Allows time course measurements in individual subjects; samples well suited for subsequent analytical procedures</td>
<td>Technically involved; in vivo probe calibration required for valid quantitative evaluation; local damage to BBB integrity</td>
</tr>
<tr>
<td>CSF sampling</td>
<td>Influx/efflux</td>
<td>Readily accessible for sampling; applicable to humans</td>
<td>Reflects permeability of B-CSF-B and CSF fluid dynamics rather than BBB</td>
</tr>
<tr>
<td><strong>In vitro methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh isolated brain microvessels</td>
<td>Binding, uptake, efflux</td>
<td>Representing the in vivo expression of transporters and efflux systems at the BBB</td>
<td>Tranceellular passage cannot be measured</td>
</tr>
<tr>
<td>EC membrane Vesicles</td>
<td>Carrier-mediated transport</td>
<td>Allows distinction of luminal versus abluminal transport activity</td>
<td>Large amounts of source material required, laborious preparation</td>
</tr>
<tr>
<td>Endothelial cell cultures, Primary cultures, cell lines</td>
<td>Receptor binding; uptake; luminal to abluminal transfer (and opposite direction)</td>
<td>Permeability screening experiments (feasible with primary EC from bovine/porcine sources); effect of culture conditions on BBB transport properties may be studied (e.g., astroglial factors, serum effects, inflammatory stimuli, hypoxia/aglycemia)</td>
<td>No system yet able to represent in vivo condition with respect to barrier tightness and BBB specific transporter expression; multitude of models makes comparison of results between studies Difficult</td>
</tr>
<tr>
<td><strong>In silico models</strong></td>
<td>CNS active (+/-); Log BB; Log PS</td>
<td>Screening of large compound libraries (depending on model selection and computational resources); screening of virtual libraries</td>
<td>Many current models based on data, which may not represent BBB permeability as such (log BB; CNS activity); still very limited data bases for BBB transport (log PS models)</td>
</tr>
</tbody>
</table>

PhD (Pharmaceutics), Jamia Hamdard 45
1.4. NEED FOR NANO-CARRIERS

Nanoparticles are receiving considerable attention for the delivery of therapeutic drugs. From the last few decades nanoparticles have attracted considerable interest in targeting drug molecules to brain. Nanoparticles (NPs) consist of different biodegradable materials such as natural or synthetic polymers, lipids, or metals. The term nanoparticles refers to well defined particles ranging in size approximately from 1 to 1000 nm (< 1 μm) (Kaur et al., 2008) with a core-shell structure (nanocapsules) or a continuous matrix structure (nanospheres). The unique size-dependent properties make them very attractive for pharmaceutical applications. Nanoparticles are taken up by cells more efficiently than larger micromolecules and therefore, could be used as effective transport and delivery systems. Nanoparticles have been used as a vector for delivery of a number of therapeutic agents. The use of nano-sized delivery mechanism enhances the absorption and distribution to the important organs (Tiwari et al., 2006). The effective transport and delivery of drugs into the brain by nanoparticulate carriers can be described by different mechanisms.

Mechanism of transport of nanoparticle-bound drug to the brain

Numerous mechanisms are reported by which nanoparticles attain maximum drug concentration in brain -

The nanoparticles make possible an opening of the tight junctions between the endothelial cells (an endocytotic event occurs due to upfolding of the cell membrane). This leads to increased drug paracellular permeation in the free and/or nanoparticulate-bound form.

The transcytosis through the endothelial cell layer leads to direct delivery of nanoparticles bound drug into the brain parenchyma. This causes an increase in retention of the nanoparticles in the brain-blood capillaries, and/or their adsorption to the capillary walls. This creates a higher concentration gradient enhancing the transport across the BBB and leading to drug delivery to the brain.

The membrane fluidization due to the solubilization of the endothelial cell membrane lipids by surfactants enhances drug permeability through the BBB.

The nanoparticles may be selectively endocytosed by the endothelial cells followed by the release of the drugs intracellularly and delivery to the brain.
Chapter 1

Introduction

Inhibiting the P-glycoprotein efflux system (e.g., by polysorbate coated nanoparticles) may enhance the brain drug delivery.

Permeabilization of brain endothelial cells caused due to induction of local toxic effects at the brain vasculature (Mistrya et al., 2009; Barbu et al., 2009).

1.4.1. Need for nanostructured lipid carriers (NLC)

Intensive research demonstrated the potential of polymeric nanoparticles. However, despite almost 30 years of research, nanoparticulate products do practically not exist on the pharmaceutical market. To overcome problems of polymeric nanoparticles (e.g., lack of large scale production, use of organic solvents), nanoparticles based on lipids were developed (e.g., SLN, NLC).

Lipid-based drug delivery systems gained increased attention during the last few years. At the beginning of the 1990s there were only the research groups of Muller (Berlin, Germany), Gasco (Turin, Italy) and Westesen (Braunschweig, Germany) working on lipid nanoparticles. Currently more than 20 research groups are working on lipid nanoparticles world wide, estimated by the published articles. This proves the increasing interest in the field of lipid nanoparticles. In November 2007, the US pharmaceutical company Brookwood acquired the worldwide exclusive rights for the NLC® technology from Pharmasol to turn NLC® into pharmaceutical products (Muchow et al., 2008).

Lipid nanoparticles exploit the absorption enhancing properties of lipids, which are now commonly used for new improved delivery systems. Lipid nanoparticles fulfill essential prerequisites for entering the market with a new formulation. Such prerequisites are low cost production, clinical and large-scale production facilities, and accepted status of excipients. Lipid nanoparticles have been investigated for various pharmaceutical applications e.g., parenteral (Blasi et al., 2007; Bondi et al., 2007), peroral (Müller et al., 2006), dermal (Müller et al., 2002; Priano et al., 2007), ocular (Attama et al., 2008) and pulmonary (Liu et al., 2008) administration.

After the first generation lipid carriers in the form of lipid pellets (e.g., Mucosolvan retard, Boehringer-Ingelheim), the second generation was developed by P. Speiser, so-called "lipid nanopellets for oral administration" in the middle of the eighties (Speiser 1990). At the
beginning of the nineties the third generation has been developed the so-called “solid lipid nanoparticles” (SLN).

Solid lipid nanoparticles (SLN) were developed at the beginning of the 1990s as an alternative carrier system to emulsions, liposomes and polymeric nanoparticles. SLN are produced by replacing the liquid lipid (oil) of an o/w emulsion by a solid lipid or a blend of solid lipids, i.e., the lipid particle matrix being solid at both room and body temperature (Rainer et al., 2009).

In the fourth generation of the lipid nanoparticle technology, the particles are produced using blends of solid lipids and liquid lipids (oils). To obtain the blends for the particles matrix, solid lipids are mixed with liquid lipids (oils), preferably in a ratio of 70:30 up to a ratio of 99.9:0.1. Due to the oil in these mixtures a melting point depression compared to the pure solid lipid is observed, but the blends obtained are also solid at body temperature (Muller et al., 2007). This fourth generation of nanoparticles is called nanostructured lipid carriers (NLC). The overall solid content of NLC could be increased up to 95% (Muller et al., 1999).

Being lipophilic in nature SLN has been extensively studied and investigated for the transport of therapeutic substances to the brain (Kaur et al., 2008). Consequently, SLN has proved its brain targeting potential with improved bioavailability and targeting efficiency. However, there are also some potential limitations (Mehnert and Mader 2001) which might occur including:

(i) Limitation in drug loading capacity

(ii) Drug expulsion during storage due to crystallisation process in lipid to perfect crystal: SLNs are produced from solid lipids only and after preparation at least a part of the particles crystallizes in a higher energy modification (α or β'). During storage, these modifications can transform to the low energy, more ordered β modification. Due to its high degree of order, the number of imperfections in the crystal lattice is reduced leading to drug expulsion (Figure 1.11).

![Figure 1.11: Crystallisation process during storage to perfect crystal in SLN leading to drug expulsion.](image-url)
(iii) High water content of aqueous SLN dispersions (70–95%)

(iv) Aggregation of SLN dispersions: The low concentrated dispersions aggregated during storage time, the particles were freely diffusible, collided and led to aggregate formation (Figure 1.12).

![Figure 1.12: Aggregation phenomenon in low concentrated SLN dispersions.](image)

(v) Burst release of the drug: Phase separation process during cooling in SLN production leading to a drug-enriched shell and consequently burst release (zur Muhlen et al., 1998) (Figure 1.13).

![Figure 1.13: Separation of phases in SLN results in drug-free core and a drug-enriched outer shell.](image)

Due to these limitations the formulators were looking for better options. Finally, the nanostructured lipid carrier (NLC) came into existence as an advance form of lipid carrier with better loading capacity and storage. NLC has already proved their credentials in oral as well as transdermal drug delivery systems and the same competence is expected for the CNS delivery of drugs also. It can be summarized that SLN are particles produced from a solid lipid (fat) only and NLC are particles produced from a blend of solid lipid with a liquid lipid (oil). This results in differences of the particle matrix structure. NLC posses many “imperfections” increasing drug loading capacity and minimizing or avoiding drug expulsion during storage (Muchow et al., 2008).
The distinguishing characteristic of the NLC includes –

1. Controlled nanostructuring of the lipid particle matrix, i.e., creation of a lipid particle matrix as imperfect as possible. To accomplish this, spatially very different molecules are mixed. In general a solid lipid is mixed with a liquid lipid (oil). This blend is used to produce the lipid particles that are still solid at temperatures up to about 40°C (Muchow et al., 2008).

2. By creating a less ordered solid lipid matrix, i.e., by blending a solid lipid with a liquid lipid (in NLC), a higher drug load of the particles can be achieved. In general, the drug can be located in between the fatty acid chains or in between the lipid layers and also in imperfections of the lipid matrix (e.g., amorphous drug clusters) (Pardeike et al., 2009). Also, the solid matrix of the lipid nanoparticle contains tiny liquid nanocompartments of oil (Figure 1.14). In these oil compartments the drug solubility is higher, thus increasing the total drug loading capacity.

![Figure 1.14: NLC containing oil nanocompartments.](image)

3. By using special mixtures of solid lipids (fat) and liquid lipids (oil) the particles become solid after cooling but do not crystallize (amorphous state), thereby limiting the drug expulsion from NLC (Figure 1.15).

![Figure 1.15: Conversion of lipid melt to amorphous form in NLC.](image)
4. The spherical lipid nanoparticles (NLC) form a pearl-like network possessing the advantage of stabilising the lipid nanoparticle dispersion against aggregation. In the pearl-like network the particles are fixed, diffusion is minimized and thus aggregation avoided (Lippacher et al., 2002) (Figure 1.16).

![Diagram: High concentrated NLC dispersions in pearl-like network.](image)

Figure 1.16: Fixation of high concentrated lipid particle dispersions in pearl-like network (lower), dilution (e.g. in gastric fluid) leads to non-aggregated, definite nanoparticles.

In addition, NLCs have many features that are advantageous for brain targeting –

*Since the brain is protected with BBB and only lipophilic molecules can cross the barrier. Being lipophilic in nature NLC has been expected for the transport of therapeutic substances to the brain.*

*They are colloidal carriers providing controlled release profiles for many substances.*

*They are composed of physiological and biodegradable lipids exhibiting low toxicity and low cytotoxicity that means an excellent tolerability.*

*The small size ensures a close contact to the BBB and can increase the amount of drug penetrated into the brain.*

*The lipid nanoparticles are able to enhance the chemical stability of compounds sensitive to light, oxidation and hydrolysis (Pardeike et al., 2009).*
1.4.1.1. Methodology

1.4.1.1.1. General ingredients:

The general ingredients that are required for the preparation of NLC include solid lipid (fat), liquid lipid (oil), emulsifier and water. The solid lipids (fat) employed include monoglycerides (glycerylmonestearate), diglycerides (glyceryl dipalmitate), triglycerides (Triacetin - glycerin triacetate, Trimeystin – glycerin trimyristate, Trilinolein - Glycerol trilinoleate) or mixture of mono-, di-, tri-glycerides. All classes of emulsifiers (with respect to charge and molecular weight) or combination of emulsifiers have been used to stabilize the lipid dispersion (prevent particle agglomeration). An overview of ingredients which are commonly used is provided in Table I.6.

Table I.6: Different ingredients (fat, oil & emulsifier) used in preparation of NLC

<table>
<thead>
<tr>
<th>Type of ingredients</th>
<th>Name of ingredients</th>
<th>Fat</th>
<th>Oil</th>
<th>Emulsifier</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tricaprin, Trilaurin, Trimyristin, Tripalmitin, Tristearin Softisan 142, Witepsol W35, Witepsol H35, Witepsol H42, Witepsol E 85, Glyceryl monostearate (Imwitor 900), Glyceryl behenate (Compritol 888 ATO), Glyceryl palmitostearate (Precirol ATO 5), Cetyl palmitate, Stearic acid, Palmitic acid, Decanoic acid, Behenic acid, Acidan N12.</td>
<td>Miglyol, Capmul, Capryol 90, Capryol PGMC, Labrafil, Labrasol, Tocopherol</td>
<td>Soybean lecithin (Lipoid S75, Lipoid S100), Egg lecithin (Lipoid E 80), Phosphatidylecholine (Epikuron 170, Epikuron 200), Poloxamer (182, 188, 407, 08), Polysorbate (20, 60, 80), Tyloxapol, Sodium cholate, Sodium glycocholate, Taurocholic acid sodium salt, Taurodeoxycholic acid sodium salt, Butanol, Butyric acid, Dioctyl sodium sulfosuccinate, Monooyctylphosphoric acid sodium.</td>
</tr>
</tbody>
</table>

1.4.1.1.2. Preparation/Production:

Many different techniques for the production of lipid nanoparticles have been described in the literature. These methods are high pressure homogenization, microemulsion technique, emulsification-solvent evaporation, emulsification-solvent diffusion method, solvent injection...
(or solvent displacement) method, phase inversion, multiple emulsion technique, ultrasonication and membrane contractor technique.

1.4.1.1.2.1. High shear homogenization and ultrasonication

High shear homogenization and ultrasonication are dispersing techniques which were initially used for the production of solid lipid nanodispersions. Both methods are widespread and easy to handle. However, dispersion quality is often compromised by the presence of microparticles. Furthermore, metal contamination has to be considered if ultrasound is used. Ahlin et al., 1998 used a Lak Tek rotor-stator homogen-techizer (Omni International, Gainesville, USA) to produce SLN by melt-emulsification. They investigated the influence of different process parameters, including emulsification time, stirring rate and cooling conditions on the particle size and the zeta potential. Lipids used in this study include trilinolenin (Dynasan 114), tripalmitin (Dynasan 116), tristearin (Dynasan 118), a mixture of mono-, di- and triglycerides (Witepsol W35, Witepsol H35) and glycerol behenate (Compritol 888 ATO), poloxamer 188 was used as steric stabilizer (0.5 w%). For witepsol W35 dispersions the following parameters were found to produce the best SLN quality: stirring for 8 min at 20 000 rpm, the optimum cooling conditions: 10 min at 5000 rpm at room temperature. In contrast, the best conditions for dynasan 116 dispersions were a 10-min emulsification at 25 000 rpm and 5 min of cooling at 5000 rpm in cool water (T=16 °C).

Higher stirring rates did not significantly change the particle size, but slightly improved the polydispersity index. No general rule can be derived from differences in the established optimum emulsification and cooling conditions. Average particle sizes in the range of 100–200 nm can be obtained by using this method.

1.4.1.1.2.2. High pressure homogenization

High pressure homogenization (HPH) is a reliable and powerful technique for the preparation of lipid nanoparticles. This technique has many advantages compared to the other methods, e.g., easy scale up, avoidance of organic solvents and short production time. High pressure homogenizers are widely used in many industries including the pharmaceutical industry, e.g., for the production of emulsions for parenteral nutrition. High pressure homogenizer pushes a liquid with high pressure (100–2000 bar) through a narrow gap (in the range of a few μm) to accelerate
Chapter 1

Introduction to very high velocity (over 1000 km/h). Very high shear stress and cavitation forces disrupt the particles down to the submicron range. Typical lipid contents are in the range 5–10% and represent no problem to the homogenizer.

For the production of lipid nanoparticles by high pressure homogenization there are many machines available on the market. With a minimum batch size of 3 ml an EmulsiFlex-B3 or C5 (Avestin, Ottawa, Canada) can be used for the laboratory scale production with limited new chemical entities or very expensive active materials. The Micron Lab 40 (APV Deutschland GmbH, Unna, Germany) is a laboratory scale high pressure homogenizer with a maximum batch size of 40 ml if operated discontinuously and a batch size range from 200 ml to 1000 ml when modified to work continuously. Another laboratory scale homogenizer is the Panda (tabletop homogenizer) (Niro Soavi, Lübeck, Germany). It is used for feasibility testing and process development. The batch size ranges from 500 ml to 2 l. Medium scale batches (up to 10 l) can be produced using a Micron Lab 60 (APV Deutschland GmbH, Unna, Germany). Examples of high pressure homogenizers for large scale production are the Gaulin 5.5 (APV Deutschland GmbH, Unna, Germany) and the Rannie 118 (APV Deutschland GmbH, Unna, Germany). These machines have a homogenization capacity of 150 l/hr and 2000 l/hr respectively (also depending on the pressure applied).

Two general approaches of the homogenization step, the hot and the cold homogenization techniques, can be used for the production of NLC and require a preparatory step involving the drug incorporation into the bulk lipid by dissolving or dispersing in the lipid melt.

1.4.1.1.2.2.1. Hot homogenization technique

Hot homogenization is carried out at temperatures above the melting point of the lipid and can therefore be regarded as the homogenization of an emulsion (Figure 1.17). A pre-emulsion of the drug loaded lipid melt and the aqueous emulsifier phase (same temperature) is obtained by high-shear mixing device (Ultra-Turrax, IKA, USA). HPH of the pre-emulsion is carried out at temperatures above the melting point of the lipid. In general, higher temperatures result in lower particle sizes due to the decreased viscosity of the inner phase. However, high temperatures may also increase the degradation rate of the drug and the carrier. The homogenization step is repeated several times. High pressure homogenization increases the temperature of the sample approximately 10°C for 500 bar (Jahnke et al., 1998). Increasing the homogenization pressure or
the number of cycles often results in an increase of the particle size due to particle coalescence which occurs as a result of the high kinetic energy of the particles (Siekmann et al., 1994) therefore 3–5 homogenization cycles at 500–1500 bar are sufficient.

Initially, due to the liquid state of the lipid, the product of the hot homogenization results in nanoemulsion. When the sample is cooled to room temperature or to temperatures below it then solid particles are formed. The sample may remain as a supercooled melt for several months due to the small particle size and the presence of emulsifiers which retards lipid crystallization (Bunjes et al., 1996).

![Diagram](image)

**Figure 1.17: Schematic procedure of homogenization techniques for NLC production.**
1.4.1.2.2. Cold homogenization technique

The cold homogenization is carried out with the solid lipid and represents a high pressure milling of a suspension (Figure 1.17). Effective temperature control and regulation is needed in order to ensure the unmolten state of the lipid due to the increase in temperature during homogenization (Jahnke et al., 1998). Cold homogenization overcomes the following three problems of the hot homogenization technique:

Temperature-induced drug degradation

Drug distribution into the aqueous phase during homogenization

Complexity of the crystallization step of the nanoemulsion leading to several modifications and/or supercooled melts

In the preparatory step the solubilisation or dispersing of the drug in the melt of the bulk lipid is carried out. The drug containing melt is rapidly cooled (e.g., by means of dry ice or liquid nitrogen) as higher cooling rate favors homogenous distribution of the drug within the lipid matrix. The solid, drug containing lipid is milled to microparticles. Typical particle sizes obtained by means of ball or mortar milling is in the range of 50–100 μm. Low temperatures increases the fragility of the lipid and favors particle comminution. The solid lipid microparticles are dispersed in a chilled emulsifier solution. The pre-suspension is subjected to high pressure homogenization at or below room temperature. As compared to hot homogenization, larger particle sizes and a broader size distribution are observed in cold homogenized samples.

1.4.1.2. Characterization

Particle size and zeta potential analysis

The particle size of aqueous NLC dispersions may be performed by photon correlation spectroscopy (PCS) with a Zetasizer (Malvern Instruments, Malvern, UK) and by laser diffractometry (LD), using a Coulter LS 230. PCS yields the mean particle size and the polydispersity index (PI) as a measure of the width of the distribution. The LD data is evaluated using the diameter d90%, which means that 90% (volume distribution) of the measured particle is below the obtained value. LD analysis is performed using 1.456 35 as the real refractive index and 0.01 as the imaginary refractive index applying the Mie theory. The zeta potential is
determined by Laser Doppler Anemometry (Zetasizer IV, Malvern Instruments, UK) using the Helmholtz–Smoluchowsky equation.

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

Differential scanning calorimetry (DSC)

It is performed in order to determine the degree of crystallinity of the lipid nanoparticles. Samples containing NLC aqueous dispersions accurately weighed in 40 ml aluminium pans and cold sealed. The reference pan kept empty and sealed in the same manner. Heating curves are recorded and the crystallization index (CI) is calculated using the following equation.

$$CI\ (%) = \frac{\Delta H_{NLC\ aqueous\ dispersion}}{\Delta H_{bulk\ material} \times Concentration_{lipid\ phase}} \times 100$$

Thermal gravimetry analysis

It is carried out for the determination of chemical stability of the drug at higher temperatures. Samples of ~15mg are heated in an aluminium oxide crucible (Mettler TG-DTA analyser) and the loss of weight is recorded.

X-ray diffraction

X-ray diffraction investigations have been most valuable in the elucidation of the manner of arrangement of lipid molecules, their multiple-melting phenomena, phase behaviour and the characterization and identification of the structure of lipid and drug molecules.

X-ray measurements is performed by wide-angle X-ray scattering (WAXS). Aqueous dispersions are transformed into a paste using a special gum (locust bean gum) as a thickening agent. Briefly, a small amount of gum was primarily mixed with the aqueous dispersion obtaining a paste which was placed into a thin X-ray glass fibre and then transferred to the camera for analysis.
Chapter 1

Introduction

Polarized light microscopy
To investigate drug crystals in the lipid melts (i.e. solubility studies), a polarized light microscope equipped with a digital camera at the magnification powers of 100x, 400x and 1000x is applied. The absence of drug crystals indicates that the active ingredient is completely dissolved in the lipid. Only lipid mixtures showing complete dissolution in the solid lipid state at room temperature are used for NLC production.

Scanning electron microscopy
The size and the shape of NLC are also evaluated by scanning electron microscopy (SEM). Prior to analysis, samples are diluted with ultrapurified water and sonicated to obtain a suitable concentration. Then, the samples are spread on a sample holder and dried using vacuum. They are subsequently coated with gold (SCD 040) and examined by a scanning electron microscope.

Loading capacity
The loading capacity of NLC, expressed as amount of loaded drug in percent related to the lipid phase (matrix lipid + drug). Typically, accurately weighed amount (e.g., 5 mg) of each batch of freeze-dried drug-loaded NLC is solubilized in a suitable solvent (e.g., dichloromethane, methanol). The organic solution is filtered through filters (e.g., 0.45 µm PTFE membranes) and analyzed to determine the amount of compounds.

Drug release
In vitro release may be evaluated using a dialysis bag diffusion technique, Franz diffusion cells or USP type II dissolution apparatus. For dialysis bag diffusion technique the NLC-suspension (with human plasma and/or without human plasma) is placed in dialysis bags (molecular weight cut off 12000—14000) and immersed in phosphate-buffered saline at pH 6 with paddle rotation at 50 rpm. Aliquots of 0.5 ml dissolution medium are removed and the same volume of fresh dissolution medium is added periodically. The aliquots may be diluted with suitable solvent before analysis by HPLC.
1.4.1.3. NLC in Brain Targeting

Lipid nanocarriers (e.g., NLC) are considered to be a promising strategy for drug delivery without any modification to the drug molecule. These are interesting candidates for brain targeting because of their rapid uptake by the brain, bioacceptability and biodegradability. Moreover, the feasibility in scale-up and absence of burst effect make them promising carriers for drug delivery (Alam et al., 2011). The lipid-based colloidal carriers are well tolerated in living systems because they are made of physiological compounds and the metabolic pathways exist decreasing the risk of acute and chronic toxicity. The low toxicity of lipid nanocarriers has already been shown in human promyelotic cells (Garcia-Fuentes et al., 2005). However, the nature of the lipid matrix (fat and oil) and concentration of nanoparticles may induce cytotoxic effects on macrophages (Scholer et al., 2002). The consideration of toxicity of the emulsifiers is observable (Mehnert and Mader 2001). In a nutshell, the emulsifiers (surfactants) used for the preparation of lipid nanocarriers (e.g., NLC) may induce irritative or sensitizing action (Zhang et al., 2010). NLC have been investigated for the delivery of numerous therapeutic substances including antifungal agent (Souto and Muller 2005), antioxidant (Teeranachaideekul et al., 2007), anti-inflammatory molecule (Joshi et al., 2008), antitumor (Zhang et al., 2008), cosmetic product (Pardeike et al., 2009) and antidepressant (Alam et al., 2011). Effective therapeutic effects were observed in the studies performed in animals when therapeutic substances were delivered encapsulated in NLC. Zhao et al., (2011) developed docetaxel-loaded nanostructured lipid carrier to lead to a long blood circulating effect and targeting ability for the delivery of antitumor drug in cancer. The results indicated that the drug loaded NLC led to significant differences in pharmacokinetic profile and tissue distribution. The protective effects and brain:plasma concentration ratio was much higher after intranasal administration of NLC containing valproic acid than the positive control group (Eskandari et al., 2011). NLCs respectively revealed 7.5- and 4.7-fold higher baicalein accumulations compared to the aqueous solution in the cerebral cortex and brain stem after the intravenous administration to rats. Moreover, the plasma level of baicalein in NLCs was much higher and the half-life much longer than those in the free control (Tsai et al., 2012). The mean residence time was prolonged and AUC (area under tissue concentration-time curve) value was also improved on parenteral delivery of oridonin loaded NLC compared with oridonin solution (Jia et al., 2012).
In addition, NLCs have many features that are advantageous for brain targeting;

*Since the brain is protected with BBB and only lipophilic molecules can cross the barrier. Being lipophilic in nature NLC has been expected for the transport of therapeutic substances to the brain.*

*They are colloidal carriers providing controlled release profiles for many substances.*

*They are composed of physiological and biodegradable lipids exhibiting low toxicity and low cytotoxicity that means an excellent tolerability.*

*The small size ensures a close contact to the BBB and can increase the amount of drug penetrated into the brain.*

*The lipid nanoparticles are able to enhance the chemical stability of compounds sensitive to light, oxidation and hydrolysis (Pardeike et al., 2009).*