COMPARATIVE BIOAVAILABILITY OF PROCHLORPERAZINE EDISYLATE IN PLASMA AND BRAIN TISSUE AFTER INTRAVENOUS, ORAL AND INTRANASAL ADMINISTRATION

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Abstract

Background and aim: The nasal administration of drugs offers advantages over administration by intravenous injection. Drugs can be rapidly absorbed through the nasal mucosa, resulting in a rapid onset of action, and also avoiding degradation in the gastro-intestinal tract and first-pass metabolism in the liver. Targeting the brain via nasal administration offers potential for the development of new drug products. The olfactory cells are in direct contact with both the environment and the central nervous system (CNS). The olfactory pathway thus circumvents the blood-brain barrier (BBB), which prevents many systemically administered drugs from entering the brain. A literature study concerning the anatomy and physiology of the nose, factors affecting the absorption of nasally administered drugs; different mechanisms to enhance nasal drug absorption as well as the general characteristics of prochlorperazine edisylate were performed. As a result of the literature study, it was concluded that the nasal route is suited for administration and absorption of prochlorperazine edisylate. The aim of this study was to compare the concentrations of prochlorperazine found in plasma and brain tissue of rats, after intravenous, oral and intranasal administration. **Methods:** In order to investigate the objective, a dose of 0.167 mg/kg prochlorperazine edisylate was administered intravenously (100 µl), nasally (50 µl) and 1.333 mg/kg was administered orally (100 µl). The concentrations were corrected for the different dosages in order to compare the respective bioavailabilities. The nasal bioavailability of prochlorperazine was investigated in a rat model: uptake in the brain tissue and plasma levels were compared after intravenous, oral and intranasal administration. A liquid-liquid extraction method for the quantitative determination of prochlorperazine in brain tissue and a solid-phase extraction method for the quantitative determination of prochlorperazine in plasma were used. The concentrations of prochlorperazine in plasma and brain tissue were measured with high performance liquid chromatography. **Results:** The results indicate that the nasal administration of prochlorperazine is an easy and workable alternative to intravenous injections, which may enhance patient compliance.

Nasal absorption: The concentration-time profile achieved after nasal administration of prochlorperazine is similar to that achieved after intravenous administration. The absorption of prochlorperazine edisylate from the nasal cavity into the systemic circulation was rapid and almost complete. The AUC-values, used as an indication of the extent of absorption, for the intravenous (3371.47 ± 173.79 ng/ml/h), intranasal (2936.71 ± 189.65 ng/ml/h) and oral routes of administration (718.07 ± 42.74 ng/ml/h) were compared. Compared to the intravenous route of administration (100%), the nasal route showed an absolute bioavalability of 87.10% and the oral route 21.30%. A low oral bioavailability was achieved, as expected, due to degredation in the gastro-intestinal tract and first-pass metabolism in the liver. *Uptake into the brain tissue:* The concentration-time profiles of prochlorperazine in brain tissue showed no increased maximum concentrations of drug after nasal administration compared to intravenous administration. However, this concentration was retained longer after nasal administration compared to intravenous administration. No direct evidence for transfer along the olfactory pathway was shown with prochlorperazine. The intranasal/intravenous brain tissue concentration ratio exceeded one after 30 and 45 minutes after nasal administration at a pH of 6.6 and 4.65 respectively, indicating that after these time intervals the concentrations of nasally administered prochlorperazine in the brain tissue were higher than those after intravenous administration. Prochlorperazine concentrations in the brain tissue were significantly higher after nasal administration than after oral administration. Significant concentrations of prochlorperazine were found in the brain tissue as early as 5 minutes after nasal administration. The AUC-values after nasal (96745.32 ± 3649.65 ng/g/h), intravenous (90051.71 ± 6189.75 ng/g/h) and oral administration (12507.20 ± 1248.01 ng/g/h) indicated that the nasal/intravenous AUC ratio in brain tissue was found to be greater than one. **Conclusion:** Nasal administration of CNS-active anti-emetic drugs with low oral bioavailability could be used as an alternative for the intravenous route of administration. The lipophilic drug, prochlorperazine was rapidly and almost completely absorbed after nasal administration. These molecules appeared rapidly in the brain tissue. Although

hard evidence of direct transfer from the nose remains elusive, the fact tht a higher AUC-valuewasobtained after nasal than after intravenous administration was evidence enough that the olfactory route does contribute to the delivery of drugs to the brain after nasal administration.

Uittreksel

Agtergrond en doelstelling: Die nasale toediening van geneesmiddels kan meer voordelig wees as intraveneuse geneesmiddeltoediening. Geneesmiddels kan vinnig deur die nasale mukosa geabsorbeer word wat lei tot 'n vinnige aanvang van die terapeutiese effek terwyl gastro-intestinale geneesmiddelafbraak en eerste deurgangseffek deur lewer kan vermy word. Die nasale roete van toediening van geneesmiddels met die brein as teikenorgaan, bied potensiaal vir die ontwikkeling van nuwe geneesmiddelprodukte. Die olfaktoriese selle in die neus is in direkte kontak met beide die eksterne omgewing en die sentrale senuweestelsel (SSS). Die olfaktoriese roete omseil dus die bloedbreinskans wat baie sistemies toegediende geneesmiddels verhoed om die brein binne te dring. 'n Literatuurstudie aangaande die anatomie en fisiologie van die neus, faktore wat die absorpsie van nasaal toegediende geneesmiddels beinvloed, verskillende meganismes om die absorpsie van nasaal toegediende geneesmiddels te bevorder asook die algemene eienskappe van prochloorperasien edisilaat is gedoen. Uit die literatuurstudie is afgelei dat die nasale roete geskik is vir toediening en absorpsie van prochloorperasien edisilaat. Die doel van diè studie was om die konsentrasies prochloorperasien in plasma en breinweefsel as 'n funksie van tyd te bepaal na intraveneuse, orale en nasale toediening. **Metode:** Om die doelwit te bereik is 'n dosis van 0.167 mg/kg prochloorperasien edisilaat intraveneus (100 µl), nasaal (50 µl) en 1.333 mg/kg oraal (100 µl) toegedien. Die konsentrasies is gekorregeer vir die verskillende dosisse om sodoende die onderskeie biobeskikbaarhede te kon vergelyk. Die nasale biobeskikbaarheid van prochloorperasien is ondersoek in 'n rotmodel: geneesmiddelopname in breinweefsel en plasmavlakke is vergelyk na intraveneuse, orale en nasale toediening. 'n Vloeistof-vloeistof ekstraksie metode vir die kwantitatiewe bepaling van prochloorperasien in die breinweefsel en 'n soliede fase ekstraksie metode vir die kwantitatiewe bepaling van prochloorperasien in die plasma is gebruik. Die konsentrasies prochloorperasien in plasma en breinweefsel is bepaal deur hoë druk vloeistof

chromatografie. **Resultate:** Die resultate is 'n aanduiding daarvan dat die nasale toediening van prochloorperasien 'n interessante en werkbare alternatief bied vir intraveneuse inspuitings. *Nasale absorpsie:* Die konsentrasie-tyd profiel wat verkry is na nasale toediening is soortgelyk aan diè verkry na intraveneuse toediening. Die absorpsie van prochloorperasien vanuit die nasale holte was vinnig en byna volledig. Die AUC-waardes, wat as 'n indikasie vir die mate van absorpsie gebruik word, vir die intraveneuse (3371.47 ± 173.79 ng/ml/h), nasale (2936.71 ± 189.65 ng/ml/h) en orale roetes van toediening (718.07 ± 42.74 ng/ml/h) is vergelyk. In vergelyking met die intraveneuse roete van toediening (100%), het die nasale roete 'n absolute biobeskikbaarheid van 87.10% en die orale roete 21.30% getoon. 'n Lae orale biobeskikbaarheid is verkry soos verwag, as gevolg van gastro-intestinale afbraak en die eerste deurgangseffek in die lewer. *Geneesmiddelopname in die brein:* Die konsentraie-tyd profiel van prochloorperasien in die breinweefsel het geen verhoogde maksimum geneesmiddelkonsentrasies getoon na nasale toediening in vergelyking met intraveneuse toediening nie. Diè konsentrasies is egter vir 'n langer periode gehandhaaf na nasale toediening in vergelyking met intraveneuse toediening. Geen bewyse van direkte verplasing via die olfaktoriese roete is gevind met prochloorperasien nie. Die nasale/intraveneuse konsentrasie verhouding in die breinweefsel was groter as een, 30 en 45 minute na toediening by onderskeie pH waardes van 6.6 en 4.65, wat aandui dat die konsentrasies van nasaal-toegediende prochloorperasien na bg. tydsintervalle hoër is as na intraveneuse toediening. 'n Betekenisvolle hoër konsentrasie prochloorperasien is in die breinweefsel gevind na nasale toediening in vergelyking met orale toediening. Beduidende prochloorperasien konsentrasies is so vinnig as 5 minute na nasale toediening in die breinweefsel gevind. Die AUC-waardes na nasale (96745.32 ± 3649.65 ng/g/h), intraveneuse (90051.71 ± 6189.75 ng/g/h) en orale toediening (12507.20 ± 1248.01 ng/g/h) het aangedui dat die nasale/intraveneuse AUC verhouding in die breinweefsel groter as een is. **Gevolgtrekking:** Die nasale toediening van anti-emetikums wat aktief is in die SSS en 'n lae orale biobeskikbaarheid toon, gebruik kan word as 'n alternatiewe roete vir die

intraveneuse toediening van geneesmiddels. Die lipofiele geneesmiddel, prochlooperasien, is vinnig en amper volledig geabsorbeer na nasale toediening. Hierdie molekules het vinnig in die breinweefsel verskyn. Alhoewel daar geen konkrete bewyse van direkte oordrag vanaf die neus na die brein is nie, dien die feit dat 'n groter AUC waarde na nasale as na intraveneuse toediening verkry is, as genoegsame bewys dat die olfaktoriese roete wel bydra tot geneesmiddelaflewering in die brein.

Statement of the problem

The bioavailability of a drug and hence its therapeutic effectiveness are often influenced by the route of administration. For a medication to achieve its maximal efficacy it should be easily administered in order to achieve better patient compliance; and it should be efficiently absorbed in order to achieve greater bioavailability (Chien *et al.*, 1989:1).

Continuous intravenous infusion of a drug at a programmed rate has been recognised as a superior mode of delivery, since it is capable of bypassing gastrointestinal incompatibility and hepatic "first pass" metabolism, and results in a constant, prolonged plasma drug level within the therapeutically effective range. However, such mode of delivery entails certain potential risks and, therefore, necessitates hospitalisation of the patient for close medical supervision (Chien *et al.*, 1989:iii).

Oral dosage forms are usually intended for systemic effects resulting from drug absorption through various epithelial layers of the gastrointestinal tract (York, 1988:4). This is the most popular and convenient route of administration for those drugs that can survive the acid environment of the stomach and which are absorbed across the gastrointestinal membranes (Florence & Attwood, 1988:348). The oral route is the simplest, most convenient and the safest route of administration, however, it has a relatively slow onset of action, possibilities of irregular absorption, destruction of certain drugs by the enzymes and secretions of the gastrointestinal tract as well as the "first-pass" effect (York, 1988:4).

During the past 20 years, advances in drug formulations and innovative routes of administration have been made. Understanding of drug transport across tissues has increased. These changes have often resulted in improved patient

adherence to the therapeutic regimen and pharmacologic response. The administration of drugs by the transdermal and transmucosal routes offers the advantage of being relatively painless, as well as flexibility in a variety of clinical situations. The development of alternative methods of drug administration has improved the ability of physicians to manage specific problems. Practitioners recognise the rapid onset, relative reliability, and the general lack of patient discomfort when drugs are administered by the transmucosal and transdermal routes.

Drug absorption through a mucosal surface is generally efficient because the stratum corneum epidermis, the major barrier to absorption across the skin, is absent. Mucosal surfaces are usually rich in blood supply, providing the means for rapid drug transport to the systemic circulation and avoiding, in most cases, biotransformation by first-past hepatic metabolism (American academy of pediatrics, 2000).

The importance of effective management of chemotherapy induced nausea and vomiting has been realised for some time. The standard antiemetic agents first used were the phenothiazines. Most of these are very familiar and a good deal has been published about them since they were introduced in the early 1950's. Some of the most common phenothiazines are chlorpromazine prochlorperazine, triethylperazine, promethazine, triflupromazine and perphenazine (Laszlo, 1983:5).

In this study prochlorperazine edisylate is used as a model drug to compare the bioavailability after oral, intravenous and intranasal administration to rats. Prochlorperazine, a piperazine derivative of the phenothiazine family, is an effective antiemetic agent for chemotherapy and radiotherapy treatment and is frequently prescribed for the treatment of vertiginous disorders (Bond, 1998:1).

However, the combination of reduced blood flow in the gastric mucosa, accelerated mucus production and a decreased parietal cell output resulting from vertigo, together with a reduction in gastric emptying rate, tone and contraction displayed consistently in physically-induced vertigo, may reduce the absorption of the oral preparation. In addition, following administration of oral prochlorperazine, pharmacokinetic studies have reported a bioavailability as low as 12.5 % owing to metabolism in the intestinal wall and first-pass metabolism in the liver. In addition, the increased likelihood of regurgitation in the nauseous patient further reduces the potential efficacy of the oral preparation (Bond, 1998:1).

The aim of this study was to compare the bioavailability of prochlorperazine edisylate following oral, intravenous and intranasal administration to rats.

The objectives were to:

1. Perform a literature study including:
   - the bioavailability of prochlorperazine edisylate after administration at various absorption sites and its viability for intranasal administration;
   - the physical and chemical characteristics of prochlorperazine edisylate;
   - the anatomy and physiology of the nasal cavity;
   - factors influencing nasal absorption and strategies to improve nasal absorption and
   - high-performance liquid chromatography (HPLC) methods for the determination of prochlorperazine in biological fluids;
2. Develop a sensitive and specific HPLC method for the determination of prochlorperazine concentrations in brain tissue;
3. Perform in vivo drug delivery studies in rats to:

- compare the intravenous, oral and intranasal absorption of prochlorperazine edisylate into systemic circulation ;
- compare the intravenous, oral and intranasal absorption of prochlorperazine edisylate into the brain tissue;
- compare the primary pharmacokinetic parameters (AUC, C_{max} and t_{max}) obtained with each of the routes of administration and to
- determine whether prochlorperazine is a potential candidate to be incorporated into a nasal therapeutic system.

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Chapter 1

Transnasal drug delivery

1.1 Introduction

In the last decade, there has been much interest in the nasal route for delivery of drugs to the brain via the olfactory region in order to circumvent the blood brain barrier (BBB). In recent studies, nerve growth factor (Frey II *et al.*, 1997:87), local anaesthetics (Chou & Donovon, 1998a:137), dihydroergotamine (Wang *et al.*, 1998:571) and 2',3'-didehydro-3'-deoxythymidine (Yajima *et al.*, 1998:272) have been transported into the central nervous system (CNS) via the nasal route.

The BBB that segregates the brain interstitial fluid from the circulating blood consists of two plasma membranes in series, the luminal and the anti luminal membranes of the brain capillary epithelium. These two membranes are separated by about 0,3 mm of endothelial cytosol. The cells of the capillary endothelium are closely connected via intercellular connections; the tight-junctions that act as zips closing the inter-endothelial pores that normally exists in endothelial membranes. This makes the BBB resistant to the free diffusion of molecules across the membrane and prevents most molecules from reaching the CNS from the blood stream (Illum, 2000:1).

Several different approaches have been attempted in order to circumvent the BBB and to deliver drugs efficiently to the brain for therapeutic or diagnostic applications. According to Pardridge (1991), one of the approaches to increase the permeability of a drug, is to create a more lipophylic molecule, often in the form of a prodrug that is converted to the parent drug once in the brain. Other approaches include the binding of drugs to carrier molecules such as transferrin

or to polycationic molecules such as cationised proteins that will bind preferentially to the negatively charged endothelial surface. Targeting the brain via nasal administration of drugs also offers potential for drug development since the olfactory receptor cells are in direct contact with both the environment and the CNS. The absence of a strict nose-brain barrier could, then, allow air-borne substances, viruses, metals or drugs to be delivered into the CNS.

1.2 Anatomy and physiology of the nose

1.2.1 Anatomy and function

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 (Figure 1.1). The nasal vestibule, the respiratory region and the olfactory region are the three main regions of the nasal cavity (Chien *et al.*, 1989:2).

In cross section the nasal cavity is very narrow, with a diameter of around 1-5mm. The turbinates provide a very large surface area, which in combination with the convoluted air stream provide intimate contact between inspired air and the nasal mucosa (Ridley *et al.*, 1992:14).

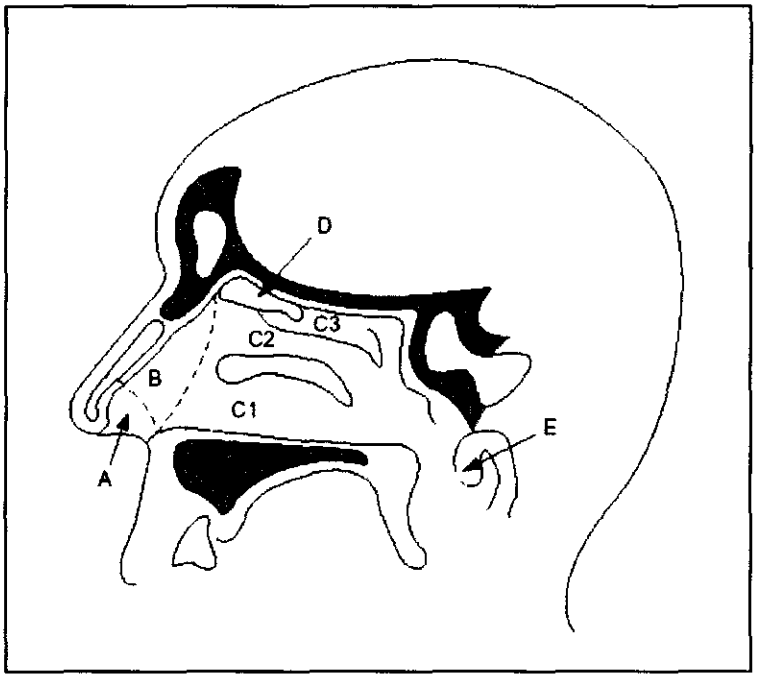

Figure 1.1: Schematic of saggital section of the nasal cavity showing the nasal vestibule (A), atrium (B), respiratory area: Inferior turbinate (C1), middle turbinate (C2) and the superior turbinate (C3), the olfactory region (D) and the nasopharynx (E) (Arora *et al.*, 2002:969).

During inspiration, 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; this conditioning of the inhaled air is facilitated by the fluid secreted by the mucosa and the high blood supply in the nasal epithelium The sub mucosal zone of the nasal passage is extremely vascular and this network of veins drains blood from the nasal mucosa directly to the systemic circulation, thus avoiding first-pass metabolism (Dahlin, 2000:8). Another, perhaps more familiar, major function of the nose is olfaction. The olfactory region is located on the roof of the nasal cavity. The nasal cavity is covered with a mucous membrane, which can be divided into non-olfactory, and olfactory epithelium areas (Dahlin, 2000:8). The non-olfactory area includes the nasal vestibule, which is lined with skin-like cells, and the respiratory region, which is typical airway epithelium.

1.2.2 Respiratory region

The respiratory epithelium is very important for nasal drug absorption, because it is the barrier through which a drug must pass before entering the systemic circulation (Verhoef & Merkus, 1994:12). 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 respiratory epithelium consists of four types of cells (Figure 1.2):
Basal cells: the basal cells, which are progenitors of the other cell types, lie on the basement membrane and do not reach the airway lumen. Among their morphological specialisations are desmosomes, which mediate adhesion between adjacent cells, and hemidesmosomes, which help anchor the cells to the basement membrane. Currently, basal cells are believed to help in the adhesion of columnar cells to the basement membrane.

Columnar cells: the columnar cells, which could be ciliated and non-ciliated, are covered by about 300 microvilli, uniformly distributed over the entire apical surface. These short and slender fingerlike cytoplasmic expansions increase the surface area of the epithelial cells, thus promoting exchange processes over and across the epithelium. The microvilli also prevent drying of the surface by retaining moisture essential for ciliary function. The cilia have a typical ultra structure, each ciliated cell containing about 100 cilia, 0,3μm wide and 0.5μm in length. The anterior one-third of the nasal cavity is non-ciliated. Cilia start occurring just behind the front edge of the inferior turbinate, and the posterior part of the nasal cavity, as well as the para nasal sinuses is densely covered by cilia. The distribution pattern of ciliated cells corresponds well with the map of nasal airflow, indicating that the density of ciliated cells is inversely proportional to the linear velocity of inspiratory air in the nasal cavity. Consequently there are less cilia in the upper part of the nasal cavity than along the floor. It is also assumed

that low temperature and humidity contribute to a reduced number of ciliated cells. Understandably, the anterior part of the nasal cavity, hit by strong currents of cold, dry and polluted air has a low density of ciliated cells.

Goblet cells: the goblet cells are characteristic to the airway epithelium. There are slight topographical differences, with a larger number in the posterior than in the anterior part of the nasal cavity. The mean concentration of goblet cells (4000 – 7000 cells per mm^2) is similar to that in the trachea and the main bronchi. The goblet cell contribution to the volume of nasal secretion is probably small, compared to that of the sub mucosal glands. Little is known of the release mechanisms from goblet cells but it probably responds to physical and chemical irritants in the microenvironment. Surface epithelial cells are bound together by tight junctions located on the apical part of the intracellular connection. Ultra structural studies have shown fragmentation and discontinuity of tight junctions around filled goblet cells. This finding may be of relevance for the absorption of aerosolized drugs, deposited on the airway epithelium (Mygind & Dahl, 1998:5).

The protrusions of the different cell types vary in different regions of the nasal cavity. In the lower turbinate area about 15–20% of the total number of cells are ciliated and 60–70% are 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 non-ciliated cells indicates their importance for absorption across the nasal epithelium. The large number of microvilli increases the surface area and this is one of the main reasons for the relatively high absorption capacity of the nasal cavity (Dahlin, 2000:8)

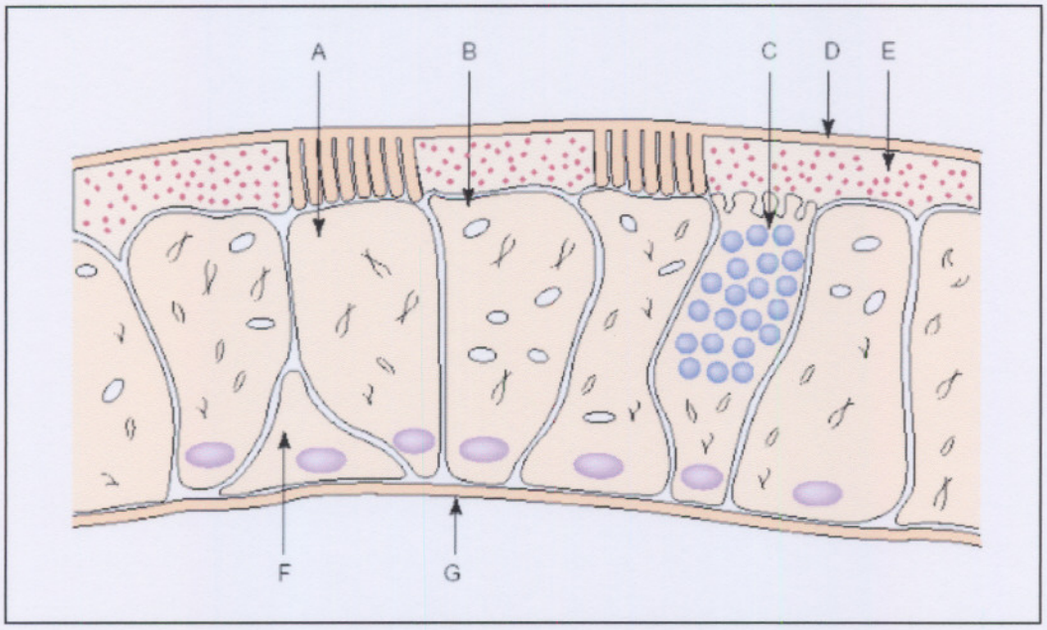

Figure 1.2: Cell types of the nasal epithelium showing a ciliated cell (A), non-ciliated cell (B), goblet cells (C), gel mucus layer (D), sol layer (E), basal cell (F) and basement membrane (G) (Arora *et al.*, 2002:969).

The thickness of the respiratory epithelium is approximately 100 µm (Verhoef & Merkus, 1994:121). There are two types of mucus covering the surface of the mucous membrane; one adheres to the tips of cilia, and the other fills the space among the cilia (Chien *et al.*, 1989:4).

The ciliated nasal epithelial cells present a barrier to the absorption of drugs and contaminants. This barrier is relatively thin and well perfused by nasal blood vessels. However, any drug substance applied nasally would rarely come into prolonged intimate contact with the epithelial cell surface owing to the presence of mucus glands and goblet cells (Rogerson & Parr, 1990:1).

Adequate moisture is required to maintain the normal functions of the nasal mucosa. Dehydration of the mucous blanket increases the viscosity of the

secretions and reduces ciliary activity. Thus, the recovery of heat and moisture from expired air by the nasal membranes is of fundamental importance for retaining its normal functions (Chien *et al.*, 1989:6).

1.2.3 The olfactory region

In humans, 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. The olfactory tissue is often yellow of color, in contrast to the surrounding pink tissue (Chien *et al.*, 1989:2). Humans have relatively simple noses since the primary function is breathing, while other mammals have more complex noses better adapted for the primary function of olfaction. The olfactory region is about $10cm^2$ in man, as compared to $170cm^2$ in the German shepherd dog. These size differences in the olfactory area reflect the importance of the sense of smell for different species (Chien *et al.,* 1989:2). The human olfactory organ is similar in organization and cell morphology to that of vertebrate species. The olfactory epithelium rests upon thick connective tissue, the lamina propria, which contains blood vessels, olfactory axon bundles and Bowman's glands. Like the epithelium of the respiratory region, the olfactory epithelium comprises pseudo-stratified columnar cells of three principal types: olfactory receptor cells, supporting cells and basal cells (Figure 1.4).

The basal cells are flattened to an elongated ovoid shape, and are located close to the epithelial side of the basal lamina. The olfactory neurons are interspersed between the supporting cells that form a distinct layer in the upper third of the olfactory epithelium (Dahlin, 2000:10).

The olfactory receptor cells are specialised for the detection of odorants. It is estimated that there are 10 to 20 millions of these cells in humans. Near the epithelial surface, the dendrites terminate in ciliated olfactory knobs of various shapes, which usually extend above the epithelial surface. The number of cilia varies, but there are about 10 to 25 extending from each knob (Dahlin, 2000:11).

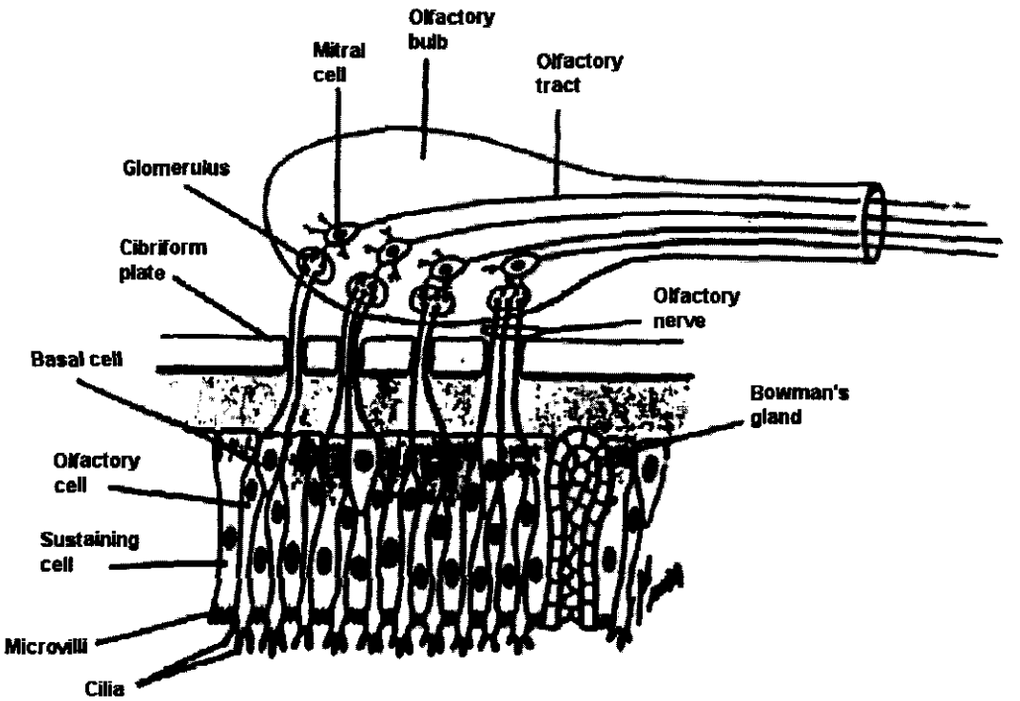

Figure 1.3: Schematic illustration of the various cell types in the olfactory region in the vault of the human nose (Illum, 2000: 4).

The nasal mucosa is the only location in the body that provides a direct connection between the central nervous system and the atmosphere. Drugs sprayed onto the olfactory mucosa are rapidly absorbed by three routes namely, by the olfactory neurons, by the supporting cells and the surrounding capillary bed, and directly into the cerebrospinal fluid (CSF) (American academy of pediatrics, 2000).

1.3 Absorption across the nasal epithelium

Intranasal administered drugs have to pass through the epithelial layer to reach their site of pharmacological action via the bloodstream. A drug administered through the nasal cavity can permeate either passively by the paracellular pathway or both passively and actively via the transcellular pathway. This basically depends on the lipophilicity of the compound. Apart from the passive transport pathways, carrier mediated transport transcytosis and transport through intercellular tight junctions are other possible pathways for a drug to permeate across the nasal mucosa.

1.3.1 Barriers to drug absorption

The nasal membrane is the first line of defense against inhaled micro-organisms, allergens and irritating substances from the environment. There are various barriers in the nasal membrane for protection from these unwanted substances which must be overcome by drugs, before they can be absorbed into systemic circulation.

Lipophilic drugs are generally well absorbed from the nasal cavity. However, despite the large surface area of the nasal cavity and the extensive blood supply, the permeability of the nasal mucosa is normally low for polar molecules, including low molecular weight drugs and especially large molecular weight peptides and proteins (Illum, 2002:2).

The most important factor limiting the nasal absorption of polar drugs and especially large molecular weight polar drugs such as peptides and proteins is

the low membrane permeability (Illum, 2002:3). Absorption through the nasal mucosa decreases exponentially with increases in molecular size if the molecular size is greater than 400 Da. The nasal rate-limiting molecular weight was found to be 1000 Da in comparison to the 300 Da for the oral route (Dondeti *et al.*, 1996:116).

The general rapid clearance of the administered formulation from the nasal cavity due to the mucociliary clearance mechanism is another factor of importance for low membrane transport. This is especially the case for drugs that are not easily absorbed across the nasal membrane. It has been shown that the half life of clearance is in the order of 15-20 min for both liquid and powder formulations that are not mucoadhesive (Illum *et al.*, 1987:133). It has further been suggested that the deposition of a formulation in the anterior part of the nasal cavity can decrease clearance and promote absorption, as compared to deposition further back in the nasal cavity (Harris *et al.*, 1986:1085).

Another contributing (but normally considered less important) factor to the low transport of especially peptides and proteins across the nasal membrane is the possibility of enzymatic degradation of the molecule either within the lumen of the nasal cavity or during passage across the epithelial barrier. The enzymes present are both oxidative (e.g. cytochrome P-450, aldehyde dehydrogenases, carboxy esterase and carbonic anhydrase) and conjugative (e.g. glucuronyl, sulfate and glutathione transferases). Cytochrome P-450 activity in the olfactory region of the nasal cavity is even higher than in the liver (Verhoef & Merkus, 1994:127). Cytochrome P-450 dependant monooxygenases have been reported to catalyse the metabolism of different xenobiotics. It has also been observed to metabolize many compounds in the nasal mucosa, such as nasal decongestants, nicotine and cocaine, phenacetin and progesterone (Chien *et al.*, 1989:16).

The enzymatic activities cleaving peptides and proteins are also endopeptidases (e.g. aminopeptidases, carboxypeptidases, trypsin-like activities, cathepsins),

which are present at the surface of the nasal mucosa and/or within the epithelial cells. Among these enzymes, amino peptidase activity is the most predominant. The enzymatic characteristics of the nasal mucosa create a pseudo-first-pass effect, which may hamper nasal drug absorption (Verhoef & Merkus, 1994:127). Insulin (zinc free) was found to be hydrolysed slowly by leucine amino peptidase in the nasal epithelium (Chien *et al.*, 1989:16).

1.3.2 Factors affecting nasal drug absorption

Figure 1.4 gives a schematic representation of the different factors affecting the nasal absorption of drugs.

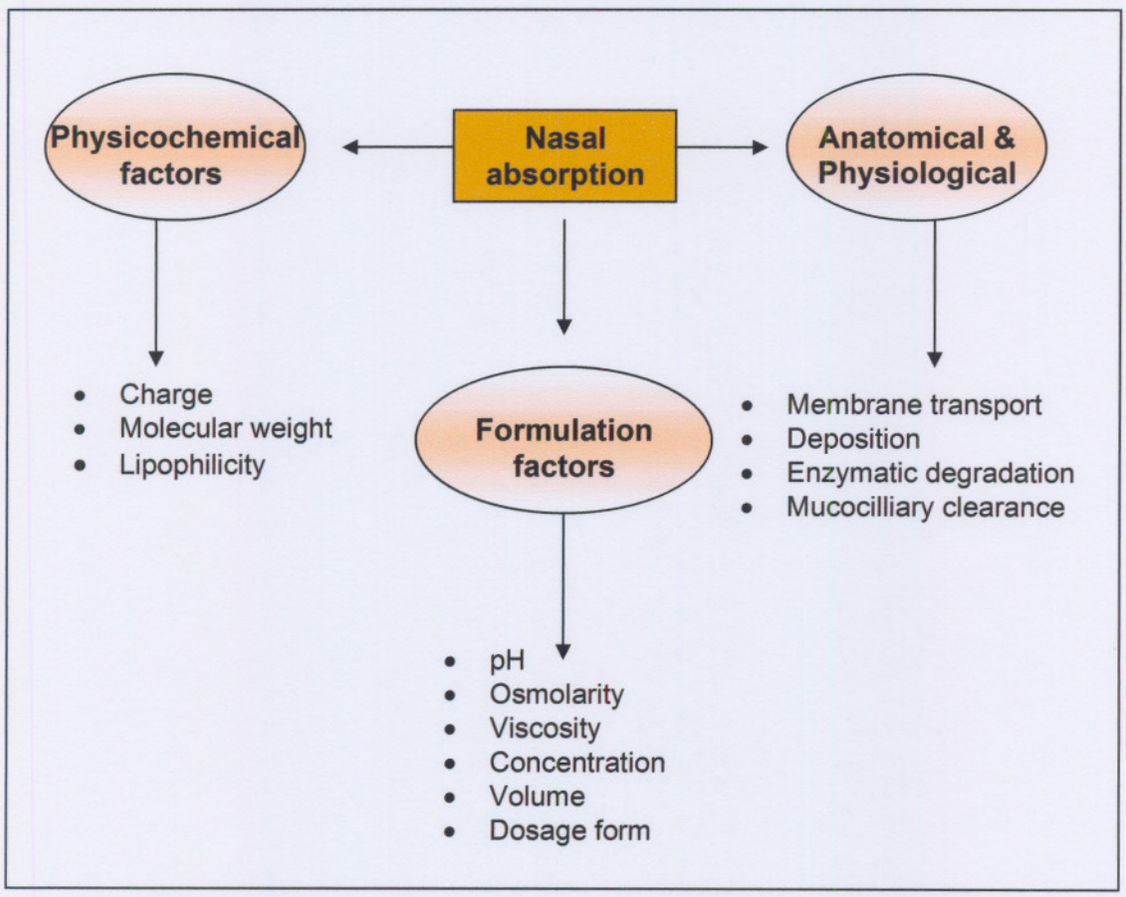

Fig. 1.4: The physicochemical, anatomical, physiological and formulation factors affecting the nasal absorption of drugs (Illum, 2002:1186).

The extent of absorption from the nasal cavity depends partly on the size of drug molecules, a factor that is most important for hydrophilic compounds. The nasal route, as mentioned, appears to be suitable for the efficient rapid delivery of molecules with a molecular weight of less than 1000 Da. This means that the bioavailability of larger polypeptides like insulin (2.5kDa) will be too low when administered nasally. However, formulation additives (absorption enhancers) may increase the bioavailability of these compounds (Dahlin, 2000:12).

Lipophilic drugs, such as propranolol, progesterone, pentazocine and fentanyl, generally demonstrate rapid and efficient absorption when given nasally. For such drugs, it is possible to obtain pharmacokinetic profiles similar to those obtained after intravenous injection, with the bioavailability for some drugs approaching 100% (Illum, 2002:2).

The pKa of a substance and the pH in the surrounding area are two factors that decide the ratio of dissociated to undissociated molecules of a drug. Several studies have shown that the amount of absorbed drug is increased with increasing fraction of undissociated molecules (Hussain *et al.*, 1985:128).

A linear relationship between the rate constant of absorption and the log P (chloroform/water) has been demonstrated with Barbiturates in rats (Table 1.1).

Table 1.1: Comparison of the extent of nasal absorption of barbiturates after 60 minutes at pH 6.0 in rats and the partition coefficients (chloroform / water) of the undissociated drug (Huang *et al.*, 1985:611).

Barbiturate	Partition coefficient	Percentage absorption	
		Nasal	Oral
Barbital	0.7	5.0 ±3.00	12
Phenobarbital	4.8	10.6±3.88	20
Pentobarbital	28.0	20.3±4.65	30
Secobarbital	50.7	23.9±9.38	40

1.4 The olfactory pathway

Every day we use our noses to help make sense of our environment. We may not be as dependent on our olfactory capabilities as animals, but we are able to recognize thousands of chemicals in our environment.

Odor molecules that enter the nose are detected by odor receptors located on the surface of olfactory neurons. There are about 5 million olfactory neurons, which are located in the olfactory epithelium on the wall of the nasal cavity. Each of these neurons extends a long process, called an axon, to the olfactory bulb of the brain. Once in the olfactory bulb, the axon enters a spherical structure called a glomerulus, where it makes contact with the neurons in the bulb. The bulb neurons, in turn, extend axons to make contact with neurons located in the olfactory cortex. When odor receptors on the olfactory neuron detect an odorant, the neuron is activated. This sets off a chain reaction, whereby signals are

transmitted from the neuron in the nose to connected neurons in the bulb and olfactory cortex (Fig. 1.5) (Zou *et al.*, 2001:173).

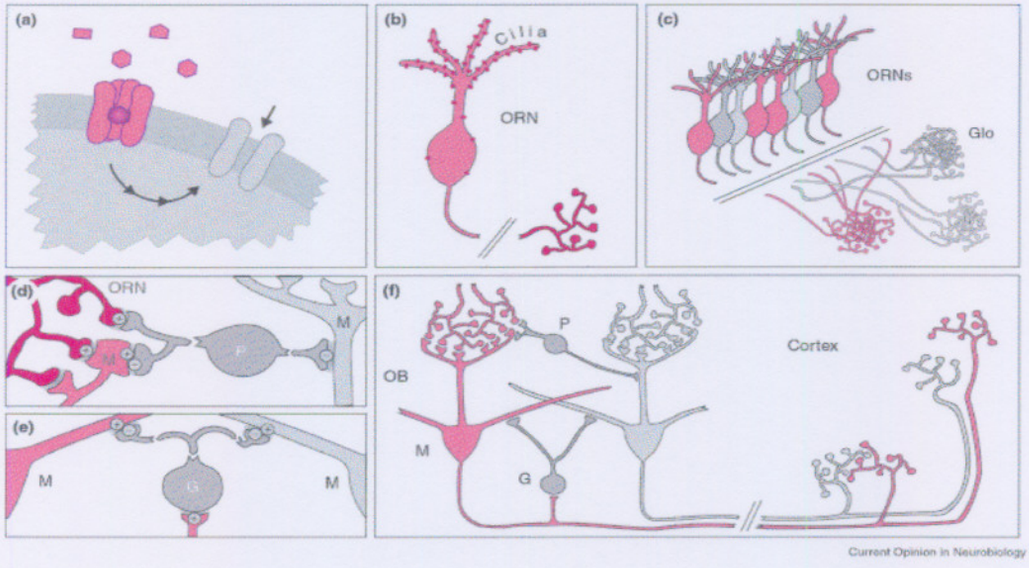

Fig 1.5: Schematic representation of the vertebrate olfactory information pathway. **(a)** Odorant receptors (heptahelicals) transduce their activation by a signaling cascade involving several steps, culminating in the opening of ion channels. Odorants fit to a different degree into the binding pocket of the receptor. **(b)** An olfactory receptor neuron (ORN) contains only same type odorant receptor molecules. Activated ORNs relay signals to axon terminals in the olfactory bulb. **(c)** ORNs of the same kind segregate from unrelated ORNs and converge onto a glomerulus (Glo) in the olfactory bulb. **(d)** and **(e)** Mitral cells (M) are activated by ORNs but are inhibited by various horizontal inhibitory interneurons — periglomerular (P) and granule (G) cells. **(f)** Mitral cells deliver their signal via branched axons to pyramidal neurons in several cortical projection areas. Partial overlap for termination areas of mitral cells connecting to different glomeruli is observed (Korsching, 2002:388).

1.4.1 The central nervous system (CNS)

The central nervous system is protected from trauma by the scull and vertebra. The brain is surrounded by the subarachnoid space in which runs the cerebrospinal fluid (CSF). This space is again surrounded by the meninges which consist of three membranes, the dura mater, which lies directly beneath the scull, the pia mater, which lies directly over the brain, and in between the arachnoid. Between the pia mater and the arachnoid is the subarachnoid space (Fig. 1.6) (Illum, 2000:5).

The CSF is not a filtrate of plasma, but rather a secretory fluid produced mainly by the choroids plexus. Each choroids plexus comprises a secretory epithelium that is perfused by blood at a local high perfusion rate. The ependyma is the lining membrane of the choroids plexus and the lateral ventricles. This membrane consists of cubic cells joined in close apposition by apical junctional complexes, thus forming a barrier to the CSF. The blood-CSF-barrier, however, is not as formidable as the BBB, since many compounds that are restricted by the BBB can fairly easily pass the cellular ependymal layer (Pardridge,1993,as quoted by Dahlin, 2000:13).

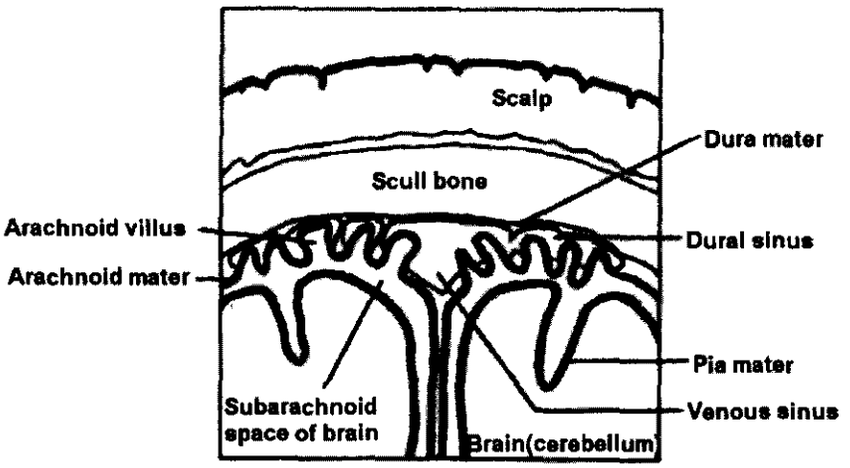

Fig 1.6: Relationship of meninges and cerebrospinal fluid to brain and spinal cord. The frontal section in the region between the two cerebral hemispheres of the brain, depicting the meninges in greater detail (Illum, 2000:5).

The rate of CSF production, which equals the rate of CSF absorption into the peripheral bloodstream at the arachnoid villi, varies from 21 ml/hour in humans to 0,18ml/hour in rats and 0.018ml/hour in mice. It can hence be calculated that for a rat the entire CSF volume would be totally replaced every hour (i.e. 24 times a day) whereas in humans the CSF is turned over every 5 hours, 4-5 times a day. Thus, the CSF is constantly formed at the choroid plexi and subsequently drained into the peripheral bloodstream at the arachnoid villi (Illum, 2000:6).

According to Pardridge (1991), the distinct difference in CSF bulk flow properties and the diffusional flow rates of drugs in brain tissue (and interstitial fluid (ISF)) creates a functional barrier between the CSF and the cells of the brain tissue to include the ISF. This prevents complete equilibration between the two fluid compartments and a significant drug concentration difference exists between CSF and brain ISF. A graphic representation of these two central extracellular compartments of the brain and their functional interaction with the bloodstream is given in Fig. 1.7. Hence, although no anatomical barrier exists between the CSF and the brain it can be concluded that a drug administered nasally which successfully reaches the CSF (and available drug receptors at the site) cannot

automatically be considered to distribute further into the brain parenchyma (Illum, 2000:6).

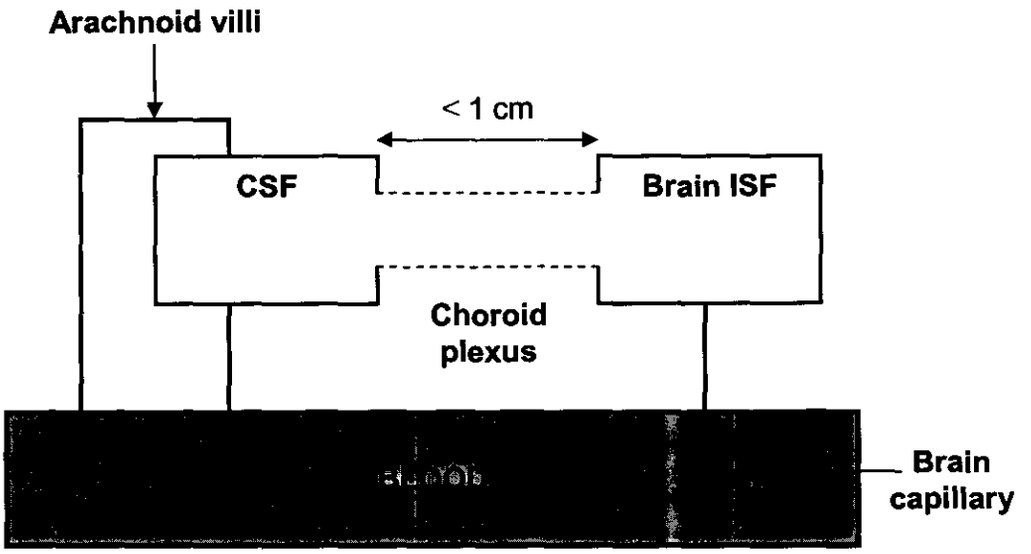

Figure 1.7: The relationship between the cerebrospinal fluid and the interstitial fluid/brain tissue and their functional interaction with the bloodstream (Illum, 2000).

1.4.2 Transport mechanisms along the olfactory pathway

The different routes by which a drug delivered nasally can reach the CSF and the brain are shown schematically in Fig. 1.8, where the thickness of the arrows indicates the likelihood of drugs exploiting the route in question. When drugs are administered nasally the drug will normally be rapidly cleared by the mucocilliary clearance system (Illum *et al.*, 1994: 82). Some of the drugs (for lipophylic drugs up to 100%, but normally much less) will be absorbed into the bloodstream, from where it reaches the systemic circulation directly and subsequently is eliminated from the bloodstream via normal clearance mechanisms (Illum, 2000:6). The drug can reach the brain from the blood by crossing the blood-brain barrier (the so-called systemic pathway to the brain) but can also be eliminated from the CSF into the blood. Of particular interest to this review is the fact that the drug can

also be absorbed from the nose via the olfactory region into the CSF and possibly further into the brain.

The amount of drug absorbed or lost via the different pathways has been shown to be highly dependant upon the characteristics of the drug, especially lipophilicity and molecular weight, but also the drug formulation (Sakane *et al.*, 1991b:2456, 1995:379).

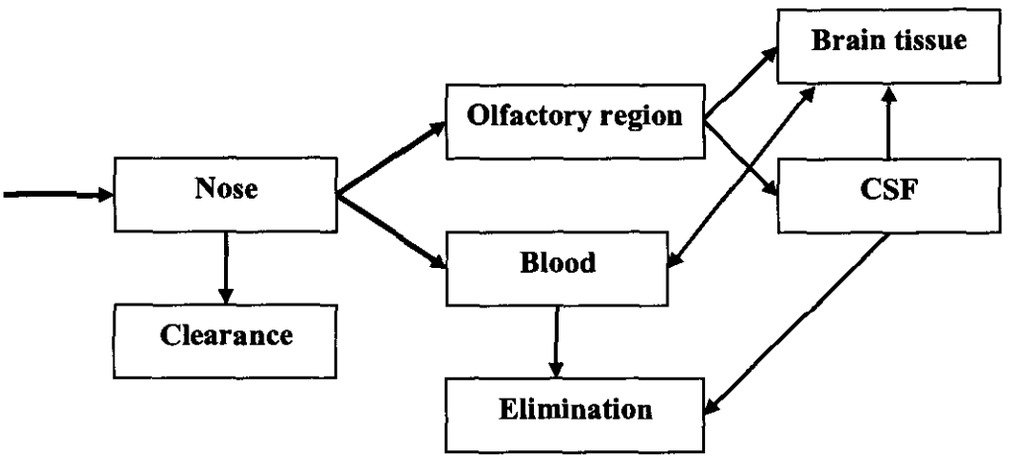

Fig 1.8: The nose to brain transport route (Illum, 2000:6).

In order for a drug to travel from the olfactory region in the nasal cavity to the CSF or the brain parenchyma, it has to transverse the nasal olfactory epithelium and, depending on the pathway followed, also the arachnoid membrane surrounding the subarachnoid space. In principle, one can envisage three different pathways across the olfactory epithelium; (1) transcellularly, especially across the sustentacular cells, most likely by receptor mediated endocytosis, fluid phase endocytosis or by passive diffusion, the latter pathway most likely for more lipophylic drugs, (2) paracellularly through the tight-junctions between the sustentacular cells or the so-called clefts between the sustentacular cells and the olfactory neurons, (3) by the olfactory nerve pathway, where the drug is taken up into the neuron cell by endocytotic or pinocytotic mechanisms and transported by intracellular axonal transport to the olfactory bulb (Illum, 2000:6).

Axonal transport of endogenous substances, in either the anterograde or retrograde direction is a well-known phenomenon. The transport rate depends on the substance being transported and also varies in different animal models (Dahlin, 2000:14). Intranasal instillation of nickel ($^{63}Ni^{2+}$) in rats resulted in an uptake of the metal in the olfactory epithelium and a migration along primary olfactory neurons to the glomeruli of the olfactory bulb. The metal was then seen to pass to the interior of the bulb and further to the olfactory peduncle, the olfactory tubercle and the rostral parts of the prepiriform, frontal and cingulate cortices. These results indicate that $^{63}Ni^{2+}$ slowly passes to secondary and tertiary olfactory neurons (Hedriksson *et al.*, 1997:153).

This extracellular pathway relies on the anatomical connection between the nasal submucosa and the subarachnoid space. The perineural space around the olfactory neurons is an extension of the subaracnoid space and the fluid in the perineural space is in direct contact with the CSF. Transport of substances into the CNS via the epithelial pathway could thus be more rapid than via axonal transport. It is likely that smaller compounds that appear rapidly in the CSF after nasal administration have been transported through this pathway (Dahlin, 2000:15). However, Frey II *et al.* (1997) showed that neuro growth factor (NGF), with a molecular weight of 37 kDa, was transported into the CNS within 20 minutes after nasal administration in a rat model. The rapid appearance of the NGF in the olfactory bulb indicated that transport was more likely to have taken place through the intercellular clefts and extracellular transport to the CSF and brain, rather than via axonal transport along the olfactory neurons.

Airborne neurotoxic metals like cadmium, nickel, mercury and manganese have been shown to enter the CNS via the olfactory epithelium in the nose. In a study by Brenneman *et al.* (2000), a unilateral occlusion model was developed. High levels of manganese were observed in the olfactory bulb and tract/turbinate on the side with an open nostril within 1-2 days following inhalation exposure.

These results demonstrate that the olfactory route contributes the majority (up to 90%) of the manganese found in the olfactory pathway (Brenneman *et al.*, 2000:238).

1.4.3 Factors affecting transport along the olfactory pathway

1.4.3.1 *Molecular weight*

The molecular weight of a substance is, as mentioned above, one deciding factor in whether or not it will be transported along the olfactory pathway, as with absorption across other epithelia in the body. Sakane and co-workers have demonstrated a linear relationship between the transport of compounds from the nose to the CSF and their molecular weight (Sakane *et al.*, 1994:379) and lipophilicity (Sakane *et al.*, 1991b: 2457) in rats.

In these studies, direct uptake into the CSF of various molecular weights of dextrans labeled with fluorescein isothiocyanate, was dependant on molecular weight after nasal administration. Dextrans with molecular weights of 4kD, 10kD and 20kD were transported directly to the CSF while those weighing 40kD were not found in the CSF (Sakane *et al.*, 1994:380).

1.4.3.2 *pKa & pH*

Nasal administration of sulphisomidine in perfusions of varying pH resulted in more extensive transport of undissociated drug molecules into the CSF (Sakane *et al.*, 1994:379). The ratio of the drug concentration in the CSF to that in the nasal perfusion fluid was dependant on the unionized fraction of the drug. Thus the drug transport from the nasal cavity into the CSF conforms to the pH partition theory. For drugs with comparatively low lipophlicity, transport into the CSF is dependant on the partition coefficient.

1.4.3.3 *Partition coefficient*

The concentration of various sulphonamides in the CSF increased linearly with the partition coefficient (between isoamyl alcohol and the phosphate buffer, pH 7.4) (Sakane *et al.*, 1991: 2457). Similar results were shown by Chow & Donovon (1998:144) who studied the distribution of local anesthetics with similar chemical structures in rats. The rank order of these local anesthetics, according to the ratios of the area under the concentration-time curve (AUC) values in the CSF for nasal and parental administration, correlated well with their ranking by distribution coefficients.

1.5 Drug transport along the olfactory pathway

1.5.1 Experimental methods used in nose to brain transport studies

In the most basic studies, for example in mice and rats, the animals are dosed with the drug nasally and parentally, plasma samples are taken for a dedicated time period and the animal then sacrificed at certain time points. The dosing of these animals is normally performed when anaesthetized and placed on their backs. The dose is given as nose drops in volumes as high as 100 μl, given over extended periods of time. Such high dosing volumes and the position of the animal during dosing can promote the drug formulation reaching and covering the olfactory region. It should be noted, however, that volumes of 50 μl and larger will fill the nasal cavity of the rats and the surplus will disappear into the back of the throat. It has also been reported that some anesthetics can have an inhibitory effect on the retrograde and anterograde exoplasmic transport (Illum, 2000:8).

In most studies the brain is removed and then either counted as whole tissue for radioactivity or measured for drug content (Hussain *et al*,. 1990:771, Girurarson *et al*,. 1996:79), or sliced into vertical slices and counted for radioactivity (Javaid & David, 1993:358) or separated in various brain sections and counted individually (Wang *et al*., 1998: 572). In certain studies the CSF is collected, either as a one off sample at the end of the experiment (Illum, 2000: 8) or as several samples during the period of the study (Chou & Donovon, 1998:139). The collection of CSF is most often performed by cisternal puncture with a fine needle connected to tubing, where an incision is made in the skin over the occipital bone. Collection is terminated when blood appears (Chen *et al*., 1998: 37). Volumes of 150μl and larger can be collected in this way (Seki *et* al., 1994:1135).

Some papers report technical difficulties in obtaining consistent volumes of CSF using the cisternal cannulation method, due to the slow flow rate of the CSF and blood appearing in the sample (Kumbale *et al*., 1999: 25). It has also been reported by Sakane *et al.* (1991a:380, 1991b:449) that due to the location of the CSF on the surface of the brain, the initial CSF fraction obtained often has a lower drug concentration than the later fractions collected. This problem was overcome by some researchers by sampling an early and a late fraction of the CSF (Seki *et al*., 1994:1135). A volume larger than 70 μl was recommended in order to have representative samples. In experiments where the CSF is collected throughout the entire study period, the CSF has been replaced by infusion of artificial CSF into the lateral ventricle (Chou & Donovon, 1997:337).

1.5.2 Drug transport along the olfactory pathway in animal models

It has long been known that cocaine is absorbed rapidly from the nasal mucosa. Moreover, the euphoria derived from the sniffing of cocaine in conscious subjects has been reported to occur rapidly (within 3-5 minutes) (Bromley & Hayward,

1988:356). It was speculated that the reason for such a rapid effect of cocaine on the CNS was the presence of a direct pathway for cocaine from the nasal cavity to the brain and the capacity for the drug to concentrate selectively in specific regions of the brain. In order to evaluate the theory Javaid and Davis (1993) examined the cocaine concentration in serum and discrete brain regions after intraperitonial administration in rats. They found that the absorption pattern was similar to that seen in humans after nasal administration with a peak cocaine level in the brain obtained after 10 minutes. There was no indication of cocaine disposition in selective regions of the brain. Chow *et al.* (1999) compared the uptake of cocaine in the various regions of the brain after nasal and intravenous administration in rats. These authors confirmed the similar distribution of the cocaine in the various regions of the brain after intravenous administration found by Javaid and Davis (1993). However, after nasal administration, the cocaine content in samples collected within 60 minutes after administration showed the highest concentration in the olfactory bulb, followed by the olfactory tract and then the remaining parts of the brain. For direct comparison between brain uptake, following the two routes of administration, the concentrations were normalized in relation to their respective plasma cocaine concentrations. It would be seen that for early time points (0-1 minutes) there was a significant higher ratio between 'AUC olfactory bulb/AUC plasma' after nasal administration compared to the intravenous injection of cocaine. However due to the rapid and extensive systemic absorption of the drug after nasal administration, most of the brain deposition of the drug resulted from access across the blood-brain barrier. This could be seen in very similar ratios obtained after nasal and intravenous injection after 1 min. A similar result was found for the nasal administration of a cognition enhancer in rats by Hussain *et al.* (1990:772) who showed that the ratio of brain to plasma concentrations of the drug were similar for administration via the nasal route and by intravenous injection. This suggests that a direct pathway from the nasal epithelium to the brain may be significant only for poorly absorbed solutes like proteins, peptides and metals for which it has been described. Well-absorbed solutes are apparently cleared rapidly to the systemic circulation and

any neuronal absorption or absorption into the subarachnoid space is relatively slow and insignificant. The advantage of administering the cognition enhancer nasally is that the bioavailability was fivefold greater than after oral doses.

Table 1.2 lists drug and drug related compounds that are reported to reach the CNS after nasal administration in different species.

Table 1.2: Drug and drug related compounds that are reported to reach the CNS after nasal administration in different species.

Drug	Species	Samples	Method	References
Benzoylecgonine	Rat	Brain tissue	HPLC	(Chow *et al.*, 2001)
Bupivacaine	Rat	CSF	HPLC	(Chou & Donovon, 1998a)
Cephalexin	Rat	CSF	HPLC	(Sakane *et al.*, 1991b)
Cocaine	Rat	Brain tissue	HPLC	(Chow *et al.*, 1999)
D4T	Rat	CSF	HPLC	(Yajima *et al.*, 1998)
Dextrans	Rat	CSF	HPLC	(Sakane *et al.*, 1995)
[^{14}C]Dextrometorphan	Rat	Brain tissue	Radioactivity counting	(Char *et al.*, 1991)
Dihydroergotamie	Rat	Brain tissue	Radioactivity counting	(Wang *et al.*, 1998)
Dopamine	Rat	Brain tissue & CSF	Radioactivity counting	(Dahlin *et al.*, 2001)

Hydroxyzine	Rat	CSF	HPLC	(Chou & Donovon, 1997)
Insulin	Rat	Brain tissue	Radioactivity counting	(Wang *et al.*, 1998)
L-Dopa	Rat	Brain tissue & CSF	HPLC	(Kao *et al.*, 2000)
Lidocaine	Rat	CSF	HPLC	(Chou & Donovon, 1998)
MPTP	Mouse	-	Activity	(Dluzen & Kefalas, 1996)
Nerve growth factor	Rat	Brain tissue	ELISA	(Chen *et al.*, 1998)
Sulphonamides	Rat	CSF	HPLC	(Sakane *et al.*, 1991a)
Sulphasomidine	Rat	CSF	HPLC	(Sakane *et al.*, 1994)
(S)-UH301	Rat	CSF	HPLC	(Dahlin & Bjork, 2000)
Tetracaine	Rat	CSF	HPLC	(Chou & Donovon, 1998a)
VIP	Rat	Brain tissue	HPLC	(Dufes *et al.*, 2003)
WGA-HRP	Rat	-	Electron microscopy	(Thorne *et al.*, 1995)
Zidovidine	Rat	CSF	HPLC	(Seki *et al.*, 1994)

In one of the first studies by Sakane *et al.* (1991b), the authors compared the uptake in the CSF after intranasal, intra duodenal and intravenous administration of the water-soluble antibiotic cephalexin, in a rat model. The plasma concentrations were similar after 15 and 30 minutes for the three routes, but the levels in the CSF were significantly higher at both times after nasal administration. Because of the higher concentration in CSF after 15 minutes, Sakane *et al.* postulated that cephalexin was transported from the nasal cavity to the CSF by passive diffusion, i.e. via the olfactory epithelium pathway.

Chou & Donovon (1998b) studied the disposition of lidocaine within the CNS of the rat after nasal and intra-arterial administration. Since the systemic bioavailability of lidocaine is 100% after nasal administration (Chou & Donovon, 1998a), the CSF/plasma concentration for the two administration routes (nasal and intraarterial) should be equal. However, the ratio for nasally administered lidocaine was 1.54 when measured using the direct CSF sampling technique and 1.07 when using the micro dialysis probe in the cisterna magma. The changes in disposition pattern between the two administration routes indicated that other factors or pathways in addition to the systemic circulation may play a role in the transport of lidocaine into the brain following nasal administration.

Non-oral and non-parental dosage forms for AIDS treatment have been researched to improve patient compliance and efficacy of zidovidine (AZT) over that seen with oral administration in patients with AIDS and neuropathies. Seki *et al.* (1994) examined the nasal absorption of AZT and its subsequent transport to the CSF in rats. Both rapid absorption and high CSF concentrations were observed after nasal application. The nasal bioavailability was 60% compared to the intravenous administration. It was found that at 15 minutes after drug administration, the ratio of CSF to plasma was higher after nasal than after intravenous infusion. A direct transport from the nasal cavity to the CSF was suggested.

Char *et al.* (1992) compared the plasma and brain levels after nasal and intravenous administration of dextrometorphan hydrochloride (DH) in rats. This drug has good blood-brain permeability. The results showed that the plasma and brain concentrations of DH after nasal administration were 78.8% and 65.7%, respectively, as compared to an intravenous injection. These results were based on AUCs obtained from 2-120 min after administration. They are in line with the results obtained for drugs, such as cocaine, mentioned above.

Wang *et al.* (1998) studied the brain uptake of tritium–labeled dihydroergotamine ([^{3}H]-DHE) after nasal and intravenous administration in rats. Dihydroergotamine is used for the treatment of migraine headache and, because of low oral bioavailability, it is usually administered intravenously or intramuscularly. In the same study, [^{14}C]-insulin was used as a non-BBB-permeable marker. Both [^{3}H]-DHE and [^{14}C]-insulin were transported directly into the brain. [^{3}H]-DHE penetrated the BBB, but the level of radioactivity in the olfactory bulb was approximately four times higher 30 minutes after nasal administration than it was after intravenous administration.

The delivery of proteins to the brain has been demonstrated using wheat germ agglutinin-horseradish peroxidase (WGA-HRP) (Thorne *et al.*, 1995) and more recently, radioactively labeled insulin (Gizurarson *et al.*, 1996). The distribution of [^{125}I]-insulin between blood and brain was investigated in mice after intra olfactory instillation and subcutaneous administration. The amount of radioactivity in the brain, measured with a gamma-counter, were significantly higher following intra olfactory than subcutaneous administration. Thorne *et al.* (1995: 281) designed a study to quantitively assess the anterograde transport of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) and horseradish peroxidase (HRP) to the brain via olfactory neurons and to evaluate the capacity of the potential drug delivery method to achieve biologically significant protein concentrations in the brain. According to the authors, the results indicated that the transport of protein along the olfactory pathway into the brain after nasal

administration was sufficiently high to attain therapeutic levels (Thorne *et al.*, 1995:281).

1.5.3 Drug transport along the olfactory pathway in humans

A few studies showing access to the human brain after nasal administration of drugs have been published. A research group provided functional evidence of the facilitated access of arginine-vasopressin (Pietrowsky *et al.*, 1996a) and cholecytokinin-8 (Pietrowsky *et al.*, 1996b) into the brain by this route. The substances were administered nasally or intravenously to healthy subjects and the event-related potentials (ERP) were recorded during the subject's performance on a oddball task. The P3 component of the ERP increased after nasal but not after intravenous administration and it was suggested that the substances were delivered to the brain by a direct pathway from the nose.

Intranasal administration of angiotensin II to healthy volunteers showed that the drug directly influences the CNS regulation of blood pressure It was shown that the blood pressure profiles differed with the route (intravenous or intranasal) of administration of angiotensin II, and that the plasma concentration of vasopressin were increased after intranasal but not after intravenous angiotesin II administration (Derad *et al.*, 1998: 971). The same research group also showed that nasal administration of insulin resulted in effects not seen after intravenous administration, assuming direct delivery into the CNS (Kern *et al.*, 1999:557).

Recently, the first attempt at radio isotopic assessment of the integrity of the nose–brain barrier was published. In a study by Okuyama (1997) a mixture of Technicium-diethylenetriaminepenta-acetic acid (Tc–DTPA) and hyaluronidase was sprayed onto the olfactory mucosa of anosmic patients and the cerebral radioactivity measured. A significant rise in cerebral radioactivity was observed 5 min after application of the radioisotope. The above studies have given clear evidence that even large molecules, such as the peptide insulin, can be

transported rapidly from the nasal cavity to the CNS in man where they can exert specific effects on various brain functions. Such effects are not seen after parenteral administration or placebo treatment. Due to the rapid onset of action it is most likely that the molecules are transported by an extracellular transport pathway.

1.6 Conclusion

Due to many advantages of intranasal administration such as high vascularity allowing good bioavailability, numerous microvilli, absence of hepatic first pass metabolism, low amino peptidase activity, the nasal route reveals itself to be especially interesting for the systemic administration of drugs. The nasal route of administration also provides a good alternative to patients who are too nauseous to take medication orally and it also appears that the nasal route is a useful, practical alternative for the administration of drugs that show low bioavailability after per oral administration or drugs that require parental administration.

Progress in intranasal application of drugs is increasing due to the increase in knowledge of this route of administration, the accessibility of the nose, the ease of administration and the route being well tolerated by patients.

It is evident from the results described and discussed above that when drugs are administered to the nasal cavity of rodent animal models, a proportion is transported through the olfactory epithelial region to the CSF, the olfactory lobes of the brain or, in some cases, further into the parenchyma of the brain. It has been shown that the rate and degree of transport and deposition are highly dependent on the physico-chemical properties of the drugs, especially the molecular weight and the lipophilicity. It is also evident that the choice of pathway is drug dependent and can be related to the existence (or not) of receptors on the olfactory neurons. This was seen in particular for HRP and WGA–HRP, which exploit different pathways through the epithelium (WGA–HRP binding sites on the

plasma lemma). The results from studies performed in animal models have been confirmed to some extent in human studies. Evidence has been provided that suggests that even large molecules, such as the peptide insulin, can be transported from the nasal cavity to the CNS and by doing so can exert specific effects on various brain functions. For most drugs studied the rapid appearance of the drug in the CNS or the rapid onset of effect (often within 10 min) indicates that the initial pathway used is by an extracellular route. However, since most of the published experiments last for but a few hours only, it cannot be ruled out that intracellular pathways can also take effect at later times.

A question could be raised as to the importance of the "nose to brain" transport mechanism. Is it a route of transport to the brain that could be exploited for drugs that have poor blood-brain barrier permeability or should it be considered an undesirable consequence of nasal delivery, which should be avoided?

It is evident that for drugs, where the target receptors are situated in the CNS and the therapeutic effect is related to the brain function, such as the treatment of Parkinson's disease, Alzheimer's disease, nausea or pain, an increased transport of drug to the brain could be considered beneficial. However, for other situations, where nasal delivery is used only for its convenience and a means of obtaining high bioavailability, the direct delivery of drugs to the CNS could possibly result in side effects.

It should be remembered that the quantities of drugs reported to access the brain are very low. In many case, for the effective exploitation of the nose to the CNS delivery route, it will be necessary to find ways of increasing the quantity of drug delivered.

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Chapter 2

General characteristics of prochlorperazine edisylate

2.1 Introduction

Prochlorperazine, a piperazine phenothiazine, is an oral, rectal and parental antiemetic and antipsychotic agent. It is closely related to the high-potency neuroleptics such as perphenazine. Prochlorperazine is mainly used for the management of nausea and vomiting, but shares many of the actions and adverse effects of the antipsychotics. Prochlorperazine was approved by the FDA in 1956 (Clinical pharmacology, 2001:2).

The phenothiazines are a group of chemical agents with neuroleptic, anti-emetic, antihistaminic, anticholinergic and sedative activities. Their main pharmacological response is determined by variations in the chemical structure of the side-chain at position 10 of the phenothiazine ring. The most widely used anti-emetics contain a basic amino group incorporated into a piperazine ring. These compounds have decreased autonomic activity, but show the greatest extrapyramidal effects of all the phenothiazine subgroups (Wampler, 1983:36).

2.2 Chemical characteristics

2.2.1 Chemical denominations

Prochlorperazine edisylate is a piperazine side chain phenothiazine also known as 2-chloro-10-(3-(4-methyl-1-piperazinyl)propyl) phenothiazine 1,2-ethaedisulfoate (1:1) or 10-*H*-Pheothiazine, 2-chloro-10-[3-(4-methyl-1-piperazinyl)-propyl]-1,2-ethodisulfonate (1:1) (USP, 2001:1405).

2.2.2 Chemical structure

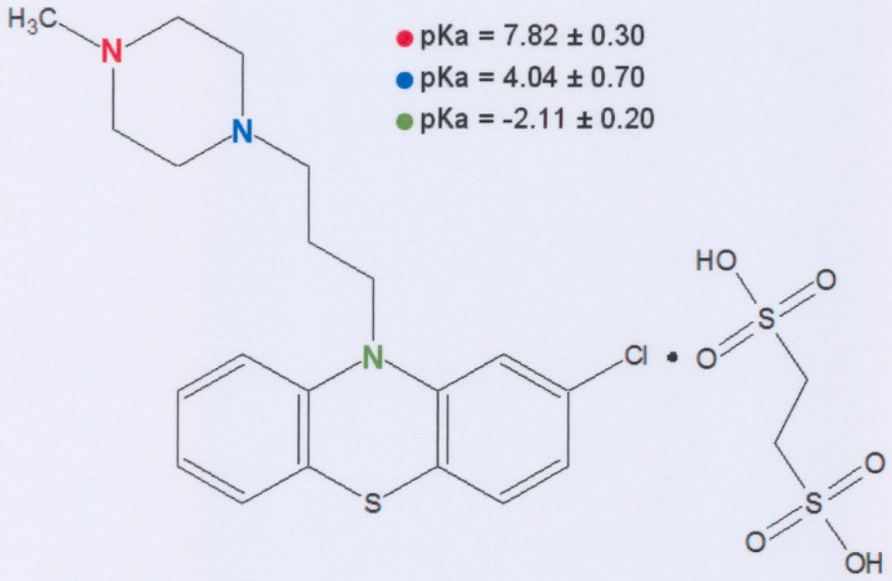

Fig 2.1: The chemical structure of prochlorperazine edisylate.

Prochlorperazine has pKa values of 7.82 ± 0.30, 4.04 ± 0.70 and -2.11 ± 0.20 (ACD/labvs Software™, Toronto, Canada). The log P value of prochlorperazine was calculated as 4.76 (ACD/labvs Software™, Toronto, Canada). The software

did not allow the calculation of prochlorperazine edisylate, but one can assume that it would be more hydrophilic (lower log P) than the free base.

2.2.3 Molecular formula and molecular weight

Molecular formula: $C_{20}H_{24}Cl_3S.C_2H_6O_6S_2$ (USP, 2003:1551)
Molecular weight: 564.14 (USP, 2003:1551)

2.2.4 Description

Prochlorperazine is commercially available as the base, edisylate salt and maleate salt. Each 7.5 mg of prochlorperazine edisylate or 8mg of prochlorperazine maleate is approximately equivalent to 5 mg prochlorperazine.

Prochlorperazine edisylate occurs as a clear, pale yellow, viscous liquid, whereas prochlorperazine occurs as a white to very light yellow, odorless crystalline powder and prochlorperazine maleate as a white to pale yellow, practically odorless, crystalline powder (AHFS Drug Information, 2002:2295).

2.2.5 Ultraviolet absorption spectra

Figure 2.2 shows the ultraviolet absorption spectra of prochlorperazine edisylate in water with maximum absorption at 255 nm.

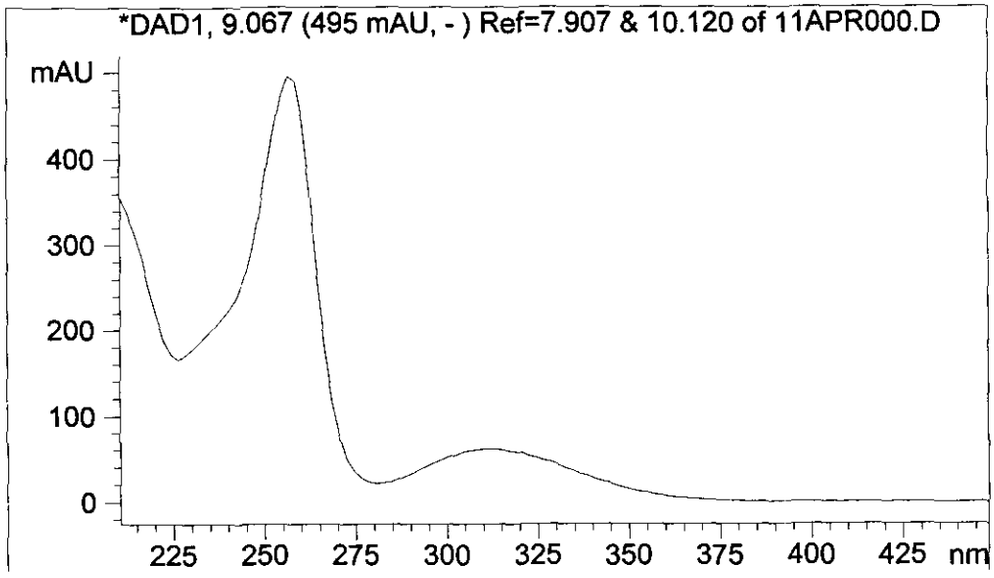

Fig 2.2: The ultraviolet absorption spectra of prochlorperazine edisylate in water with maximum absorption at 255 nm.

2.3 Physicochemical characteristics

2.3.1 Solubility

- Prochlorperazine is slightly soluble in water and freely soluble in alcohol.
- Prochlorperazine edisylate has approximate solubilities of 500 mg/ml in water and 0.67mg/ml in alcohol
- Prochlorperazine maleate is practically insoluble in water and has a solubility of approximately 0.83mg/ml in alcohol (AHFS Drug Information, 2002:2295)

2.3.2 Stability

Commercially available preparations of prochlorperazine should be stored in tight, light-resistant containers. Prochlorperazine preparations should preferable be stored between 15-30 °C; freezing of oral solutions and injections should be avoided. Slight yellowish discolorations of the oral solution or injection will not affect potency or efficacy, but should not be used if markedly discolored or if a precipitate is preset (AHFS Drug Information, 2002:2814).

2.4 Pharmacology

2.4.1 Indications

- *Psychotic Disorders*

Prochlorperazine is used for the symptomatic management of psychotic disorders. Drug therapy is integral to the management of acute psychotic episodes and accompanying violent behaviour in patients with schizophrenia and generally is required for long-term stabilization to improve symptoms between episodes and to minimize the risk of recurrent acute episodes (AHFS Drug Information, 2002:2295).

- *Nonpsychotic anxiety*

Prochlorperazine also is used for the short-term management of nonpsychotic anxiety. Because of the risks of toxicity associated with its use, prochlorperazine should be used only as an alternative to other less toxic anxiolitic agents (e.g., benzodiazepines) in most patients (AHFS Drug Information, 2002:2295).

- *Nausea and vomiting*

Prochlorperazine is used for the control of severe nausea and vomiting of various etiologies. The drug is effective for the management of postoperative nausea and vomiting, and nausea and vomiting caused by toxins, radiation, or cytotoxic drugs. Prochlorperazine is not effective in preventing vertigo and motion sickness, or for the managing of emesis caused by the action of drugs on the nodose ganglion or locally on the GI tract. Because safety of prochlorperazine for the prevention and treatment of nausea and vomiting associated with pregnancy has not been established, use of the drug during pregnancy is not recommended (AHFS Drug Information, 2002:2813).

2.4.2 Dosage and administration

2.4.2.1 Administration

For psychiatric use, prochlorperazine edisylate is administered orally or by deep IM injection. Subcutaneous administration of the drug is **not** recommended because of local irritation. Prochlorperazine maleate is administered orally. Prochlorperazine is administered rectally. Prochlorperazine edisylate is also administered by direct IV injection or by IV infusion in the management of severe nausea and vomiting (AHFS Drug Information, 2002:2295).

2.4.2.2 Dosage

Psychotic disorder

For the symptomatic management of psychotic disorders in outpatients with a relatively mild symptomatology, the usual adult dosage of prochlorperazine is 5 to10 mg three to four times daily. For hospitalized or well-supervised patients with moderate to severe symptomatology, the usual initial adult oral dosage is 10 mg three to four times daily. Dosage is then gradually increased every 2 or 3 days until symptoms are controlled or adverse effects become troublesome.

Although some patients exhibit optimum response with 50-75 mg daily, dosages up to 150 mg may be required in severely disturbed patients (AHFS Drug Information, 2002:2295).

Nonpsychotic anxiety

For the short-term management of anxiety, the usual adult oral dosage of prochlorperazine is 5 mg three to four times daily. Alternatively dosage of 15 mg (extended release) once daily or 10 mg (extended release) every 12 hours may be used. Dosage should not exceed 20 mg daily nor should the drug be administered for longer than 12 weeks, since the use of higher dosages for longer periods may result in the development of persistent (and possibly irreversible) tardive dyskinesia (AHFS Drug Information, 2002:2295).

Nausea and vomiting

For the control of severe nausea and vomiting in patients who can tolerate oral administration of the drug, the usual adult oral dosage of prochlorperazine is 5 mg three to four times daily. Alternatively a dose of 15 mg (extended release) once daily or 10mg (extended release) every 12 hours may be used; some patients subsequently may require a dosage of 30 mg. Oral dosages exceeding 40 mg should be used only in resistant cases.

The usual adult rectal dosage for the control of severe nausea and vomiting is 25 mg twice daily. The usual adult IM dose is 5-10 mg. If necessary, the initial dose may be repeated every 3-4 hours not exceeding 40 mg daily. The usual adult IV dose is 2.5 -10 mg; single IV doses should not exceed 10 mg and the total IV dosage should not exceed 40 mg daily. For IV infusion, an infusion containing prochlorperazine 20 mg/L can be used (AHFS Drug Information, 2002:2813).

2.4.3 Mechanism of action

Prochlorperazine blocks postsynaptic dopamine receptors in the mesolimbic system and increases dopamine turnover by blockade of the D_2 somatodendritic autoreceptor. After about 12 weeks of chronic therapy, depolarization blockade of the dopamine tract occurs. The decrease in dopamine neurotransmission has been found to correlate with the antipsychotic effects. This D_2 – blockade is also responsible for the strong extrapyramidal effects observed with this drug. Dopamine blockade in the chemoreceptor trigger zone accounts for the anti-emetic effects. Prochlorperazine possesses moderate anticholinergic and alpha-adrenergic receptor blocking effects. Blockade of $alpha_1$ – adrenergic receptors produces sedation; muscle relaxation; and cardiovascular effects such as hypertension, reflex tachycardia, and minor changes in ECG patterns (Clinical Pharmacology, 2001:2).

2.4.4 Contraindications

Prochlorperazine should be avoided in patients with blood disorders, liver or renal dysfunction, glaucoma, Parkinson's disease, pheochromocytoms, myasthenia gravis, prostrate hypertrophy, epilepsy and uncontrollable movement disorders. It should also be avoided in patients known to be hypersensitive to phenothiazines (Clinical Pharmacology, 2000:2).

2.4.5 Side effects

Side effects that may need medical attention:

- blurred vision
- breast enlargement in men or women
- breast milk in women who are not breast feeding

- chest pain, fast or irregular heartbeat
- confusion & restlessness
- dark yellow or brow urine
- difficulty breathing or swallowing
- dizziness
- rigidity
- fever or chills
- involuntary or uncontrollable movements
- seizures

Side effects that usually do not require medical attention:

- constipation
- difficulty passing urine
- difficulty sleeping
- drowsiness
- dry mouth
- headache
- nasal congestion
- skin rash
- weight gain

(Clinical Pharmacology, 2000:2).

2.4.6 Pharmacokinetics

Prochlorperazine is rapidly absorbed following oral administration, but there is considerable individual variation in peak plasma concentrations. This is due to metabolism in the gastric mucosa and on first pass through the liver. Following IM or p.o. administration, absorption is rapid. Onset of action occurs in about 30-

60 minutes. Antipsychotic effects are gradual, with considerable individual variation.

Prochlorperazine is widely distributed into body tissues and fluids, and crosses the blood-brain barrier. The drug is highly plasma protein-bound (91-99%), predominantly to alpha$_1$ – acid glycoprotein. The drug crosses the placenta and is excreted into breast milk. Metabolism is extensive, but metabolites have not been shown to have pharmacological activity. Some conjugation with glucuronides occurs, and these along with the unconjugated metabolites accounts for most of the drug found in urine. Metabolites and unchanged drug can be detected for some months after discontinuation of the drug. Some excretion may occur via the biliary tract and feces. The duration of activity is 4-6 hours (Clinical Pharmacology, 2001:2).

In a study conducted by Isah *et al.*, (1991) the plasma concentration of prochlorperazine (PCZ) in seven healthy volunteers, declined bi-exponentially with a terminal half-life of 9.3 ± 1.2 hours, following intravenous administration. The plasma clearance averaged 0.98 ± 0.1 l $h^{-1}kg^{-1}$. The apparent volume of distribution was 12.9 ± 1.6 l kg^{-1}. Peak plasma concentrations (C max) of oral prochlorperazine was 3.9 ± 1.0 ng ml^{-1} (n=5), and occurred at 5.4 ± 1.2 hours. In the five patients in whom PCZ was measurable the oral bioavailability was 12.5% ± 4.3% (range 4.8 – 25.3%) (Isah *et al.*, 1991:679).

It was found that a buccal preparation achieved higher plasma concentrations by direct absorption into the systemic circulation compared to the oral dosage form. Thus, indicating that the buccal route is a viable means of delivering phenothiazine drugs such as prochlorperazine. This route allows reduction in both the dose and dosing frequency over oral administration as well as avoiding possible poor absorption due to associated gastric stasis or vomiting (Hessell *et al.*, 1989:161).

Simpson (2001:97) compared the plasma levels of prochlorperazine after intravenous, oral and intranasal administration to rats. The results indicated that prochlorperazine is a potential candidate for delivering via the intranasal route. From the results it was evident that the nasal route is superior to the oral route and comparable to the intravenous route of administration in the amount of drug absorbed, thus showing much potential for the delivering of anti-emetic drugs (Simpson, 2001:97).

2.5 Preparations

The table below (Table 2.1) gives a list of the different preparations of prochlorperazine available.

Table 2.1: Preparations of prochlorperazine available. (AHFS Drug Information, 2002:28: 16.08).

Prochlorperazine		
Rectal	*Suppositories*: 2.5 mg	**Compazine®**, GlaxoSmithKline
	5.0 mg	**Compazine®**, GlaxoSmithKline
	25 mg	**Compazine®**, GlaxoSmithKline **Compro®**, Paddock **Prochlorperazine Suppositories,** Cheshire, G&W, PD-RX, Quality care

Prochlorperazine Edisylate		
Oral	*Solution*: 5 mg (of prochlorperazine) per 5 ml	**Compazine®Syrup,** GlaxoSmithKline
Parental	*Injection*: 5 mg (of prochlorperazine) per 5 ml*	**Compazine®** (with benzyl alcohol 0.75% in multiple-dose vials), GlaxoSmithKline

Prochlorperazine Maleate		
Oral	*Capsules*, extended release: 10 mg (of prochlorperazine)	**Compazine® Spansule,** GlaxoSmithKline
	15 mg (of prochlorperazine)	**Compazine®Spansule,** GlaxoSmithKline
	Tablets, film coated: 5 mg (of prochlorperazine)*	**Compazine®,** GlaxoSmithKline
	10 mg (of prochlorperazine)*	**Compazine®,** GlaxoSmithKline

• available by nonproprietary name

2.6 Conclusion

The intranasal delivery of prochlorperazine could be of great benefit in the treatment of nausea and vomiting. The oral route for administration of drugs has the disadvantage that it may lead to additional nausea and vomiting in patients already nauseous. Intranasal administration of drugs is advantageous in this respect.

Prochlorperazine fulfils the criteria for a compound which should be efficiently absorbed through the nasal mucosa: it is a highly lipid soluble base with a pKa of 8.1 (Motwani & Lipworth, 1991:89).

It has already been shown that higher prochlorperazine plasma levels were reached after buccal and nasal administration, than after oral administration (Hessell *et al.*, 1989:161). This increase in bioavailability will enable the formulator to reduce the administered dose and thus increasing the side effects as well.

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Chapter 3

Experimental procedures and validation of techniques

3.1 Introduction

In this chapter the breeding conditions of the experimental animals, the formulation and the administration of prochlorperazine edisylate and the sample collection are discussed in detail.

A liquid-liquid extraction method and high pressure liquid chromatographic method for the quantitative determination of prochlorperazine in brain tissue following intravenous, oral and intranasal administration to Spraque-Dawley rats were developed and validated. A validated high pressure liquid chromatographic and solid-phase extraction method were used for the quantitative determination of prochlorperazine in plasma following intravenous, oral and intranasal administration to Spraque-Dawley rats.

3.2 *In vivo* procedures for studying intravenous, oral and intranasal drug delivery

3.2.1 Selection of animals

Generally, it is neither practical nor economically viable to test nasal preparations routinely in human subjects: the spiralling costs of the clinical trials dictate that only a few formulations may be tested in this manner. Thus, it is essential that alternative methods of screening potential drugs and formulations are utilised (Rogerson & Parr, 1990:5).

The use of several animals for nasal absorption studies is described in literature e.g. rabbits, sheep, dogs and monkeys but rats are the animals most commonly used for *in vivo* absorption studies (Chien *et al.*, 1989:27). Rats are easy to obtain and are relatively lower in cost to care for than other animals and are easy to handle (Rogerson & Parr, 1990:5).

An application for the use of Spraque-Dawley rats in this study was approved by the ethics committee at the PU for CHE (No. 01D14)(Annexture 1). In this study healthy male Spraque-Dawley rats with a body weight of 250-350 grams were used. The rats were fasted 12 hours prior to drug administration, but water was supplied *ad libitium.*

3.2.2 Breeding conditions

Rats were bred and kept under artificial conditions to create the ideal environment for optimum growth and health. Infection with pathogenic organisms

was minimised and variables were kept as constant as possible. Conditions in the Laboratory Animal Centre (LAC) are as shown in table 3.1.

Table 3.1: Breeding conditions for rats.

CONDITION	RECOMMENDED VALUE*	VALUE IN LAC
Temperature	19 ± 2°C	21 ± 2°C
Relative humidity	55 ± 15%	55 ± 10%
Rate of ventilation / air movement	15-20 changes per minute	18 changes per minute
Light intensity	350-400 lux one meter above floor level	350-400 lux one meter above floor level
Light period	12 hours light and 12 hours dark	12 hours light and 12 hours dark

*** Values recommended according to international standards.**

3.2.3 Sample collection

Sixty (60) Spraque-Dawley rats were used per formulation, i.e. sixty (60) rats for each of the three formulations, namely intravenous, oral and intranasal formulations. Each group of sixty (60) rats were devided into ten (10) groups of six (6) rats each. Each group of six (6) rats were exposed to one of ten (10) different time intervals 2; 5; 10; 15; 20; 30; 45; 60; 90; 120 minutes post administration before the collection of blood and brain tissue samples.

3.2.4 Preparations of formulations containing prochlorperazine edisylate

A dose of 1.333 mg/kg prochlorperazine was given orally and 0.167 mg/kg intravenously and intranasally to the rats. The three different experimental formulations are shown in table 3.2.

Table 3.2: The formulations used for intravenous, oral and nasal administration

Formulation	Route of administration	Concentration prochlorperazine edisylate(mg/ml)	Dosage volume (µl)
A	Intravenous	0.5	100
B	Oral	4	100
C	Intranasal	1	50

3.2.5 Methods of administration

Administration of the three different formulations:

- *Intravenous:* 100 µl of the drug solution was administered directly into the *vena coudalis*.
- *Oral:* 100 µl of the drug solution was administered by gastric intubation.

- *Nasal:* 50 µl of the drug solution was administered to the nasal cavity by means of an Eppendorf® micropipette through the left nostril.

3.2.6 Preparation of rats, drug administration and sample collection

Blood and brain tissue sampling at a specific time interval for the three respective routes was routinely done on the same day. Rats were fasted for 12 hours prior to the administration of each formulation (water *ad libitium*).
A closed plastic container was filled with 4% halothane by spraying
0.8 ml Fluothane® (halothane) on paper towel below a metal grid. Each rat was separately sedated by placing it in the closed plastic container. The rat was removed at loss of consciousness and coupled to an anaesthetic apparatus.

The anaesthesia apparatus consisted of two 5 litre plastic bags connected to the two ends of a three-way valve. Rubber latex was connected to the middle opening of the three-way valve. The rubber latex was fitted securely over the head of the rat to maintain anaesthesia. The plastic bags were filled with 4% (1.2 ml) halothane for induction and 2% (0.6 ml) halothane for maintenance, in medical oxygen respectively. Sodium lime was placed in both bags to absorb the exhaled carbon dioxide.

Blood samples (± 5 ml) were collected directly from the heart of each rat with a 10 ml syringe and added to a 10 ml glass tube containing 1000 i.u. heparine sodium per litre. After blood collection the rat was decapitated for the removal of the brain. The brain tissue was placed in a 5 ml plastic tube and stored at -74°C until analysis. The tubes containing the blood samples were immediately subjected to centrifugation at 717.7 g's for 10 minutes. The plasma was collected and also stored at -74°C until analysis.

3.3 Quantitative analysis of prochlorperazine in brain tissue

3.3.1 Reagents and raw materials

Prochlorperazine edisylate was purchased from Sigma (Midrand, RSA). Chlorpromazine was supplied by the University (PU for CHE, RSA). HPLC grade water was prepared with a Milli-Q 50 purification system (Microsep, Bramley, RSA). All reagents were supplied by Merck NT laboratory supplies (Midrand, RSA), and were analytical grade except for the acetonitrile and 1-octanesulphonic acid sodium salt, which were HPLC grade.

3.3.2 Chromatographic conditions

Analytical instrument: Hewlett-Packard (HP) 1050 series HPLC equipped with a HP 1050 quaternary pump, HP 1050 autosampler, HP 1050 diode array detector and Chemstation Rev. A.04.02 data acquisition and analysis software.

Column: L una C18(2), 250 x 4.6 mm, 5 micron (Phenomenex, Seperations, Randburg, RSA).

Mobile phase: 50/10/40 Acetonitrile / Methanol / 1-Octanesulphonic acid in water adjusted to pH 3.5 with 1% phosphoric acid (H_3PO_4).

Flow rate: 1 ml/min.

Injection volume: 100 µl.

Detection: 254 nm.

Retention time: ± 6.5 minutes and ± 7.8 minutes for chlorpromazine(internal standard) and prochlorperazine respectively.

Temperature: ± 25 °C.

3.3.3 Preparation of standard solutions

The preparations of prochlorperazine edisylate and chlorpromazine standard solutions were done as follows:

3.3.3.1 Preparation of chlorpromazine (internal standard) solution

- Ten milligrams of chlorpromazine was weighed and dissolved in 100 ml of HPLC water (100 µg/ml stock solution).
- The stock solution was sonicated for 1 minute to aid dissolution.
- Ten millilitres of this solution was diluted to 100 ml with HPLC water (10 µg/ml).

3.3.3.2 Preparation of prochlorperazine edisylate solutions

- Ten milligrams of prochlorperazine edisylate was weighed and dissolved in 100 ml of HPLC water (100 µg/ml stock solution).
- The stock solution was sonicated for 1 minute to aid dissolution.
- The following concentrations of prochlorperazine edisylate solutions were prepared by diluting the stock solution:
  1. 100 µg/ml
  2. 50 µg/ml
  3. 10 µg/ml

4. 5 µg/ml
5. 1 µg/ml
6. 0.5 µg/ml

3.3.4 Calibration curve

The calibration curve concentrations were chosen to encompass the full range of prochlorperazine concentrations that were expected in the rat brain tissue.
The whole brain, weighing 2.0 ± 0.4 grams, was spiked with 200 µl of each of the prepared solutions in order to establish a calibration curve (Table 3.3).

Table 3.3: The concentrations used for establishing a calibration curve.

Concentration (ng/g)	Brain tissue (g)	Volume of solution (µl)	Concentration of solution used (µg/ml)
50	2.0 ± 0.4	200	0.5
100	2.0 ± 0.4	200	1
500	2.0 ± 0.4	200	5
1000	2.0 ± 0.4	200	10
5000	2.0 ± 0.4	200	50
10000	2.0 ± 0.4	200	100

3.3.5 Sample extraction

A liquid-liquid extraction method was used for the extraction of prochlorperazine. To each brain tissue sample 200 µl of the 5 µg/ml internal standard solution and 3 ml of a 25% ammonium hydroxide solution were added. The brain tissue was homogenised with a polytron homogeniser and 5 ml of organic solvent (ethyl acetate) was added to the homogenate. After 10 minutes of shaking and 15 minutes of centrifugation at 5411 g's the organic phase was transferred to new test tubes. The organic phase was evaporated to dryness, under a gentle stream of nitrogen at ±40°C. The residue was redissolved in 250 µl of the mobile phase, sonicated, vortexed and transferred into 250 µl glass inserts. The samples were centrifuged once more for 10 minutes at 7257.6 g's to remove particles that might have been left in the samples.

3.3.6 Validation of the HPLC analytical method

The aim was to develop an assay system to determine prochlorperazine concentrations in brain tissue with sufficient accuracy and precision. A literature review yielded no methods for analysis of prochlorperazine in brain tissue. The general validation was done by Simpson (2001:67). For the purpose of this study the following performance parameters were validated:

- Selectivity
- Recovery
- Repeatability
- Linearity
- Sensitivity
- Accuracy and precision

3.3.6.1 Selectivity

Selectivity is the ability of an analytical method to determine the analyte selectively and accurately in the presence of other compounds that may interfere. A test for selectivity is imperative when the drug is unstable and degradation products are formed which may interfere with the analysis or when any other compound in the sample may cause interference. It is good practice to inject a placebo of the specific extraction which is being analysed, to ensure that there are no interfering peaks.

Figure 3.1 shows a chromatograph of a blank brain tissue sample. Figure 3.2 shows a chromatograph of brain tissue spiked with prochlorperazine edisylate and chlorpromazine (internal standard) and figure 3.3 shows a chromatograph of a brain tissue sample where prochlorperazine was administered to a male Spawgue Dawley rat.

No interfering peaks were encountered at the retention times of ± 6.5 minutes for chlorpromazine and of ± 7.8 minutes for prochlorperazine.

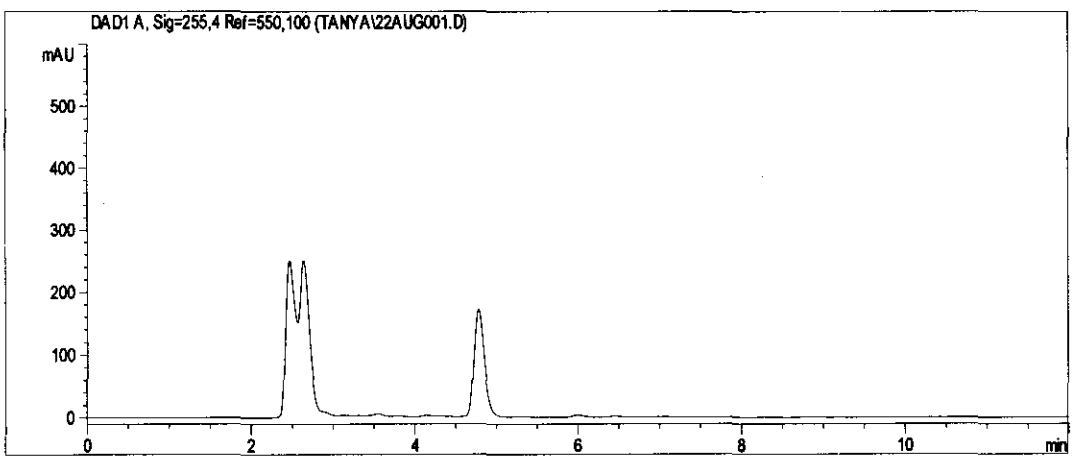


Figure 3.1: A chromatograph of a blank brain tissue sample.

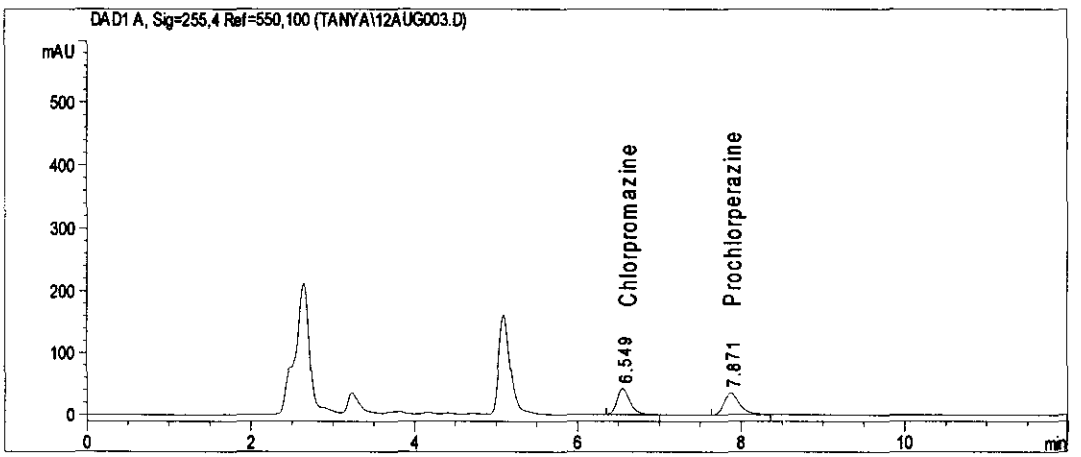


Figure 3.2: A chromatograph of a brain tissue sample spiked with prochlorperazine and chlorpromazine (internal standard).

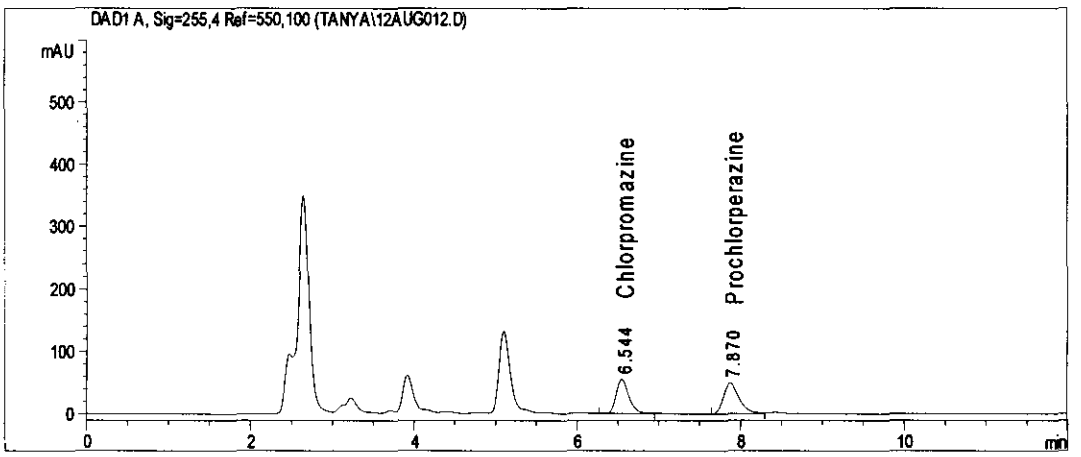


Figure 3.3: A chromatograph of a brain tissue sample after administration of prochlorperazine to a rat, spiked with chlorpromazine (internal standard).

3.3.6.2 Recovery

Recovery is determined by comparing detector response from an amount of analyte added to and recovered from the biological matrix, to detector response obtained from the pure authentic standard. Recovery determination should be done at high, medium and low regions of the expected calibration range. Although values of 50, 80 and 90% have all been used as acceptable limits, it is

more important that recovery be reproducible (Karnes *et al.*, 1991:421). Although it is desirable to obtain recovery close to 100%, there is no universally accepted value for minimum recovery. Table 3.5 gives a summary of the experimental recovery of prochlorperazine from brain tissue.

Table 3.4: Experimental recovery (%) of prochlorperazine from brain tissue samples.

Prochlorperazine[2] (ng/g)	Recovery[1] (%)		
	MEAN	SD	%RSD
500	47.3	7.5	15.0
1000	43.4	4.8	11.0
10000	45.8	3.0	6.5

1. **Mean of six samples**
2. **Prochlorperazime standard solutions were made up in water**

Table 3.5: Experimental recovery (%) of chlorpromazine in brain tissue samples.

Chlorpromazine[2] (ng/g)	Recovery[1] (%)		
	MEAN	SD	%RSD
500	47.66	4.35	9.13

1. Mean of 12 samples

2. Chlorpromazine standard solutions were made up in water

3.3.6.3 Repeatability

Six samples of 100 ng/g were analysed on the same day to determine the intra-day-repeatability (Table 3.6).

Table 3.6: Intra-day repeatability.

Sample number	Peak area ratio (Prochlorperazine/Chlorpromazine)
1	0.42
2	0.43
3	0.41
4	0.42
5	0.34
6	0.46
MEAN	**0.41**
SD	**0.036**
%RSD	**8.8**

3.3.6.4 Linearity

The linearity for prochlorperazine analysis was determined by performing linear regression analysis of the peak area ratios of prochlorperazine over the internal standard (chlorpromazine) *versus* concentration over the range 100 ng/g to 10 000 ng/g. The daily standard curve was obtained using at least five standards in the range. The data are best described by a linear equation

$y = mx + c$.

Where:

y = peak area ratios of prochlorperazine to chlorpromazine

m = slope

x = concentration of proclorperazine (ng/g)

c = y-intercept

Each rat brain was spiked with a different concentration of prochlorperazine as described in table 3.3. A triplicate of each concentration was analysed and the average of the three concentrations (Table 3.7) was used to calculate the regression statistics (Table 3.8).

Table 3.7: The mean peak area ratio of prochlorperazine/chlorpromazine as a function of concentration.

Concentration (ng/g)	Peak area ratio (prochlorperazine/chlorpromazine)					
	1	2	3	MEAN	SD	%RSD
50	0.28	0.11	0.13	0.14	0.030	21.03
100	0.27	0.32	0.19	0.31	0.030	9.50
500	0.57	0.62	0.54	0.58	0.033	5.72
1000	1.4	1.64	1.16	1.4	0.196	13.99
5000	7.72	6.98	7.33	7.34	0.302	4.12
10000	14.17	16.2	16.2	15.52	0.957	6.16

Table 3.8: Regression statistics of the average (Table 3.7).

R squared (R^2)	**0.9964**
Y intercept	**-0.0938**
Slope	**0.0015**

3.3.6.5 Sensitivity

Based on a 150 µl sample volume, the limit of quantification (LOQ) (the lowest concentration of an analyte in a sample that can be determined with acceptable accuracy and precision) for prochlorperazine was 100 ng/g (%RSD<15)

The limit of detection (LOD) (the lowest concentration of analyte that could be detected, but not necessarily quantitated) for prochlorperazine was 60 ng/g (Limit of detection was calculated on the peak that has the height of three times the baseline disturbances).

3.3.6.6 Accuracy and precision

Three sets of six samples each were spiked with specific concentrations (Table 3.9). The samples were analysed and the amount of drug in each was determined from the calibration curve. Accuracy is given as the % recovery, and precision as the %RSD between the values obtained.

Table 3.9: Concentration found (ng/g) after the brain tissue was spiked with a specific concentration.

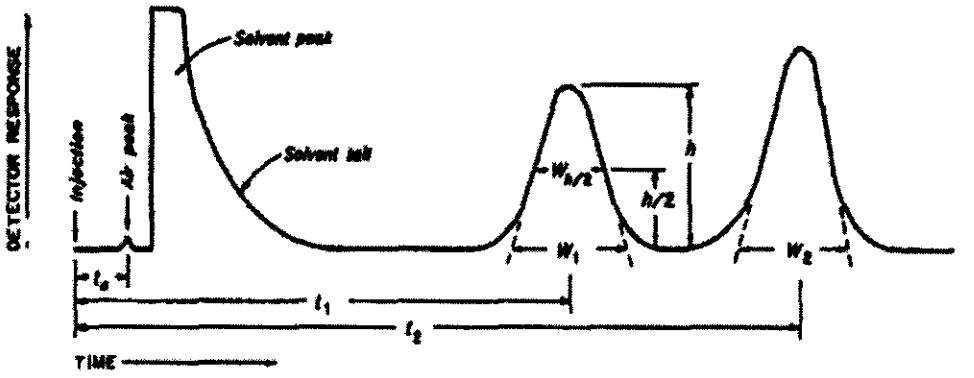
Concentration spiked (ng/g)	Concentration found (ng/g)	Accuracy (% recovered)	Precision (%RSD)
100	45.3	45.3	12.9
1000	434.2	43.4	13.71
10000	4582.1	45.8	12.07

3.3.7 System suitability

System suitability is used to ensure that the analytical method meets with the requirements of the analytical method to determining samples with the necessary resolution and reproducibility. The following parameters were determined: Resolution, peak symmetry and theoretical plate number.

3.3.7.1 Resolution (R)

Resolution is used to determine the effectivity of separation between two peaks. Figure 3.5 demonstrates the different parameters used in equation 3.1 to calculate the resolution, R. The equation for resolution takes into account both peak width and distance between the peak maxima:



Fi

gure 3.4: Chromatographic separation of two components (USP, 2003:2134).

$$R = \frac{2(t2 - t1)}{W1 + W2}$$ Equation 3.1

Where: t_2 = prochlorperazine retention time

t_1 = chlorpromazine retention time

W1 = chlorpromazine corresponding width at the base of the peak

W2 = prochlorperazine corresponding width at the base of the peak

The larger this value, the better the separation between two components. If R=1, the resolution between two equal sized peaks is considered to be about 98%. R=1.5 will mostly give base line separation. The USP usually requires the resolution to be at least 2.

The resolution between the chlorpromazine and prochlorperazine peaks were calculated at R=5.09. According to the resolution determined, the peaks were well resolved.

3.3.7.2 Peak symmetry

The tailing factor (T) is used to determine the symmetry of a peak. Figure 3.6 demonstrates the different parameters used in equation 3.2 to calculate the tailing factor.

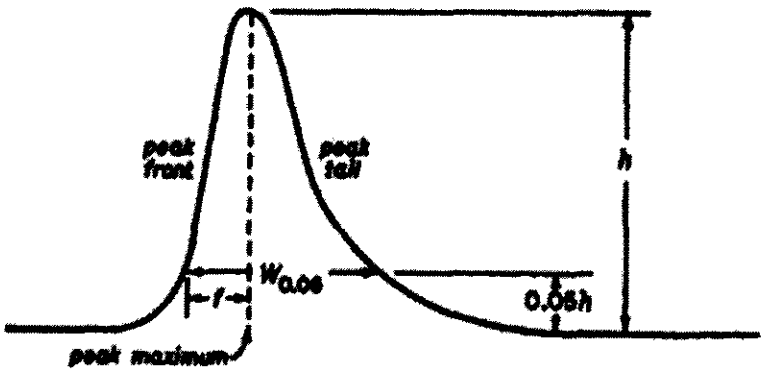


Figure 3.5: A chromatographic peak used to for the determination of peak symmetry (USP, 2003:2135).

The following equation is used:

$$T = \frac{W_{0.05}}{2f}$$ Equation 3.2

Where:

T = tailing factor.

$W_{0.05}$ = width of peak at 5% height.

f = the leading edge of the peak, the distance being measured at a point 5% the peak height from the baseline.

T > 1 indicate tailing, while T < 1 is an indication of peak fronting. For a symmetrical peak, T will equal 1.

The tailing for prochlorperazine and chlorpromazine was 1.6 and 1.25 respectively which is under the generally accepted value of 2.

3.3.7.3 Theoretical plate number (N)

The number of theoretical plates, N, is an indication of the effectivity of a column. N can be calculated by using equation 3.3. Figure 3.7 indicates the parameters used to calculate the number of theoretical plates.

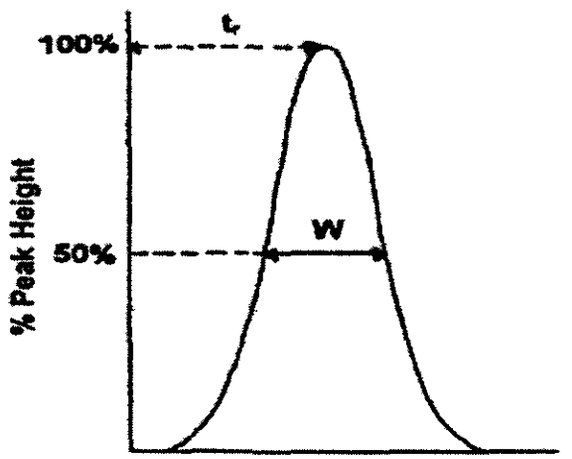


Figure 3.6: Parameters used to calculate the number of theoretical plates ($^1/_2$ peak height method)

$$N = a\left(\frac{t_r}{W}\right)^2 \qquad \text{Equation 3.3}$$

Where:

N = theoretical plate number
a = constant
t_r = retention time of peak
W = width of peak at 50% height

The theoretical plate number (N) of the column that was used to determine prochlorperazine and chlorpromazine in biological fluids was 14295 and 11078 plates/column for prochlorperazine and chlorpromazine respectively and therefore the column was considered to be efficient (USP, 1995:1176).

3.4 Quantitative analysis of prochlorperazine in plasma

A method described by Simpson (2001:67) was used for the sample extraction and analyses of the plasma. A short summary of the validation results is given in table 3.10.

Table 3.10: HPLC validation results for determination of prochlorperazine in plasma.

Test	Result
Specificity	Complies
Range	5 ng/ml to 300 ng/ml
Linearity	R^2 = 0.9985 over the range
Accuracy	94.9 to 100.4%
Precision	1.8 to 3.8%
Stability	Stable over 12 hours
Limit of quantification (LOQ)	5 ng/ml
Limit of detection (LOD)	2ng/ml

3.4.1 Chromatographic conditions

Analytical instrument: Hewlett-Packard (HP) 1050 series HPLC equipped with a HP 1050 quaternary pump, HP 1050 autosampler, HP 1050 diode array detector and Chemstation Rev. A.04.02 data acquisition and analysis software.

Column: L una C18(2), 250 x 4.6 mm, 5 micron (Phenomenex, Seperations, Randburg, RSA).

Mobile phase: 50/10/40 Acetonitrile / Methanol / 1-Octanesulphonic acid in water adjusted to pH 3.5 with 1% phosphoric acid (H_3PO_4)

Flow rate: 1 ml/min.

Injection volume: 100 µl.

Detection: 254 nm.

Retention time: ± 6.5 minutes and ± 8.2 minutes for chlorpromazine and prochlorperazine respectively.

Temperature: ± 25 °C

Figure 3.8 shows a chromatograph of plasma spiked with prochlorperazine edisylate and chlorpromazine (internal standard) and figure 3.9 shows a chromatograph of a plasma sample where prochlorperazine was administered to a male Spawgue Dawley rat.

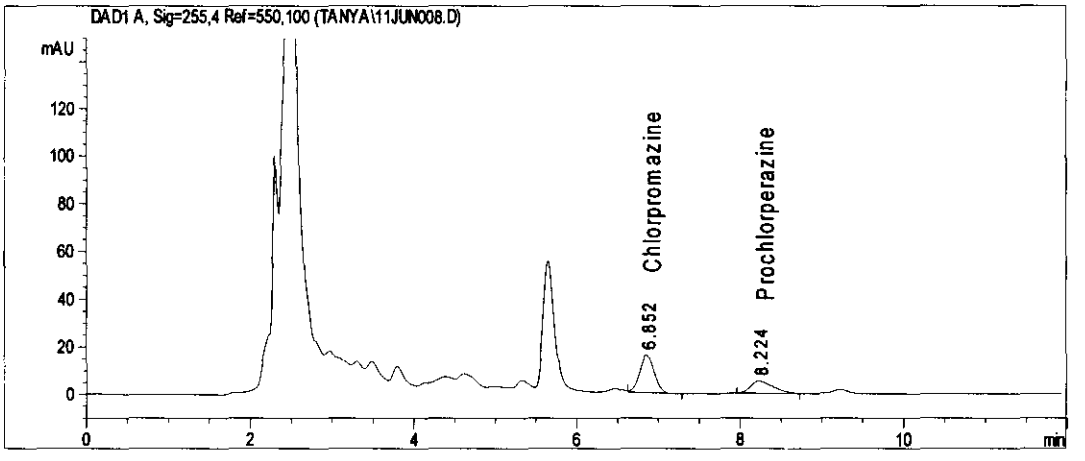


Figure 3.7: A chromatograph of a plasma sample spiked with prochlorperazine and chlorpromazine (internal standard).

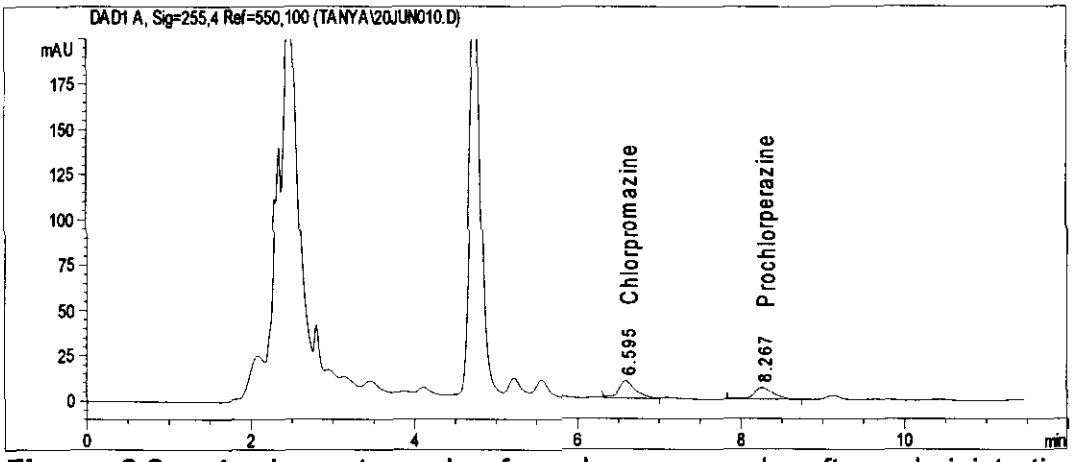


Figure 3.8: A chromatograph of a plasma sample after administration of prochlorperazine to a rat, spiked with chlorpromazine (internal standard).

3.4.2 Preparation of standard solutions

The preparations of prochlorperazine edisylate and chlorpromazine standard solutions were done as follows:

3.4.2.1 Preparation of chlorpromazine (internal standard) solutions

- Ten milligrams of chlorpromazine was weighed and dissolved in 100 ml of HPLC water (100 µg/ml stock solution).
- The stock solution was sonicated for 1 minute to aid dissolution.
- Hundred micro litres of this solution was diluted to 20 ml with HPLC water (0.5 µg/ml).

3.4.2.2 Preparation of prochlorperazine edilylate solutions

- Ten milligrams of prochlorperazine was weighed and dissolved in 100 ml of HPLC water (100 µg/ml stock solution).
- The stock solution was sonicated for 1 minute to aid dissolution.

- The following concentrations of prochlorperazine edisylate solutions were prepared by diluting the stock solution:

1. 10 µg/ml
2. 5 µg/ml
3. 1 µg/ml
4. 0.5 µg/ml
5. 0.1 µg/ml
6. 0.05 µg/ml

3.4.3 Calibration curve

The plasma (1ml) was spiked with 100 µl of each of the prepared solutions in order to obtain a calibration curve (Table 3.5).

Table 3.11: The concentrations used for establishing a calibration curve.

Concentration (ng/g)	Plasma (ml)	Volume of solution (µl)	Concentration of solution used (ng/ml)
5	1	100	5
10	1	100	10
50	1	100	50
100	1	100	100
500	1	100	500
1000	1	100	1000

3.4.4 Sample extraction

Varian Bond Elute (C18) 1ml disposable solid-phase extraction cartridges (SMM, RSA) were conditioned by sequential washing with 2 ml of methanol and one column volume of ammonium hydroxide (25% diluted to 2 ml/100 ml HPLC water). Plasma samples were thawed, vortexed and centrifuged for 5 minutes at 1614.8 g's. One millilitre of plasma was measured accurately with a calibrated Eppendorf® micropipette and transferred to a 5 ml siliconised glass tube. To each plasma sample 100 µl of the 0.5 µg/ml internal standard solution and 1 ml of HPLC water was added. The samples were vortexed for 30 seconds and pipetted into the conditioned cartridges. This was followed by another column volume of ammonium hydroxide to wash away all the plasma residue. A vacuum manifold was used to control the flow-rate at about 1 ml/minute. Full vacuum was applied to remove all the liquid from the cartridge. The cartridges was eluted with column volumes (1 ml) of a 95/5 methanol/ 5M hydrochloric acid solution, into clean siliconised 5 ml tubes, at a flow rate of 1 ml/minute. The tubes were removed and briefly vortexed and evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 150 µl of the mobile phase, vortexed and transferred to 250 µl glass inserts. Before the vials were placed on the tray for injection, the samples were centrifuged once more for 5 minutes at 7257.6 g's to remove particles that might have been left in samples.

3.5 Conclusion

The methods that were used to determine the bioavailability of prochlorperazine in plasma and brain tissue after intravenous, oral and intranasal administration were evaluated and found to be easy and effective. The analytical methods have been validated and proven to be specific and sensitive enough for the determination of prochlorperazine in plasma and brain tissue. The method that was developed, measures up to all the acceptance criteria, and was efficient for the quantitative determination of prochlorperazine in brain tissue following intravenous, oral and intranasal administration.

3.6 Bibliography

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Chapter 4

Results and discussion

4.1 Introduction

The rat is widely used in animal experiments and is established as an excellent model to study the bioavailability of drugs in general as well as the nasal absorption of drugs. The aim of this study was to compare the bioavailability of prochlorperazine edisylate in plasma and brain tissue after intravenous, oral and intranasal administration in rats. This was done to determine if the nasal route was superior to the intravenous route with regard to the delivery of central acting drugs to the brain.

By definition, bioavailability is the measurement of the rate and amount of intact drug that reaches the systemic circulation, following administration of the drug product. It may be useful to distinguish between the "absolute bioavailability" of a given pharmaceutical form as compared with that following intravenous administration (100%) and the "relative bioavailability" as compared with another form administered by any route other than intravenous (e.g. tablets) (Abdou, 1989:VI).

4.2 Bioavailability of prochlorperazine after intravenous, oral and intranasal administration without pH manipulation

Since the drug was administered at therapeutic doses, it was assumed that the drug follows linear kinetics. During this study a dose of 1.333 mg/kg of

prochlorperazine edisylate was administered orally (100 µl) and 0.167 mg/kg intravenously (100 µl) and intranasally (50 µl) respectively. The concentrations were corrected for the different dosages in order to compare the respective bioavailabilities.

The pH of the drug solutions was determined daily and varied between 4.65 and 4.75. The assumption was made that the state of ionisation as a factor to consider in the comparison of bioavailability after administration of the different solutions, was negligible.

4.2.1 Bioavaiability in brain tissue

It was postulated that the delivery of the central acting drug, prochlorperazine via the nasal route could be superior to the intravenous route because of the possibility that the drug could follow the direct route between the nose and the brain together with the contribution of drug delivery via central circulation.

The mean prochlorperazine brain concentrations and standard deviations (SD) found in the brains of rats after the intravenous, oral and intranasal routes of administration are shown in table 4.1. The prochlorperazine concentrations found in the brain are presented as a function of time in figures 4.1 to 4.3, respectively. Figure 4.4 shows the mean prochlorperazine concentrations following intravenous, oral and nasal administration simultaniously. Figure 4.5 displays the bioavailability of prochlorperazine in brain tissue after intravenous, oral and intranasal administration. The amount of drug found in the brain after intravenous administration was taken as the standard.

Table 4.1: Mean (± SD) of prochlorperazine concentrations (ng/g) found in brain tissue of rats following intravenous, oral and intranasal administration respectively as a function of time (minutes) (n=6).

Concentration in brain tissue (ng/g)			
Time (min)	Intravenous	Oral	Intranasal
2	406.19 ± 223.73	17.54 ± 6.68	229.13 ± 14.10
5	1291.36 ± 307.96	35.63 ± 9.93	583.23 ± 210.19
10	2063.63 ± 982.40	19.63 ± 5.45	978.40 ± 231.23
15	1906.14 ± 481.89	25.23 ± 5.17	896.40 ± 142.85
20	1594.35 ± 554.09	44.02 ± 10.99	950.19 ± 196.63
30	1160.06 ± 787.82	56.36 ± 18.00	989.12 ± 196.27
45	703.80 ± 180.12	58.54 ± 25.06	917.18 ± 101.17
60	411.18 ± 156.56	215.11 ± 92.08	856.25 ± 230.31
90	420.09 ± 257.15	179.59 ± 73.42	754.81 ± 209.73
120	411.18 ± 156.56	0.00 ± 0.00	658.69 ± 43.54

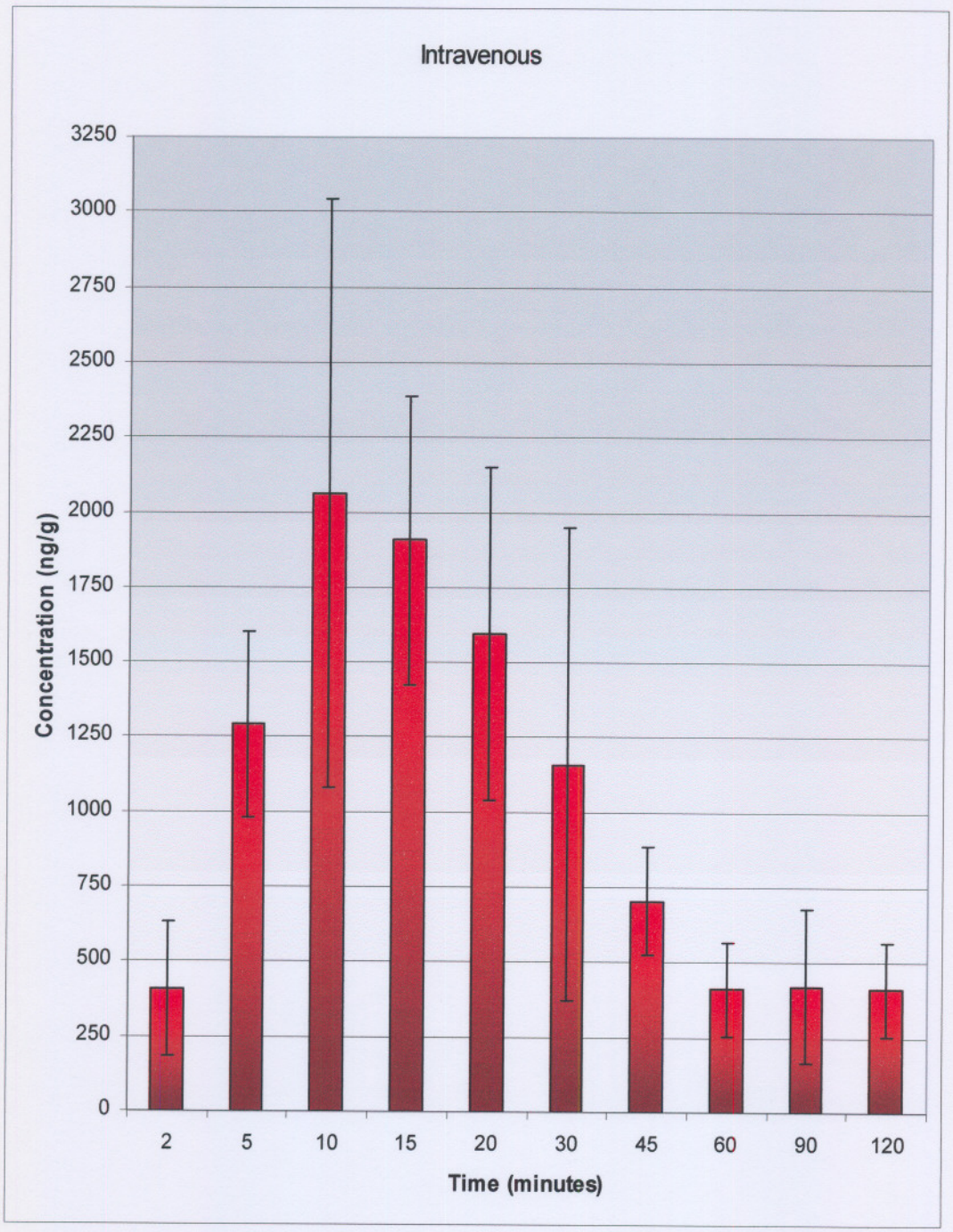

Figure 4.1: The concentration of prochlorperazine (ng/g) found in brain tissue, as a function of time (minutes) following intravenous administration (n=6).

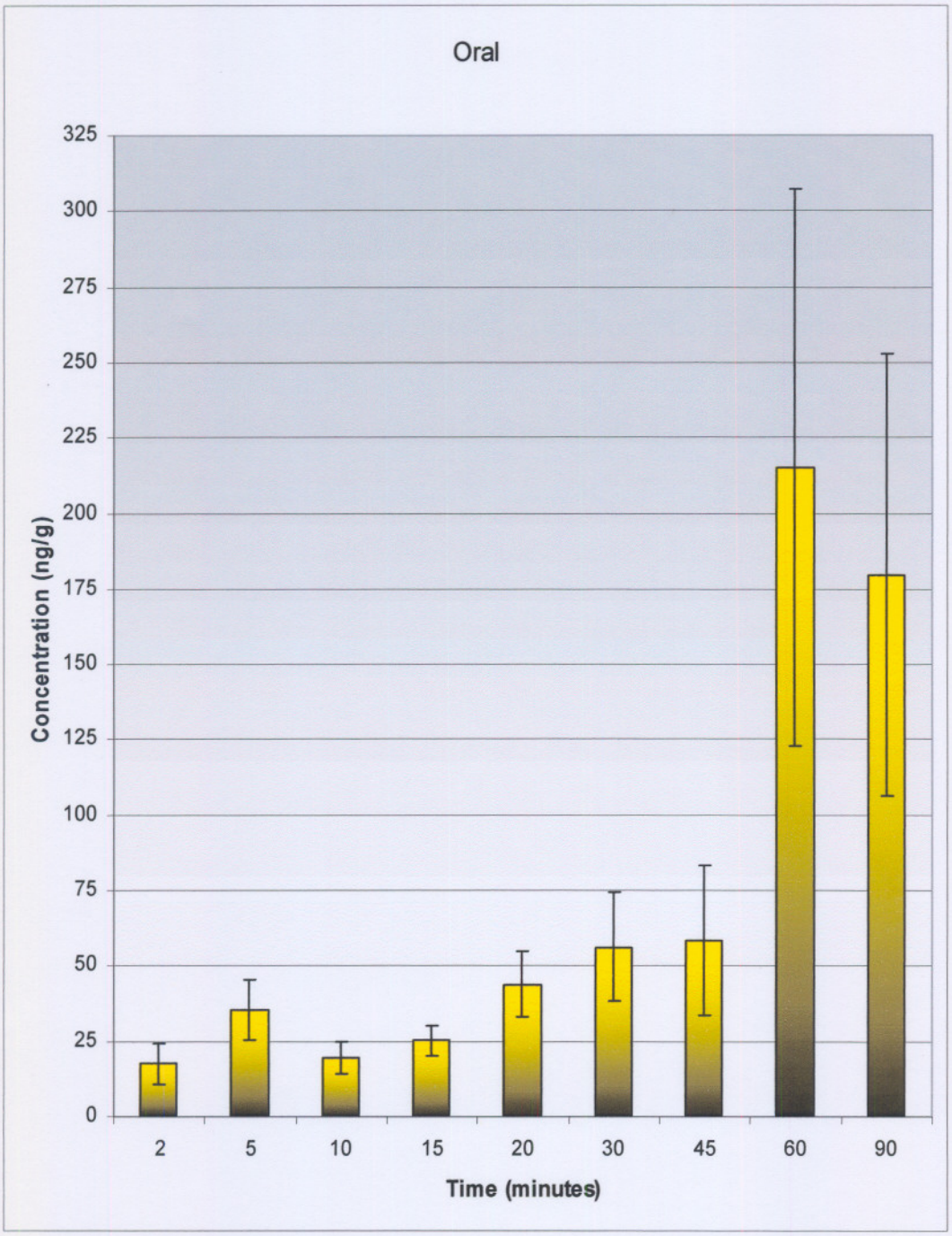

Figure 4.2: The concentration of prochlorperazine (ng/g) found in brain tissue, as a function of time (minutes) following oral administration (n=6).

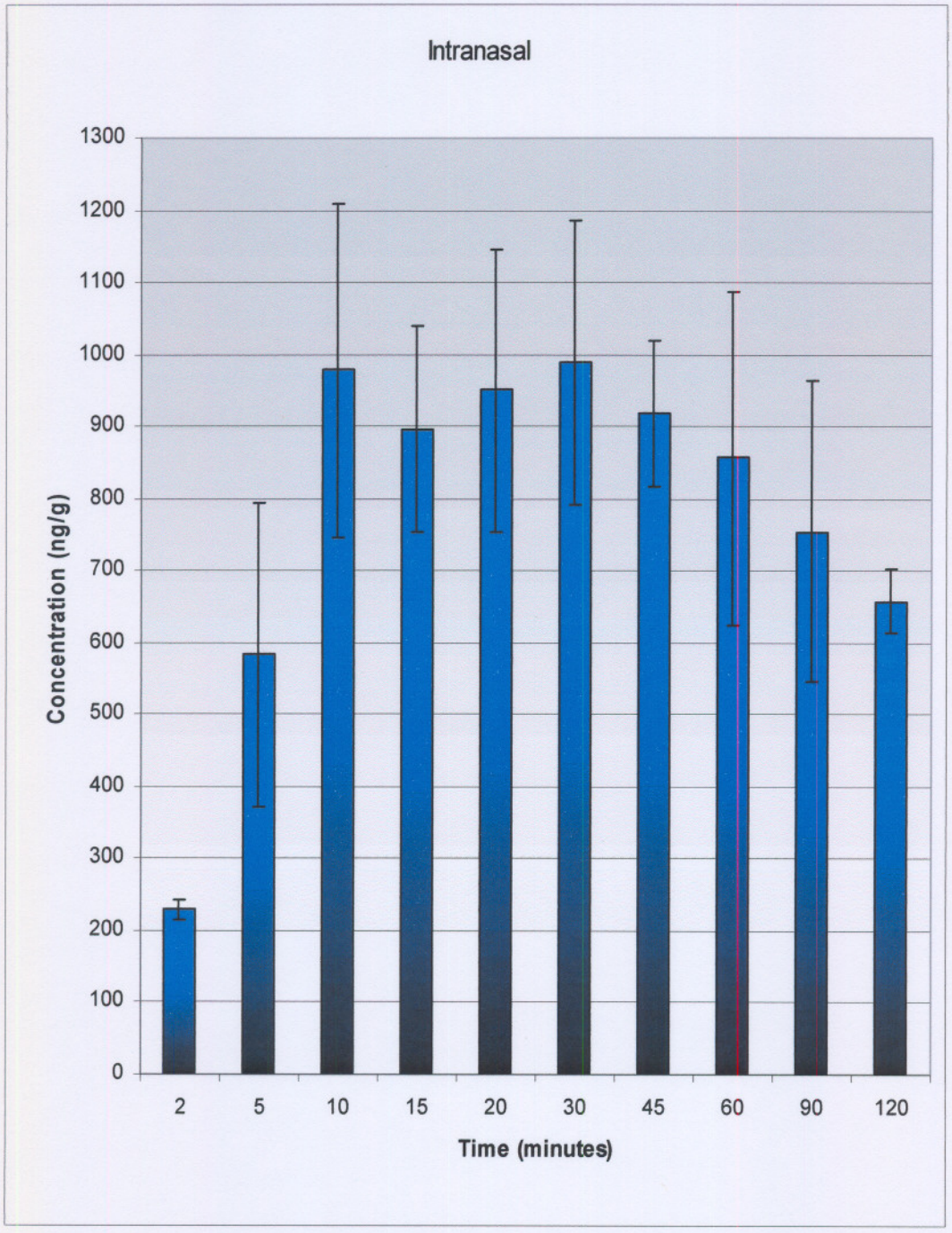

Figure 4.3: The concentration of prochlorperazine (ng/g) found in brain tissue, as a function of time (minutes) following nasal administration (n=6).

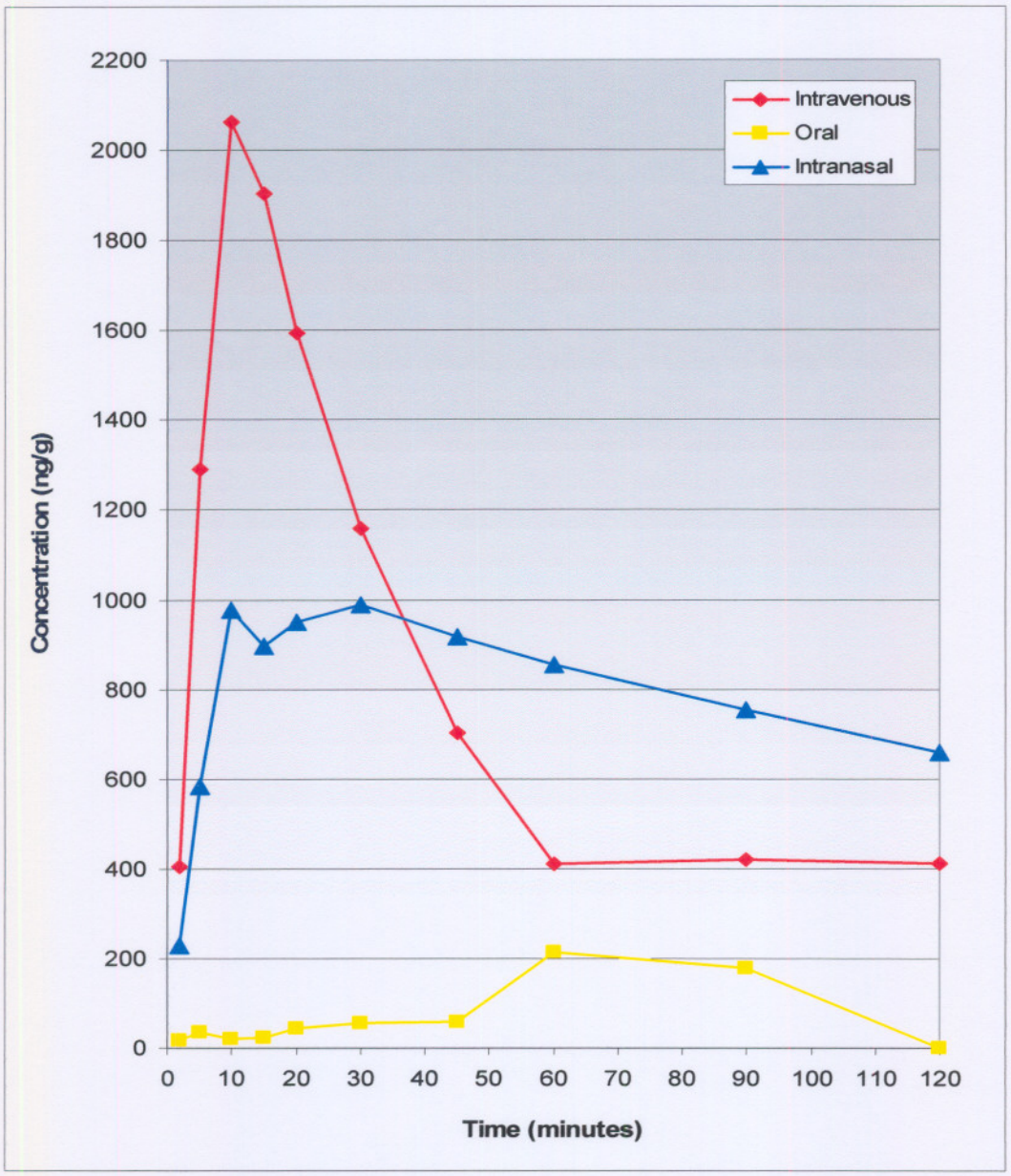

Figure 4.4: The concentration of prochlorperazine (ng/g) in brain tissue, as a function of time (minutes) following intravenous, oral and intranasal administration (n=6).

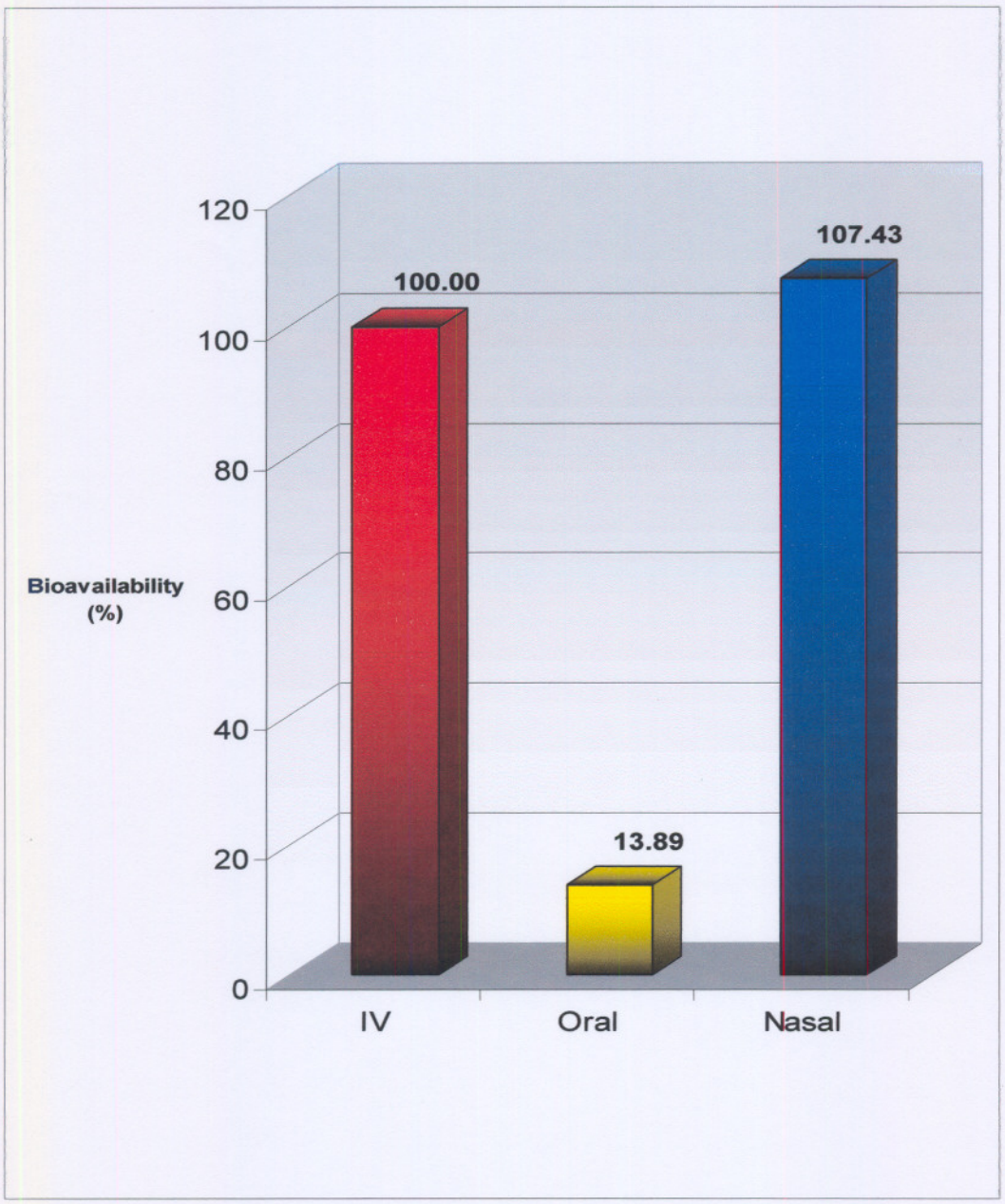

Fig 4.5: Comparison of the bioavailability (%) of prochlorperazine in brain tissue following intravenous oral and intranasal administration.

The pharmacokinetic parameters (n = 6) for the three routes of administration are summarised in table 4.2.

Table 4.2: The pharmacokinetic parameters (AUC, C_{max}, t_{max}) calculated, following intravenous, oral and intranasal administration respectively (Mean ± SD) (n=6).

Route of administration	AUC (ng/g/h)	C_{max} (ng/g)	t_{max} (minutes)
Intravenous	90051.71 ± 6189.75	2063.63 ± 982.40	10
Oral	12507.20 ± 1248.01	215.11 ± 92.08	60
Intranasal	96745.32 ± 3649.65	989.12 ± 196.27	30

The AUC-values for the three different routes of administration were calculated from the mean prochlorperazine concentrations determined in brain tissue samples (Table 4.1).

The method for calculation of the AUC values, described by Jawien (1992:484), can be used to compare two variables statistically with each other. Statistically significant differences were indicated by using a confidence interval of 95% (α = 0.05).

Tables 4.3 and 4.4 show the different confidence intervals for AUC and C_{max} respectively, calculated for the three routes of administration. If the value 0 is not included in the confidence intervals, a statistical significant difference ($p < 0.05$) between the two formulations being compared is indicated.

Table 4.3: Confidence intervals (α = 0.05) for the statistical comparison of the AUC-values for the different routes of administration.

Formulation	Confidence interval
Intravenous vs Oral	[92852.82 ; 62236.21] *
Intranasal vs Oral	[93576.23 ; 74900.02] *
Intravenous vs Intranasal	[24108.73 ; -10721.51]

*** Indicate statistical significant differences**

Table 4.4: Confidence intervals (α = 0.05) for the statistical comparison of the C_{max} – values for the different routes of administration.

Formulation	Confidence interval
Intravenous vs Oral	[2673.22 ; 1023.83] *
Intranasal vs Oral	[1598.71 ; -50.68]
Intravenous vs Intranasal	[1899.21 ; 249.81] *

*** Indicate statistical significant differences**

4.2.2 Statistical comparison of brain tissue concentrations after different routes of administration.

From table 4.1 it is evident that dramatic differences in the brain tissue concentrations were found after administration of the drug via the various routes.

4.2.2.1 Intravenous versus oral brain tissue concentrations

The AUC-value, used as an indication of the extend of absorption for prochlorperazine into the brain tissue, after oral administration was found to be 12507.20 ± 1248.01 ng/g/h which is only 13.98% of the AUC-value of 90051.71 ± 6189.75 ng/g/h found after intravenous administration.

Using 95% confidence intervals, statistical significant differences between both the AUC-values (Table 4.3) and C_{max}-values (Table 4.4) were found when comparing the intravenous and oral routes of administration. It can be seen that very low prochlorperazine concentrations were found in the brain up to 60 minutes after oral administration, and then a sudden increase in the concentration of prochlorperazine in the brain at 60 minutes, which could not be explained. Both the C_{max} and t_{max} parameters show that the rate of movement of prochlorperazine was dramatically faster after intravenous administration compared to the oral route. This was to be expected because the drug was immediately available at the BBB after intravenous administration compared to the oral route where the process of absorption first has to take place.

4.2.2.2 Intranasal versus oral brain tissue concentrations

The AUC-value after oral administration was found to be 12507.20 ± 1248.01 ng/g/h which is only 12.93% of the AUC-value of 96745.32 ± 3649.65 ng/g/h found after intranasal administration.

Statistical significant differences between the AUC-values (Table 4.4) were found, the difference found between C_{max}-values was not statistically significant (Table 4.5) when comparing the intranasal and oral routes of administration.
The rate of apearance of prochlorperazine after nasal administration was found to be higher (t_{max} = 30 minutes) than that of the oral route (t_{max} = 60 minutes). The amount of prochlorperazine found in brain tissue after nasal administration was approximately 8 fold compared to the oral route.When considering that therapeutic

concentrations are reached after oral administration of 0.07 mg/kg prochlorperazine (equivalent to a 5 mg tablet of prochlorperazine) it can be assumed that the intranasal dose could be significantly decreased (8x) to give the same therapeutic effect. A possible lower intranasal dose could be beneficiary to the patient resulting in a safer and more effective drug product.

4.2.2.3 Intranasal versus intravenous brain tissue concentrations

The AUC-value, after intranasal administration was found to be 96745.32 ± 3649.65 ng/g/h which is 107.43% of the AUC-value of 90051.71 ± 6189.75 ng/g/h found after intravenous administration.
These results indicate no statistical significant difference between the AUC-values of the mentioned routes of administration, but the C_{max}-value after intravenous administration was found to be statistically significantly higher than after intranasal administration.

The increased amount of prochlorperazine found in brain tissue after 2 hours could be as a result of the added effect of the direct route between the nose and the brain. If the bioavailability of prochlorperazine in plasma after nasal administration (87.10%) is considered, the contribution of the direct nose-to-brain route can be assumed to be ≈ 30% (107% - 87%). It is possible that for drugs that do not cross the BBB that easily, the contribution of the nose-to-brain route could become more important.

4.2.3 Bioavailability in plasma

The mean prochlorperazine plasma concentrations and standard deviations (SD) after the above mentioned routes of administration are shown in table 4.5. The data given in table 4.5 are shown as a function of time in figures 4.6 to 4.8 respectively. Figure 4.9 shows the mean prochlorperazine concentrations following intravenous, oral and intranasal administration,

simultaniously. Figure 4.10 displays the comparison of the bioavailability found with each route of administration.

Table 4.5: Mean (± SD) of prochlorperazine concentrations (ng/ml) found in plasma of rats following intravenous, oral and intranasal administration respectively, as a function of time (minutes) (n=6).

Concentration in plasma (ng/ml)			
Time (min)	**Intravenous**	**Oral**	**Intranasal**
2	99.02 ± 16.45	0.31 ± 0.17	20.62 ± 6.68
5	80.29 ± 19.60	1.17 ± 1.09	74.14 ± 50.58
10	62.41 ± 22.81	5.60 ± 2.56	97.69 ± 41.01
15	46.60 ± 20.08	6.10 ± 1.05	55.47 ± 29.29
20	37.81 ± 15.62	6.24 ± 2.95	31.51 ± 9.26
30	33.72 ± 14.99	6.33 ± 2.55	28.66 ± 9.80
45	31.89 ± 11.41	6.91 ± 0.88	21.51 ± 6.78
60	28.70 ± 2.84	7.91 ± 1.82	22.28 ± 11.26
90	16.44 ± 7.19	5.72 ± 2.79	14.26 ± 4.19
120	12.19 ± 5.33	5.05 ± 1.27	1.87 ± 2.19

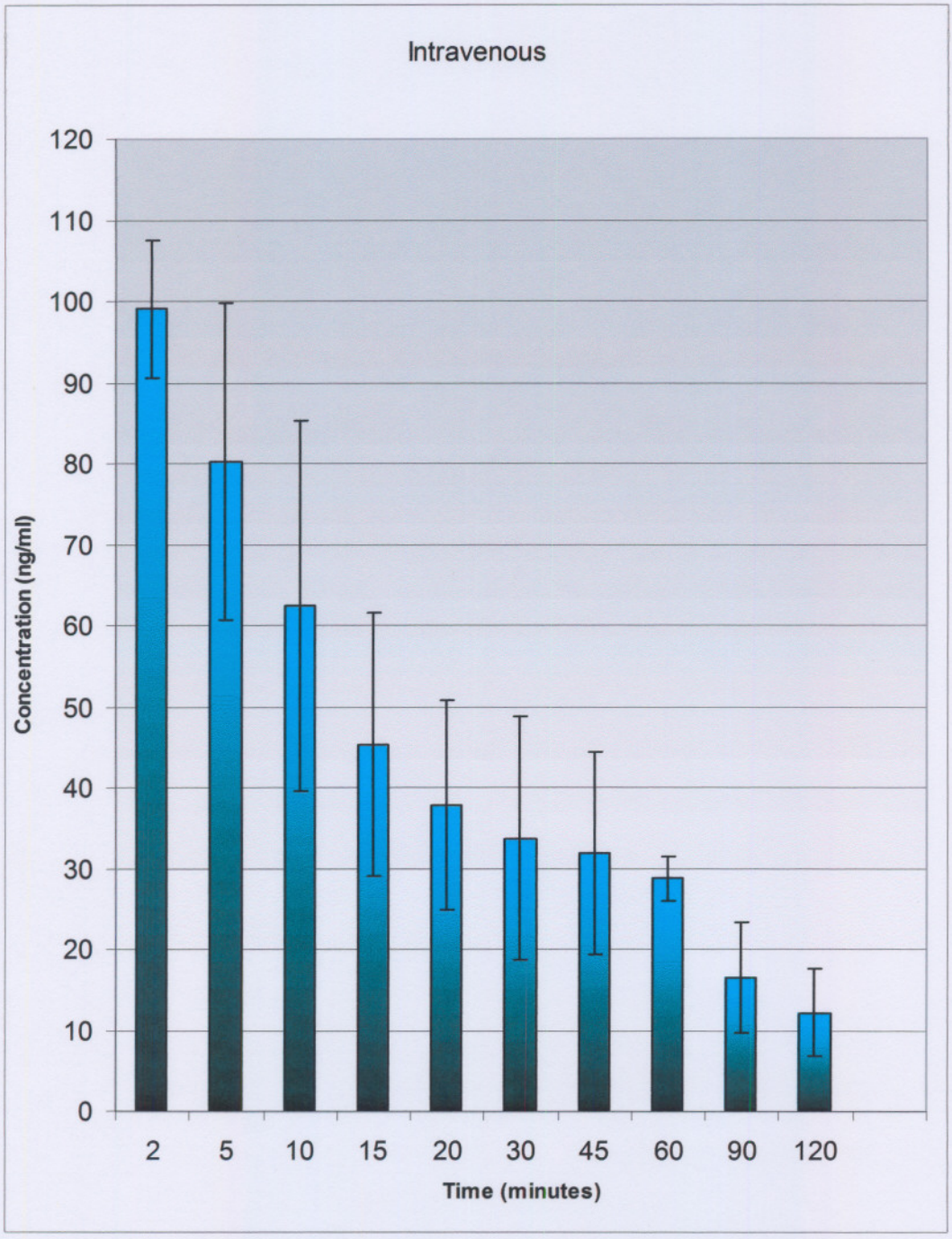

Figure 4.6: The concentration of prochlorperazine (ng/ml) in plasma, as a function of time (minutes) following intravenous administration (n=6).

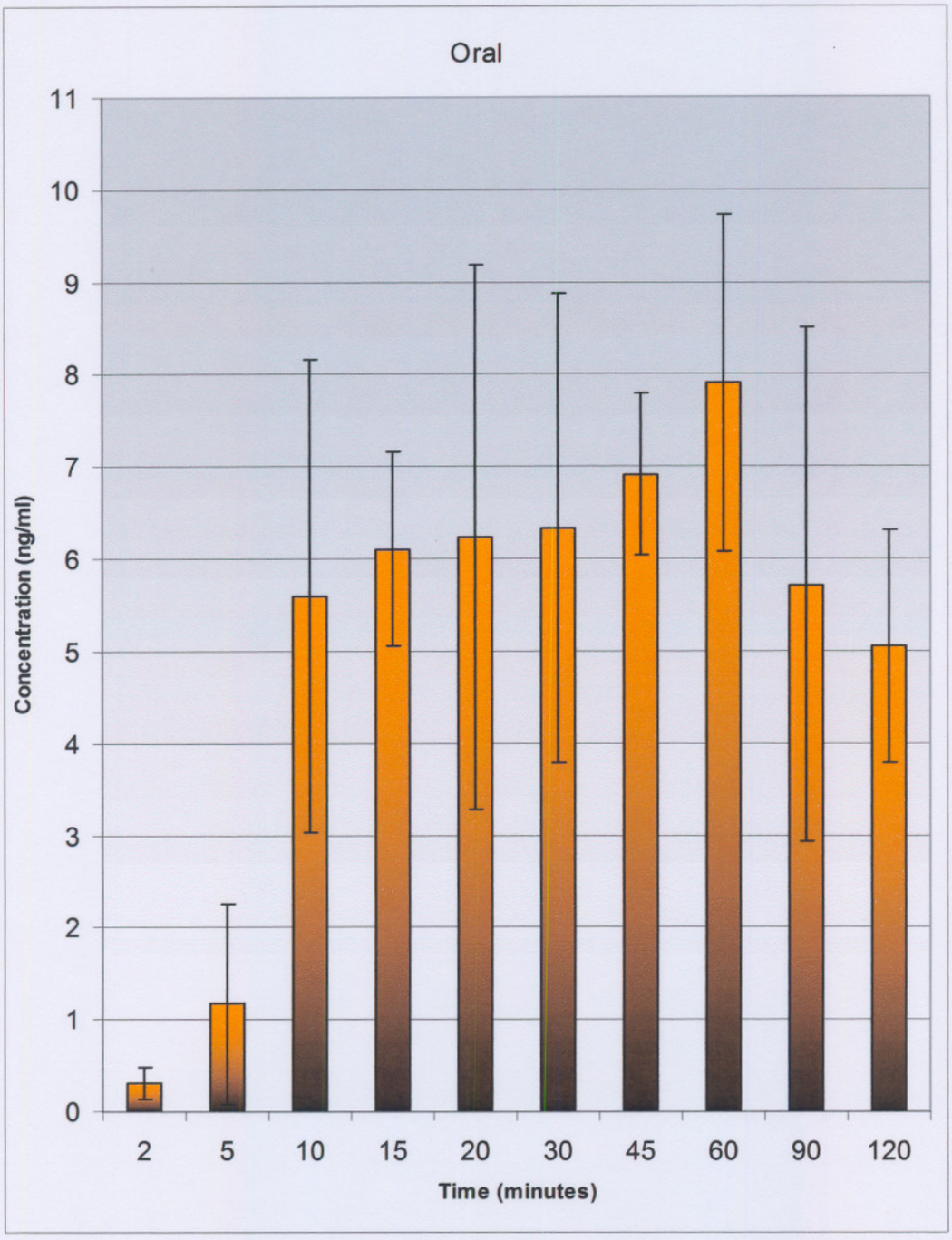

Figure 4.7: The concentration of prochlorperazine (ng/ml) in plasma, as a function of time (minutes) following oral administration (n=6).

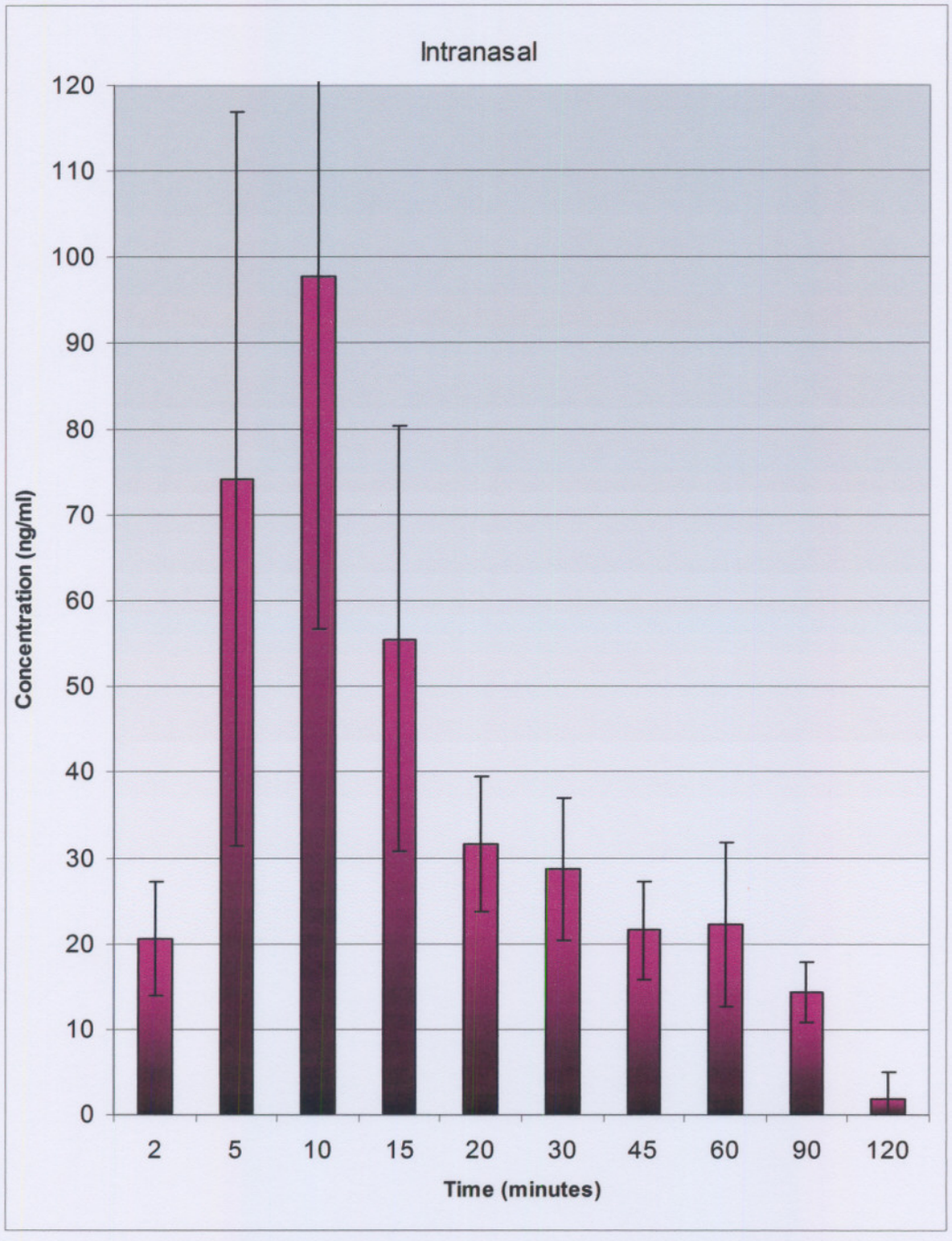

Figure 4.8: The concentration of prochlorperazine (ng/ml) in plasma, as a function of time (minutes) following intranasal administration (n=6).

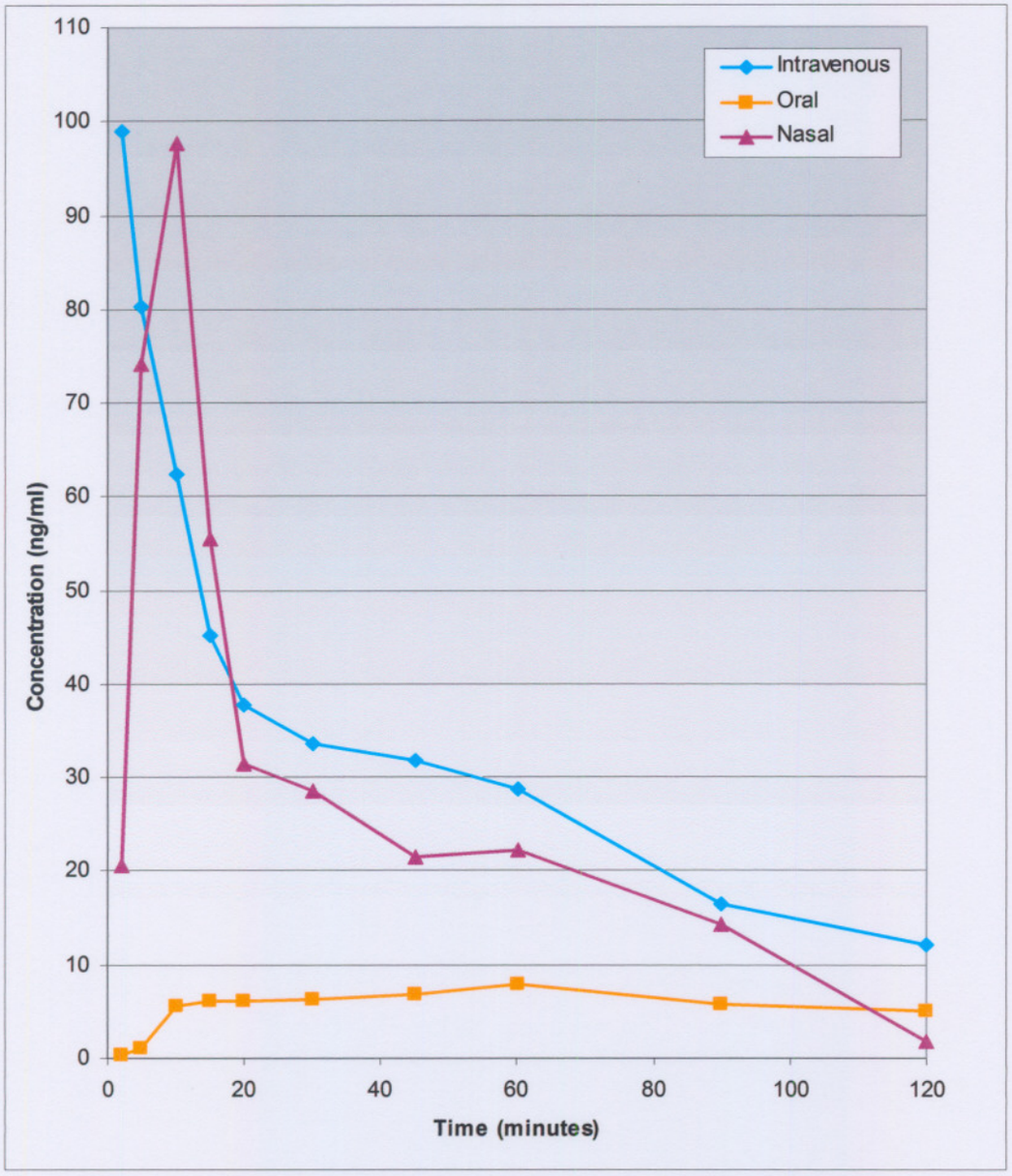

Figure 4.9: The concentration of prochlorperazine (ng/ml) in plasma, as a function of time (minutes) following intravenous, oral and intranasal administration (n=6).

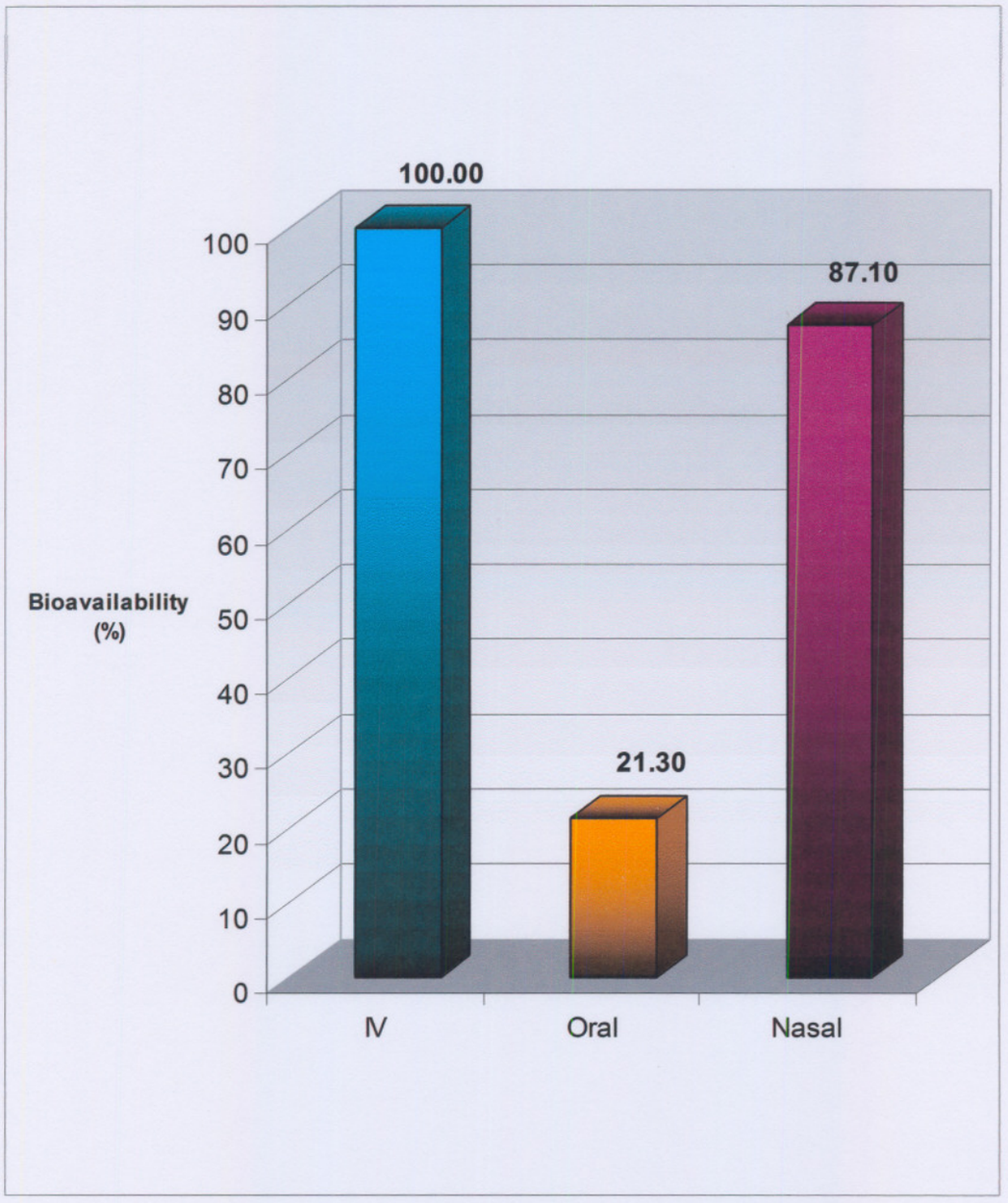

Fig 4.10: Comparison of the absolute bioavailability (%) of prochlorperazine in plasma following intravenous, oral and intranasal administration respectively.

The pharmacokinetic parameters (AUC, C_{max} and $t_{max)}$ (n = 6) for the three routes of administration were calculated and are summarised in table 4.6.

Table 4.6: The pharmacokinetic parameters (AUC, C_{max}, t_{max}) following intravenous, oral and intranasal administration respectively (Mean ± SD) (n=6).

Route of administration	AUC (ng/ml/h)	C_{max} (ng/ml)	t_{max} (minutes)
Intravenous	3371.47 ± 173.79	-- -- --	--
Oral	718.07 ± 42.74	7.91 ± 1.82	60
Intranasal	2936.71 ± 189.65	97.69 ± 41.01	10

The AUC-values for the three different routes of administration were calculated from the mean prochlorperazine concentrations determined in the plasma samples (Table 4.5). Table 4.7 and table 4.8 shows the different confidence intervals calculated for the three formulations.

Table 4.7: Confidence intervals (α = 0.05) for the statistical comparison of the AUC-values for the different routes of administration.

Formulation	Confidence interval
Intravenous vs. Oral	[3086.68 ; 2220.12] *
Intranasal vs. Oral	[2689.30 ; 1748.00] *
Intravenous vs. Intranasal	[1057.52 ; -188.00]

*** indicate statistical significant differences**

Table 4.8: Confidence intervals ($\alpha = 0.05$) for the statistical comparison of the C_{max} – values for the oral and intranasal routes of administration.

Formulation	Confidence Interval
Intranasal vs. Oral	[125.93 ; 53.63] •

• Indicate statistical significant differences

4.2.4 Statistical comparison of plasma concentrations after different routes of administration.

4.2.4.1 Intravenous versus oral plasma concentrations

The AUC-value, following oral administration was found to be 718.07 ± 42.74 ng/ml/h which is only 21.30% of the AUC-value of 3371.47 ± 173.79 ng/ml/h found after intravenous administration. These results correlate well with a study done by Isah *et al*, (1991:679) who investigated the clinical pharmacology of prochlorperazine in healthy young males and found an oral bioavailability of 12.50%.

As expected, statistical significant differences between the AUC-values (Table 4.7) were found when comparing the intravenous and oral routes of administration.

4.2.4.2 Intranasal versus oral plasma concentrations

The AUC-value for prochlorperazine following oral administration was found to be 718.07 ± 42.74 ng/ml/h which is 24.45% of the AUC-value of 2936.71 ± 189.65 ng/ml/h found after intranasal administration. Similar results were obtained in a study done by Pelser, (1999:87) on the bioavailability of doxylamine, where AUC-values for doxylamine after oral administration was found to be 34.84% of the AUC-value after nasal administration.

The rate of absorption of prochlorperazine edisylate after nasal administration was found to be much higher (t_{max} = 10 minutes, C_{max} = 97.69ng/ml) than after oral administration (t_{max} = 60 minutes, C_{max} = 7.91ng/ml).

Statistical significant differences between both the AUC-values (Table 4.7) and C_{max}-values (Table 4.8) were found when comparing the intranasal and oral routes of administration.

4.2.4.3 Intravenous versus intranasal plasma concentrations

The AUC-value for prochlorperazine following intranasal administration was found to be 2936.71 ± 189.65 ng/ml/h which is 87.10% of the AUC-value of 3371.47 ± 173.79 ng/ml/h found after intravenous administration. This value correlated well with a study done by Simpson, (2002:95) where the AUC-value of prochlorperazine after intranasal administration was found to be 90.87%. Pelser, (1999:88) indicated that the bioavalability of doxylamine after nasal administration was about 70.82% compared to the intravenous route.

No statistical significant differences were found between the AUC-values (Table 4.7) when comparing the intranasal and intravenous routes of administration.

These results show that the nasal route is superior to the oral route with regard to the systemic delivery of prochlorperazine edisylate. Further the bioavailability of prochlorperazine after nasal administration is virtvually similar to that obtained after intravenous administration. The nasal route for the delivery of anti-emetic drugs therefore shows much potential as an alternative route of administration and could have many clinical advantages compared to both the oral and intravenous route.

4.3 Bioavailability of prochlorperazine after intravenous, oral and intranasal administration with pH manipulation

In order to determine the effect of pH on the bioavailability of prochlorperazine, a pilot study was performed where the formulations were buffered at pH 6.6 using a phosphate buffer. The effect of pH was determined using the intravenous and intranasal routes at the following five time intervals: 2, 10, 30, 60 and 90 minutes.

The pKa of proclorperazine is 7.82 and the pH of the nasal epithelium varies between 5.5 and 6.5 (Chien *et al.*, 1989:17), which would cause the prochlorperazine to be in a more unionised (lipophilic) state when buffered at a pH 6.6 compared to the initial pH 4.65 of the solution. A unionised drug can be absorbed more rapidly and easily across the nasal mucosa.

4.3.1 Bioavailability in brain tissue

The mean prochlorperazine concentrations and standard deviations (SD) after the above mentioned routes of administration are shown in table 4.9 (prochlorperazine concentration in brain tissue)

Figures 4.11 - 4.14 show the mean prochlorperazine concentrations in brain tissue and plasma following intravenous and intranasal administration.

Table 4.9: Mean (± SD) of prochlorperazine concentrations (ng/g) found in brain tissue following intravenous and nasal administration.

Concentration in brain tissue (ng/g)				
Time (minutes)	**Intravenous pH = 4.65**	**Intravenous pH = 6.6**	**Intranasal pH = 4.65**	**Intranasal pH = 6.6**
2	406.19±223.73	2011.38±1144.41	229.19±14.50	139.63±33.02
10	2063.63±982.40	2020.57±372.83	978.40±231.23	785.10±106.03
30	1160.06±787.82	1153.53±293.83	989.12±196.21	1349.38±504.70
60	411.18± 56.56	808.79±481.89	856.25±230.31	1420.29±829.26
90	420.09±257.15	751.72±251.55	754.81±209.73	890.02±272.73

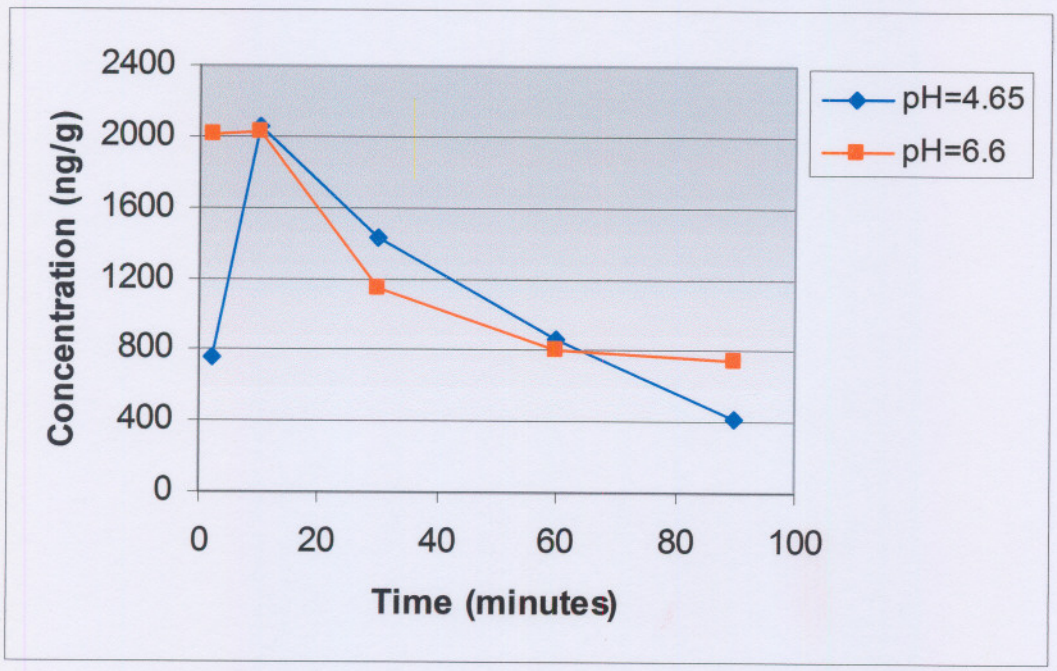

Figure 4.11: Mean prochlorperazine concentrations in brain tissue following intravenous administration at different pH values.

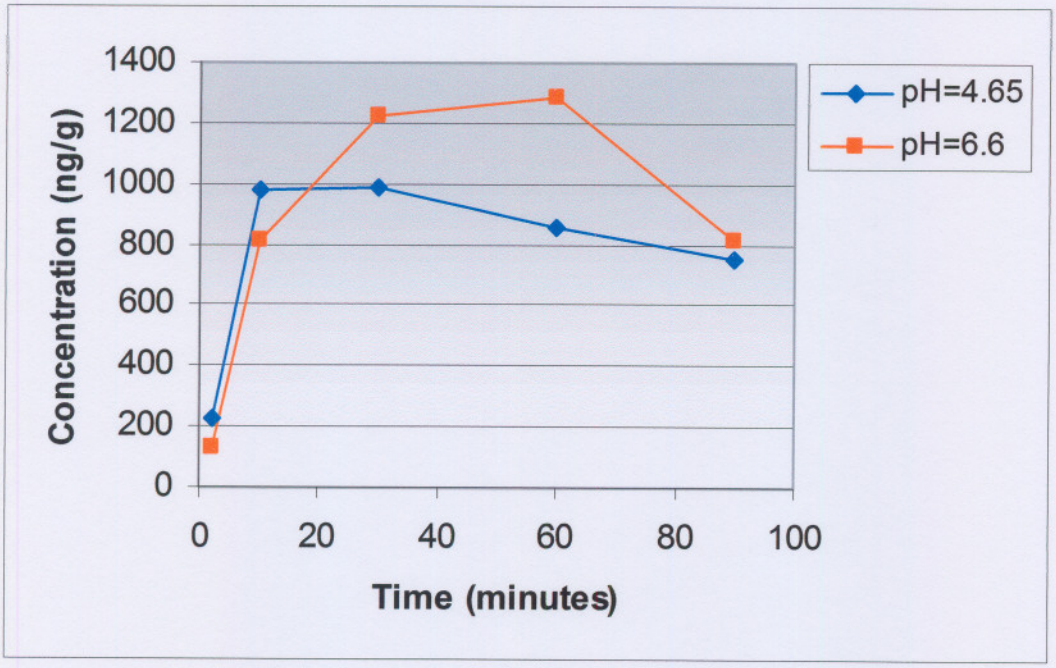

Figure 4.12: Mean prochlorperazine concentrations in brain tissue following nasal administration at different pH values.

The pharmacokinetic parameters (n = 6) for intravenous and intranasal administration of prochlorperazine buffered at pH=6.6 are summarised in table 4.10.

Table 4.10: The pharmacokinetic parameters (AUC, C_{max}, t_{max}) following intravenous and intranasal administration respectively (Mean ± SD) (n=6).

Route of administration	AUC (ng/g/h)	C_{max} (ng/g)	t_{max} (minutes)
Intravenous	92665.82 ± 7364.22	2020.57 ± 372.83	10
Intranasal	92736.02 ± 11525.80	1284.19 ± 829.26	60

The AUC-values for intravenous and intranasal administration were calculated from the mean prochlorperazine concentrations determined in brain tissue samples (Table 4.9). Table 4.13 and table 4.14 shows the different confidence intervals calculated for the formulations at different pH values.

Table 4.11: Confidence intervals (α = 0.05) for the statistical comparison of the AUC-values for the formulations at different pH values.

Formulation	Confidence interval
Intravenous(pH6.6) vs. Intravenous(pH4.65)	[46442.62 ; -14169.45]
Intranasal(pH6.6) vs. Intranasal(pH4.65)	[46774.40 ; -12172.83]
Intravenous(pH6.6) vs. Intranasal(pH6.6)	[33183.62 ; -33043.17]

Table 4.12: Confidence intervals (α = 0.05) for the statistical comparison of the C_{max} – values for the formulations at different pH values.

Formulation	Confidence interval
Intravenous(pH6.6) vs. Intravenous(pH4.65)	[867.75 ; -781.64]
Intranasal(pH6.6) vs. Intranasal(pH4.65)	[1119.77 ; -529.63]
Intravenous(pH6.6) vs. Intranasal(pH6.6)	[88.31 ; -1561.09]

4.3.2 Statistical comparison of brain tissue concentrations after intravenous and intranasal administration at different pH values of the formulation.

From the results shown in table 4.9 it is evident that the pH of a formulation does affect the bioavailability of the administered drug to a certain extent. The adjusted pH of the administered drug solution had the greatest effect on the drug absorption in the brain tissue after nasal administration (Figure 1.12).

4.3.2.1 Intravenous (pH 6.6) versus intravenous (pH 4.65) brain tissue concentrations

The AUC-values after intravenous administration at pH 6.6 and intravenous administration at pH 4.65 were 92665.82 ± 7364.22 ng/g/h and 76529.23 ± 10122.66 ng/g/h, respectively. Although there is quite a big difference in the AUC-values of the brain tissue after intravenous administration at different pH values, the difference is not of statistical significance. There is also no statistical significant difference between the C_{max}-values at the different pH values. The rate of absorption was found to be the same (t_{max} = 10 minutes) after administration of the formulations at both pH values. When prochlorperazine was administered at a pH 6.6, much higher concentrations were found in the brain tissue after 2 minutes than when it was administered at a pH 4.65, which could not be explained.

4.3.2.2 Intranasal (pH 6.6) versus intranasal (pH 4.65) brain tissue concentrations

The AUC-values after nasal administration at pH 6.6 was found to be 92736.04 ± 11525.80ng/g/h compared to the AUC-value of 75435.25 ± 3919.91 ng/g/h after nasal administration at pH 4.65. No statistical significant differences were found between the AUC-values or the C_{max}-values after intranasal drug administration at the different pH values (pH 6.6 & pH 4.65). The rate of absorption seemed to be affected by the pH differences. After nasal administration at pH 6.6 the rate of absorption seemed to be lower (t_{max} = 60 minutes) than after nasal administration

at pH 4.65 (t_{max} = 30 minutes). When looking at the concentrations in the brain tissue after 30 and 60 minutes, there is a very slight difference in concentrations obtained at these time intervals and if one compares the standard deviations (SD) at these time intervals, the SD at 60 minutes is much higher than at 30 minutes, which could explain the apparent lower rate of absorption after nasal administration of prochlorperazine at pH 6.6.

4.3.2.3 Intravenous (pH 6.6) versus intranasal (pH 6.6) brain tissue concentrations

The AUC-values after intravenous and intranasal administration were 92665.82 ± 7364.22 ng/g/h and 92736.04 ± 11525.80ng/g/h, respectively. The maximum concentrations after intravenous and nasal administrations were achieved after 10 minutes and 60 minutes respectively. Once again no statistical significant differences were found between the AUC-values after intravenous and intranasal administration, but this time there was also no statistical significant differences between the C_{max}-values. This indicates that the pH does play a role in the drug absorption of prochlorperazine after nasal administration.

4.3.3 Bioavailability in plasma

The mean prochlorperazine concentrations and standard deviations (SD) after the above-mentioned routes of administration are shown in table 4.13 (prochlorperazine concentration in plasma)

Figures 4.13 - 4.14 show the mean prochlorperazine concentrations in plasma and plasma following intravenous and nasal administration

Table 4.13: Mean (± SD) of prochlorperazine concentrations (ng/ml) found in plasma following intravenous and nasal administration.

Concentration in plasma (ng/ml)				
Time (minutes)	**Intravenous pH = 4.65**	**Intravenous pH = 6.6**	**Intranasal pH = 4.65**	**Intranasal pH = 6.6**
2	99.02±16.45	115.35±46.00	20.62±6.68	43.32±19.79
10	62.41±22.81	60.89±20.46	97.69±41.01	106.37±40.51
30	33.72±14.99	46.38±26.27	28.66±8.30	44.07±21.09
60	28.70±2.84	24.39±16.27	22.28±9.53	26.48±14.48
90	14.44±6.75	6.30±4.29	14.26±3.55	14.43±4.29

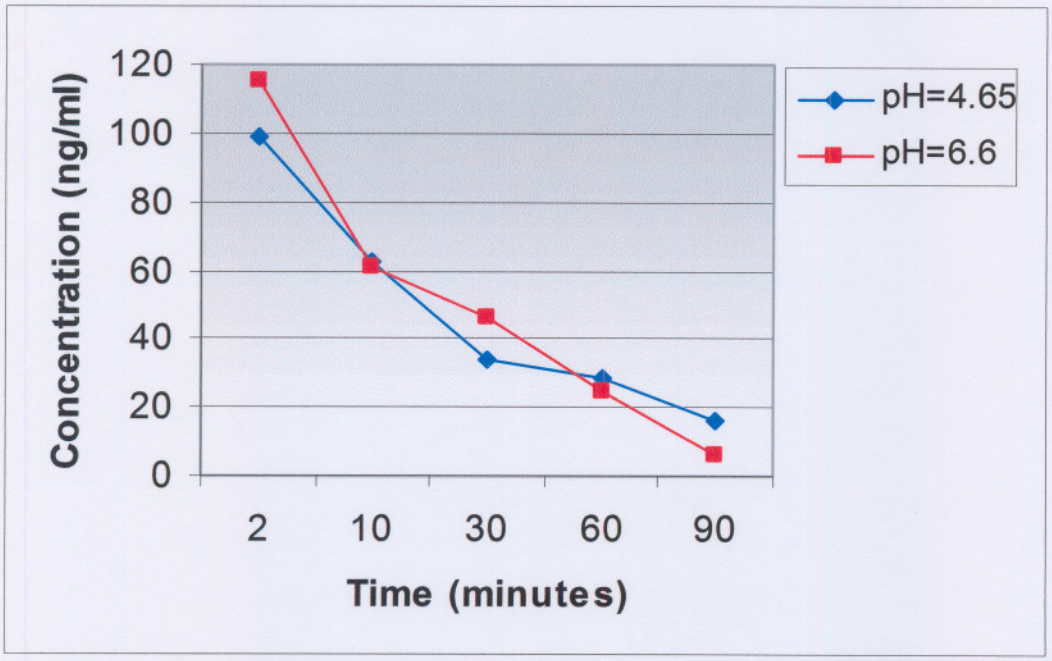

Figure 4.13: Mean prochlorperazine concentrations in plasma following intravenous administration at different pH values.

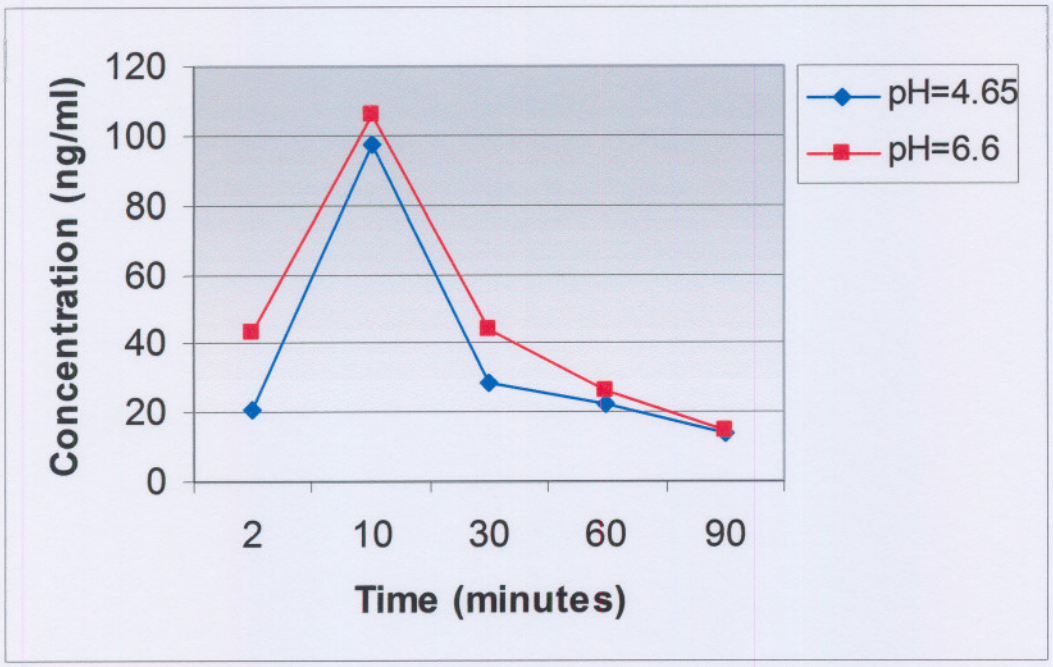

Figure 4.14: Mean prochlorperazine concentrations in plasma following nasal administration at different pH values.

Table 4.14: The pharmacokinetic parameters (AUC, C_{max}, t_{max}) following intravenous and intranasal administration respectively (Mean ± SD) (n=6).

Route of administration	AUC (ng/ml/h)	C_{max} (ng/ml)	t_{max} (minutes)
Intravenous	2838.36 ± 362.77	-- -- --	--
Intranasal	3601.76 ± 364.86	106.37± 40.51	10

The AUC-values for intravenous and intranasal administration were calculated from the mean prochlorperazine concentrations determined in plasma samples (Table 4.13). Table 4.15 and table 4.16 shows the different confidence intervals calculated for the formulations at different pH values.

Table 4.15: Confidence intervals (α = 0.05) for the statistical comparison of the AUC-values for the different routes of administration.

Formulation	Confidence interval
Intravenous(pH6.6) vs. Intravenous(pH4.65)	[30320.06 ; -30292.02]
Intranasal(pH6.6) vs. Intranasal(pH4.65)	[30108.86 ; -28838.37]
Intravenous(pH6.6) vs. Intranasal(pH6.6)	[33876.79 ; -32350.00]

Table 4.16: Confidence intervals (α = 0.05) for the statistical comparison of the C_{max} – values for the different routes of administration.

Formulation	Confidence interval
Intranasal(pH6.6) vs. Intranasal(pH4.65)	[833.38 ; -816.02]

4.3.4 Statistical comparison of plasma concentrations after intravenous and intranasal administration at different pH values of the formulation.

Very similar concentration-time profiles were obtained from the plasma concentrations after intravenous and intranasal drug administration at different pH values (Figure 4.13 & 4.14).

4.3.4.1 Intravenous (pH 6.6) versus intravenous (pH 4.65) plasma concentrations

When comparing the AUC-values after drug administration at different pH values, it was found that the AUC-value after IV administration at pH 6.6 was 2838.36 ± 362.77 ng/ml/h and the AUC-value after IV administration at pH 4.65 was 2824.34 ± 210.41 ng/ml/h. No statistical significant differences were found between the AUC-values after intravenous drug administration at the different pH values (pH 6.6 & pH 4.65).

As expected the pH of a formulation does not really affect the plasma concentrations of a drug administered intravenously.

4.3.4.2 Intranasal (pH 6.6) versus intranasal (pH 4.65) plasma concentrations

The AUC-values after nasal administration at pH 6.6 was found to be 3601.76 ± 364.86 ng/ml/h compared to the AUC-value of 2966.52 ± 276.28 ng/ml/h after nasal administration at pH 4.65. No statistical significant differences were found between the AUC-values or the C_{max}-values after intranasal drug administration at the different pH values (pH 6.6 & pH 4.65).

The difference between the AUC-values after nasal administration was greater than after intravenous administration, indicating that the pH of a formulation has a greater effect on the plasma concentrations of a drug after nasal administration than after intravenous administration.

4.3.4.3 Intravenous (pH 6.6) versus intranasal (pH 6.6) plasma concentrations

The AUC-values after intravenous and intranasal administration were 2838.36 ± 362.77 ng/ml/h and 3601.76 ± 364.86 ng/ml/h, respectively. The maximum concentration after nasal administration was achieved after about 10 minutes and

the extent of nasal bioavailability appeared to be 100%. Similar results were obtained in a study with acetylsalicylic acid, where the absolute nasal bioavailability of acetylsalicylic acid was 107.3% (Hussain *et al.*, 1992:349).

No statistical significant differences were found between the AUC-values after intravenous and intranasal drug administration pH 6.6.

4.4 Discussion

The data obtained in this study showed that the nasal route has many advantages over the oral route for the administration of, in this case, CNS-active drugs with low oral bioavailability. Rapid absorption into systemic circulation after nasal administration has been demonstrated with several drugs that are active in the CNS: physostigmine and arecoline (Hussain & Mollica, 1991:750), propiomazine (Bjerre *et al.*, 1996:217), dextrometorphan (Char *et al.*, 1992:750) and cocaine (Chow *et al.*, 1999:754). Prochlorperazine was absorbed rapid and almost complete across the nasal mucosa. The molecular weight of prochlorperazine is 564.14 g/mol and the log P value is 4.76. A linear relationship between the rate constant and the log P (octanol/water) has been demonstrated with progesterone and its monohydroxy, dihydroxy and trihydroxy derivates (Corbo *et al.*, 1989:848). Linking the extent of absorption of compounds with their molecular weight, the nasal route appears suitable for the efficient and rapid delivery of molecules with a molecular weight < 1000 (Dondeti *et al.*, 1996:116).

No direct evidence for transfer along the olfactory pathway was shown with prochlorperazine. However, a prolonged duration of the concentration in the brain tissue was seen after nasal administration and a higher AUC-value was obtained after nasal administration compared to intravenous administration. The intranasal/intravenous brain tissue concentration ratio exceeded one after 30 and 45 minutes after nasal administration at a pH of 6.6 and 4.65 respectively,

indicating that after these time intervals the concentrations of nasally administered prochlorperazine in the brain tissue were higher than those after intravenous administration. The maximum concentration of prochlorperazine in brain tissue following nasal administration did not exceed that achieved after intravenous administration, but the nasal/intravenous AUC ratio in brain tissue was found to be greater than one. Similar results were found by Chou & Donovon (1998) in a rat model of the uptake of lidocaine and by Dahlin & Bjork (1999) who investigated the absorption of (S)-UH-301 and its transport into the cerebrospinal fluid of rats.

Most studies investigating the pathway from the nose to the brain have been performed in rodents, but studies in monkeys have also been reported. Many common animal models, including those involving rodents, are macrosmatic while humans are understood to be microsmatic. Further, in humans, the olfactory region is located in the roof of the cavity, while the olfactory area in rats is spread throughout the posterior part of the cavity. It is important to take these anatomical differences between species under consideration when results are interpreted and compared (Dahlin, 2000:40).

So far, not many articles have been published regarding the influence of the formulation for the uptake of drugs into the CNS after nasal administration. Hardy *et* al. (1985) compared intranasal drug delivery by spray and drops. The spray was deposited mainly in the atrium, and cleared slowly into the pharynx. The single drop spread more extensively than the spray, while the three drops were sufficient to cover most of the walls of the nasal cavity. Clearance was faster following administration of the drops. These factors have implications when designing dosage regimens for drugs administered by the intranasal route. The uptake of midazolam into the CSF after nasal administration were compared with two devices: nasal atomiser and nasal drops. Higher bioavailability of midazolam in the CSF was obtained with the nasal atomiser (Henry *et al.* 1998

as quoted by Dahlin, 2000:40). Enhancing the delivery of drugs into the CNS along the olfactory pathway is an interesting area of research for the future.

4.5 Conclusion

The results indicate that prochlorperazine is a potential candidate for delivering via the intranasal route. The results also suggest that nasal administration of drugs active in the CNS, with a low oral bioavailability, is an easy and workable alternative to administration by intravenous injection, which may enhance patient compliance. From the results it is evident that the nasal route is superior to the oral route and comparable to the intravenous route of administration in the amount of drug absorbed in plasma and brain tissue. The lipophilic compound prochlorperazine was rapidly and almost completely absorbed into systemic circulation after nasal administration. These molecules appeared rapidly in brain tissue. Although hard evidence of direct transfer from the nose remains elusive, the fact that a higher AUC-value was obtained after nasal than after intravenous administration was evidence enough that the olfactory route does contribute to the delivery of drugs to the brain after nasal administration.

In continuation of this study the following should be investigated:

- Comparison between the concentrations of prochlorperazine in the cerebrospinal fluid after intravenous and intranasal administration.
- Whether intranasal delivery of prochlorperazine in humans will have therapeutic plasma concentrations as a result.
- The size of doses necessary to reach therapeutic plasma concentrations in humans.
- Whether intranasal delivery of prochloperazine in humans will have an irritating effect on the nasal mucosa.

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19 Junie 2001

Geagte prof Muller

GOEDKEURING VIR EKSPERIMENTERING MET DIERE

Hiermee wens ek u in kennis te stel dat u projek getiteld *"Intranasal delivery of prochlorperazin"* deur die Etiekkomitee van hierdie universiteit goedgekeur is onder die nommer: 01D14.

Gebruik asseblief bogenoemde nommer in alle korrespondensie rakende bogenoemde projek en let daarop dat daar van projekleiers verwag word om jaarliks in Junie op die voorgeskrewe vorm (wat voorsien sal word) aan die Etiekkomitee verslag te doen insake etiese aspekte van hulle projekte asook van publikasies wat daaruit voortgespruit het.

Neem asseblief kennis dat die Proefdiersentrum jaarliks in Januarie 'n kursus in die hantering van proefdiere aanbied en dat die Etiekkomitee dit sterk aanbeveel dat alle gebruikers van proefdiere hierdie kursus moet deurloop. Proefdiere word slegs verskaf aan persone wat hierdie kursus suksesvol afgelê het en u word versoek om dit in ag te neem in die beplanning van u werksaamhede.

Goedkeuring van die Etiekkomitee is vir 'n termyn van hoogstens 5 jaar geldig (volgens Senaatsbesluit van 4 November 1992, art. 9.13.2). Vir die voortsetting van projekte na verstryking van hierdie tydperk moet opnuut goedkeuring gekry word.

Die Etiekkomitee wil u graag in u werksaamhede behulpsaam wees en wens u alle voorspoed met u navorsing toe.

Vriendelike groete

JC BREYTENBACH
Sekretaris: Etiekkomitee

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