CHAPTER II

LITERATURES REVIEW

1. Nasal Route of Drug Delivery

Conventionally, the nasal route is used for delivery of drugs to treat local diseases such as nasal allergy, nasal congestion and nasal infections. However, this route can also be exploited for systemic delivery of many drugs including peptides and proteins that are not easily administered by the oral route, or where a rapid onset of action is required. The nasal route could be important for drugs that are used in the crisis treatments such as pain and migraine, and for centrally acting drugs where the putative pathway from the nose to the brain might provide a faster and more specific therapeutic effect (Illum, 2003). The possibilities for the use of the nasal cavity for drug delivery are outlined in Table1.

Table 1 Possibilities for nasal delivery of drugs

Possibilities for nasal delivery	Symptoms or diseases
Local delivery:	Nasal allergy
	Nasal congestion
	Nasal infection
Systemic delivery	
Crisis treatment :	Acute pain
	Sleep induction
	Migraine
	Erectile dysfunction
	Heart attack
	Parkinson's disease
Long-term treatment :	Diabetes
	Growth deficiency
	Osteoporosis
	Endometriosis
Peptide/proteins:	Diabetes
	Osteoporosis
Vaccine Delivery:	Antigen (Whole cells, Surface antigen)
	DNA Vaccines
Access to CNS:	

The bioavailability of a drug and its therapeutic effectiveness are often influenced by the route selected for administration. For a medication to achieve its maximal efficacy, a drug should be able to be administered easily such that better patient compliance can be achieved. It should also be able to get absorbed efficiently to accomplish greater bioavailability. The nasal route appears to be an ideal alternative to the parenteral route for administering drugs intended for systemic effect in view of the rich vasculature of the nasal membranes and the ease of intranasal administration (Hussain, 1998).

Several advantages can be achieved from delivering drugs intranasally. First, there is an avoidance of hepatic "first pass" elimination as well as gut wall metabolism and destruction in the gastrointestinal lumen. Second, the rate and extent of absorption and the plasma concentration versus time profile, in many cases, are relatively comparable to that obtained by intravenous medication. Third, the nasal mucosa is rich in vasculature and highly permeable, thereby facilitating systemic drug absorption. These advantages have made the nasal mucosa a feasible and desirable site for systemic drug delivery (Behl et al., 1998).

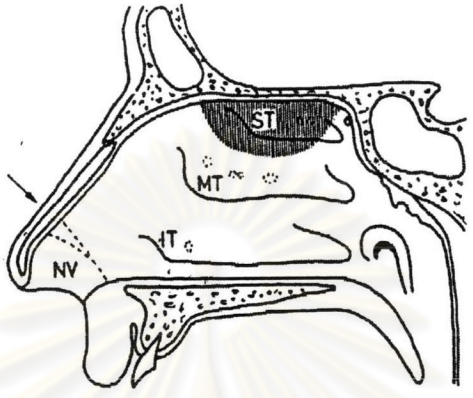
1.1 Anatomy of the Nose

In studying drug absorption from the nasal cavity, it is essential to have a clear understanding of the anatomy and physiology of the nose, and how it relates to the characteristics of the delivery system used. The human nose is characterized by an individually varying shape and caliber, which might interfere with the standard recommendations of intranasal medication.

The apparent external nose surrounds the nostrils and one-third of the nasal cavity, which in its entirety consists of a 5-cm high and 10-cm long dual chamber. The total surface area of both nasal chambers is about 150 cm^2 and the total volume about 15 mL. Approximately 1.5 cm from the nares is the narrowest portion of the entire airway, the internal ostium (or nasal valve), with a cross-sectional area of about 30 mm^2 on each side. The nasal valve accounts for approximately 50% of the total resistance to respiratory airflow from the nostril to the alveoli. Each of the two nasal cavities is limited by the septal wall and the lateral wall, and is also dominated by the inferior, middle and superior turbinates (Figure. 1 and 2).

The turbinates are important for maintaining the slit-like cavity, thus facilitating humidification and temperature regulation of the inspired air. Under and lateral to each of the turbinates are passages called the inferior, middle and superior

meatuses. The inferior and middle meatuses receive the openings of the nasolacrimal duct and the paranasal sinuses. The middle meatus is an important anatomical area in the pathophysiology of sinus disease. It has a complex anatomy of bones and mucosal folds, referred to as the ostiomeatal complex (Mygind and Dahl, 1998).



NV, nasal vestibule; IT, inferior turbinate and orifice of the nasolacrimal duct; MT, middle turbinate and orifices of frontal sinus, anterior ethmoidal sinuses and maxillary sinus; ST, superior turbinate

Figure 1 Diagram showing the lateral wall of the nasal cavity.

1.2 Nasal Passage

The upper respiratory tract is constantly influenced by the inspired air. The nasal modifications of the inspired air by filtration, humidification and warming are considered to be prime functions of the nose in man (Proctor, 1985). To carry out these functions, the nose must control the rate of airflow, remove any noxious agents, and introduce large quantities of fluid into the air stream.

The nasal passage, which runs from the nasal vestibule to the nasopharynx, has depth of approximately 12 – 14 cm. In this passage, the nasal epithelial cells are in close contact with the mucus, which protects the mucosa from the inspired air. There are three functional zones in the nasal cavities; namely, vestibular, respiratory, and olfactory areas, which are arranged anteroposteriorly in this sequence of order (Chien, 1989).

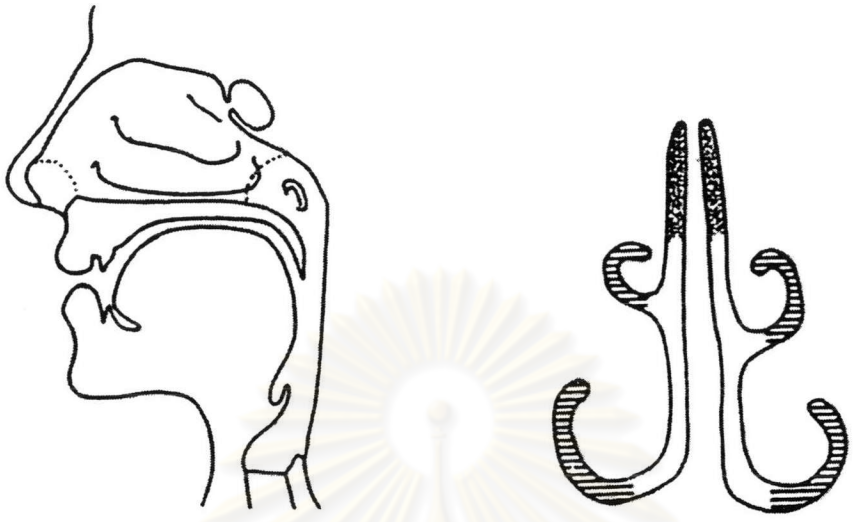


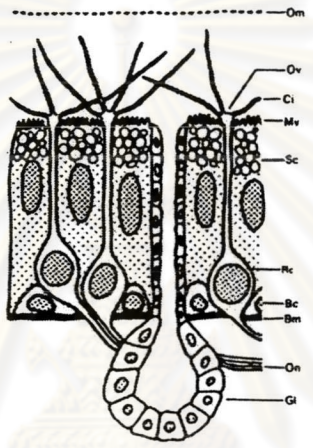
Figure 2 The upper airway seen from the midline (A) and section through the main nasal passage showing the nasal septum, folds of turbinates, and airway (B).

The vestibular area serves as a baffle system. The vestibule consists of the regions just inside the nostrils with an area of about 0.6 cm^2 interspersed with long hairs which help to filter out airborne particles. Its surface is covered with a common stratified squamous epithelium which gradually changes posteriorly into a pseudostratified columnar epithelium that covers the respiratory epithelium.

The respiratory epithelial cells are covered by microvilli and the major part of these cells is also covered with cilia. These cilia, which are long (4-6 μm) and thin projections, are mobile and beat with a frequency of 1,000 strokes per min. The beat of each cilium consists of a rapid forward movement, where the cilium is stretched and the tip of the cilium reaches into the mucus layer and carries this forward followed by a slow return beat, where the cilium is bent and moves in the sol layer that lies beneath the mucus layer. In this way the mucus layer is propelled in a direction from the anterior towards the posterior part of the nasal cavity. The mucus flow rate is in the order of 5 mm per min and hence the mucus layer is renewed every 15 – 20 min.

The olfactory region, a small patch of tissue containing the smell receptors, is located at the very top of the nasal cavity near the inner end of the upper throat. In man it covers about 10% of the total nasal area as opposed to, for example, in the dog where

the area constitutes 77% of the total nasal cavity. The olfactory area of about 10 cm^2 lies above the middle turbinate between the nasal septum and the lateral wall of the main nasal passage. The olfactory epithelium is pseudostratified columnar in type, and consists of specialized olfactory cells, as well as supporting cells and both the serous and mucous glands. The olfactory region consists of several million tiny ending of the olfactory nerves, whose bundles pass through the cabriform plate and enter the farthest forward extension of the brain. The olfactory cells are bipolar neurons and act as peripheral receptors and first order ganglion cells. A schematic diagram of the olfactory epithelium is shown in Figure 3.



Om = surface of the olfactory mucus, Ov = olfactory vesicle,
Ci = olfactory cilia, Mv = microvilli, Sc = supporting cell,
Rc = olfactory receptor cell, Bc = Basal cell,
Bm = basement membrane, On = olfactory nerve, Gl Olfactory gland.

Figure 3 Schematic diagram of olfactory epithelium.

1.3 Nasal Epithelium

The anterior one-third of the nasal cavity is covered by a squamous and transitional epithelium, the posterior upper part of the cavity by an olfactory epithelium whereas the remaining portion is covered by a typical airway epithelium, which is ciliated, pseudostratified and columnar in shape. The latter epithelium consists of the following major cell types as shown in Figure 4 (Mygind and Dahl, 1998).

Basal cells

Basal cells, which are progenitors of the other cell types, lie on the basement membrane and do not reach the airway lumen. They have an electron-dense cytoplasm and bundles of tonofilaments. Among their morphological specializations are

desmosomes, which mediate adhesion between adjacent cells, 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. This is supported by the fact that the columnar cells do not have hemidesmosomes and that they are attached to the basement membrane only by cell-adhesion molecules.

Columnar cells

Columnar cells (ciliated and non-ciliated) are related to neighboring cells by tight junctions apically and in the uppermost part, by interdigitations of cell membrane. Each columnar cell is covered by about 300 microvilli, which are uniformly distributed over the entire apical surface. These short and slender fingerlike cytoplasmic expansions increase the surface area of the epithelial cells, thus promoting the exchange processes across the epithelium. The microvilli also prevent drying of the surface by retaining moisture essential for the ciliary function. The distribution pattern of ciliated cells corresponds well with the map of nasal airflow, indicating that the density of the ciliated cells is inversely proportional to the linear velocity of the inspiratory air in the nasal cavity. Consequently, there are less cilia in the upper part of the nasal cavity than along the floor.

Goblet cells

Another cell type, characteristic of an airway epithelium, is the goblet cell. 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 (4,000–7,000 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 submucosal glands. Goblet cells probably respond to physical and chemical irritants in the microenvironments.

Basement membrane

The epithelium rests upon a layer of collagen fibrils, which in the light microscope is referred to as the "basement membrane". In the bronchi this membrane is thickened in chronic asthma, while in the nose it is thickened both in rhinitis and in symptom-free individuals.

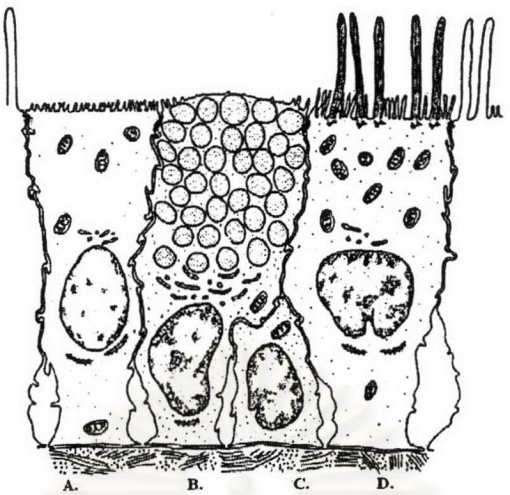


Figure 4 Microscopic diagram of the major cell types in the nasal airway epithelium: A. non-ciliated columnar cell with microvilli, B. goblet cell, C. Basal cell and D. ciliated columnar cell.

1.4 Nasal Drug Absorption

The general architecture and morphology of the human nasal cavity are shown in Figure1. The nasal absorption of drugs is considered mainly to take place in the respiratory region comprising the turbinates and part of the nasal septum.

As is the case for all biological membranes, drugs can cross the nasal mucosal membrane using two different pathways; transcellularly (across the cell) and paracellularly (between the cells). Lipophilic drugs are transported transcellularly by an efficient concentration-dependent passive diffusion process, by receptor or carrier mediation and by vesicular transport mechanisms. Polar drugs are believed to pass through the epithelium via the gaps or pores between the cells (the tight junctions). Although, the tight junctions are dynamic structures that can open and close to a certain extent, the size of these channels is less than 10 A^{o} (McMartin et al., 1987). The paracellular route will be less efficient for large molecules and is dependent upon the molecular weight of the drug with a general molecular size cut-off of ~1,000 Da.

The transport of polar drugs across the nasal mucosa can be associated with three major factors: (1) low membrane permeability, especially for the larger molecular weight drugs; (2) rapid clearance of the drug formulation from the nasal cavity as a result of the mucociliary clearance mechanism; and (3) a possible enzymatic degradation of the

drug in the nasal cavity. These factors could be important for the peptide and protein drugs (Illum, 2003).

Lipophilic drugs, such as propanolol, 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 an intravenous injection with bioavailabilities for some drugs approaching 100% (Illum, 2003). The nasal absorption of more polar compounds is poor, with bioavailabilities not exceeding 10% for small molecular weight drugs (e.g.morphine, sumatriptan) and less than 1% for peptides such as insulin, calcitonin and leuprolide.

1.5 The Site of Drug Deposition in the Nasal Cavity

Nasal deposition of particles is related to the individual subject's nasal resistance to airflow. The high linear velocity and the bend in the air stream in the anterior nares results in impaction of a large proportion of particles that are small enough to enter the nasal airway. With nasal breathing, nearly all the particles with an aerodynamic size of 10-20 μm are so deposited (Kublik and Vidgren, 1998). A significant fraction of the very small particles is also deposited in the nose, though many particles which are smaller than 2 μm pass with the inspired air into the lungs. The retaining capacity of the upper respiratory tract is 100% for particles with size larger than 10 μm, and approximately 80 % for particles of 5 μm. It drops progressively with further reduction in size and approaches zero for particles of 1-2 μm (Kublik and Vidgren, 1998). Insoluble particles, which are deposited in the main nasal passage, are likely to be carried back by the ciliary movement and dispatched to the stomach. If the drug is introduced as a vapor or a soluble particle, it may readily pass into the lining secretion and then be absorbed from there into the bloodstream.

The deposition of aerosols in the respiratory tract is a function of particle size and respiratory patterns. The aerosols are deposited mainly in the anterior and turbinate regions with little passing beyond the nasopharyngeal region. Spray droplets also deposited in spots of the middle and posterior portions of the turbinate region and this non-uniform deposition pattern may be correlated with the flow pattern (Cheng et al., 2001). The density, shape, and hygroscopicity of the particles and pathological conditions in the nasal passage will influence the deposition of particles, whereas the particle size distribution will determine the site of deposition and affect the subsequent biological response in experimental animal and man. A uniform distribution of particles

throughout the nasal mucosa could be achieved by delivering the particles in a form of fine spray using a pressurized gas propellant.

1.6 Factors Affecting Absorption of Drugs from the Nasal Mucosa

The factors affecting absorption of drug through the nasal mucosa can broadly be classified into three categories as shown in Table 2.

Table 2 Variable factors affecting the absorption of drugs through the nasal mucosa

Physiological Factors • Blood supply and neuronal regulation • Nasal secretions • Nasal cycle • pH of the nasal cavity • Mucociliary clearance and ciliary beat frequency • Pathological conditions • Environmental factors ;Temperature Humidity
Physicochemical Factors Physicochemical properties of drug • Molecular weight • Size • Solubility • Lipophilicity • pK_a and partition coefficient Physicochemical properties of formulation • pH and mucosal irritancy • Osmolarity • Viscosity/Density • Drug concentration • Dose and Volume of administration
Dosage Form and Device-related Factors • Particle size of the droplet/powder • Site and pattern of disposition

1.6.1 Physiological Factors

There are several factors, which have an effect on nasal absorption. Some of the anatomical factors, such as nasal length, the bend from the nostrils into the cavity and structure of the turbinates, can directly be related to deposition in the nasal cavity. Nasal deposition depends on the size and shape of the nose (Kublik and Vidgren, 1998). It can also be correlated to body length and weight and increases with decreasing age for a given particle size and flow rate. Absorption by the nasal route is facilitated by a highly vascularized epithelial layer with a good blood flow, the presence of venous sinusoids and arteriovenous anastomosis give the nasal mucosa being a high permeable site. The nasal cycle of congestion (increased blood supply resulting from parasympathetic stimulation) and relaxation (decreased blood supply resulting from sympathetic stimulation) regulate the rise and fall in amounts of drug permeated, respectively (Revington, 1997).

Nasal secretions

Anterior serous and seromucus glands are responsible for the production of nasal secretions. Approximately 1.5–2l mL of mucus is produced daily. The mucus layer probably exists as a double layer (5μm thick) consisting of periciliary sol phase in which the cilia beat and a superficial blanket of gel is moved forwards by the tip of the cilia. The absorption of drug through the nasal mucosa is affected by viscosity of nasal secretion. If the sol layer of mucus is too thin, the viscous surface layer will inhibit the ciliary's beating, and if the sol layer is too thick, mucociliary is impaired because contact with cilia is loss (Marttin et al., 1998). Drug needs to be solubilized before it permeates, in addition to almost of nasal secretion contain water. Aqueous solubility of drug is always a limitation for nasal drug delivery in solution. Thus, a drug needs to have appropriate physiochemical characteristics of dissolution in nasal secretions.

pH of nasal cavity

The pH of the human nasal cavity varies between 5.5–6.5 in adults and 5.0–7.0 in infants (Washington,2000). Greater drug permeation is usually achieved at a nasal pH that is lower than the drug's pK_a because under such conditions the penetrated molecules exist as unionized species (Huang et al., 1985). A change in the pH of mucus can affect the ionization and thus increase or decrease the permeation of drug, depending on the nature of the drug. Because the pH of the nasal cavity can alter the pH of the formulation or vice-versa, the ideal pH of a formulation should be within 4.5–6.5 and if possible the formulation should also have buffering capacity.

Nasal mucous and mucociliary clearance

Airway mucous is composed of water (95%), mucous glycoproteins (2%), other proteins including albumin, immumoglobulins, lysozyme and lactoferin (1%) and lipid (<1%) (Kaliner et al., 1984). The submucosal glands are the main source of mucous glycoproteins that provide mucous with its characteristic viscoelastic properties. The mucus glycoproteins consist of a protein core (20%) with oligosaccharide side chains (80%), cross-linked by disulphide and hydrogen bonds. Mucous can cross-link and produce a viscoelastic gel and can form a mechanical coupling with cilia and be transported by them. Nasal secretions have a considerably lower viscosity than tracheobronchial secretions, but a comparable elasticity, and elasticity is more important than viscosity for mucus transport.

Nasal mucociliary clearance is one of the most important physiological defense mechanisms of the respiratory tract to protect the body against any noxious material inhaled. The nasal mucocilliary clearance system transports the mucus layer that covers the nasal epithelium towards the nasopharynx by cilliary beating (Marttin et al., 1998). There are approximately five ciliated cells for each mucus cell, with the average of 200 cilia extending from every ciliated cell on the surface of psuedostratified columnar epithelium. The cilia help to remove foreign substances by transporting them posteriorly towards the nasopharynx, where they are swallowed into the stomach. The cilia also transport some materials anteriorly, which are subsequently removed by nose blowing or wiping. Therefore, any nasal drug delivery systems must be critically evaluated for its possible effects on the nasal mucociliary functions.

Whenever a substance is nasally administered, it is cleared from the nasal cavity in ~21 min (Marttin et al., 1998) by mucociliary clearance. Reduced mucociliary clearance increases the time of contact between a drug and the mucus membrane and subsequently enhances drug permeation; whereas, increased mucociliary clearance decreases drug permeation. Some drugs, hormonal changes of the body, pathological conditions, environmental conditions and formulation factors are reported to affect the mucociliary clearance and in turn exert significant influence on drug permeability (Schipper, 1991).

Pathological conditions

Diseases such as the common cold, rhinitis, atropic rhinitis and nasal polyposis are usually associated with mucociliary dysfuctioning, hypo or hypersecretions, and irritation of the nasal mucosa, which can influence drug absorption. In people

suffering from severe nasal allergies, an excessive nasal secretion can wash away the formulation before the drug has a chance to absorb through the mucosa or before acting locally (Proctor, 1985).

1.6.2 Physicochemical Factors

Specific types of dosage forms which are used to deliver formulations into the nose are important in determining the nasal absorption profiles of drugs. Choice of a certain dosage form generally depends on the physicochemical properties of the drug, the therapeutic aims, the basic compliance of the patients and marketing issues, the most suitable delivery system and formulation strategy have to be chosen. The basic formulations, i.e. solution, suspension, emulsion and dry powder systems must be considered for suitable delivery system (Chien and Chang, 1987).

Most of the nasal preparations containing solutions or suspensions are delivered by meter- dose pump sprays, which are more sophisticated drug delivery device than those in the early day. However, liquid preparations are the most widely used dosage forms for nasal administration. They are mainly on aqueous formulations. The site of deposition and deposition pattern of nasally applied liquid formulations is dependent on the delivery device, the mode of administration and the physico-chemical characteristics of formulation.

1.6.2.1 Physicochemical properties of the drug

Molecular weight and size

Molecular weight, lipophilicity and hydrophilicity act together to determine drug absorption. A large number of therapeutic agents, peptides and proteins in particular, have shown that for compounds >1 kDa, bioavailability can be directly predicted from knowledge of MW. In general, the bioavailability of these large molecules ranges from 0.5% to 5% (Donovan and Huang, 1998). In the case of lipophilic compounds, a direct relationship exists between the MW and drug permeation whereas water-soluble compounds depict an inverse relationship. Based on the reports by Fisher et al. and McMartin et al. it can be concluded that the permeation of drugs less than 300 Da is not significantly influenced by the physicochemical properties of the drug, which will mostly permeate through aqueous channels of the membrane. By contrast, the rate of permeation is highly sensitive to molecular size for compounds with MW >300 Da.

Solubility

Drug solubility is a major factor in determining absorption of drug through biological membranes. It not only limits the drug absorption rate, it can also limit a formulator's ability to formulate a product if the drug is not sufficiently soluble in the desired vehicles (Behl, 1998). As nasal secretions are more watery in nature, a drug should have appropriate aqueous solubility for increased dissolution.

Lipophilicity

On increasing lipophilicity, the permeation of the compound normally increases through nasal mucosa. In a study conducted to characterize the barrier properties of mucosal membranes, it was found that the nasal mucosa had the highest *in vitro* transport both of the model hydrophilic compound, mannitol, and the model lipophilic compound, progesterone. Although the nasal mucosa was found to have some hydrophilic character, it appears that these mucosae are primarily lipophilic in nature and the lipid domain plays an important role in the barrier function of these membranes (Corbo, 1990). However, excess hydrophilicity is also known to decrease the systemic bioavailability of many drugs (Corbo, 1989).

Partition coefficient and pK_a

As the pH partition theory, unionized species are absorbed better compared with ionized species and the same holds true in the case of nasal absorption. The nasal absorption of weak electrolytes such as salicylic acid and aminopyrine was found to be highly dependent on their degree of ionization. Although for aminopyrine, the absorption rate increased with the increase in pH and was found to fit well to the theoretical profile, substantial deviations were observed with salicylic acid. The authors concluded that perhaps a different transport pathway, along with the lipoidal pathway, existed for salicylic acid (Hirai, 1981). Similarly, when the absorption of benzoic acid was studied at pH 7.19 (99.9% of the drug existed in ionized form) it was found that >10% of drug was absorbed indicating that the ionized species also permeates through nasal mucosa (Huang, 1985). Based on all of the observations, partition coefficients were discounted as a major factor effecting nasal absorption.

1.6.2.2 Physicochemical properties of the formulation

pH and mucosal irritancy

The pH of the formulation, as well as that of nasal surface, can affect a drug's permeation. To avoid nasal irritation, the pH of the nasal

formulation should be adjusted to 4.5–6.5 (Behl, 1998). In addition to avoid the irritation, it results in obtaining efficient drug permeation and prevents the growth of bacteria.

Osmolarity

Ohwaki et al. (1985) studied the effect of osmolarity on the absorption of secretin in rats and found that absorption reached a maximum at a sodium chloride concentration of 0.462 Osmol, because shrinkage of the nasal epithelial mucosa was observed at this salt concentration. Generally, an isotonic formulation is preferred (Behl, 1998).

Viscosity

A higher viscosity of the formulation increases contact time between the drug and the nasal mucosa thereby increasing the time for permeation. At the same time, highly viscous formulations interfere with the normal functions like ciliary beating or mucociliary clearance and thus alter the permeability of drugs.

Drug concentration, dose and volume of administration

Drug concentration effects on nasal absorption are actually revealing, if the primary mechanism of absorption was passive there should be a clear positive relationship between absorption and drug concentration. However, it may not necessarily mean that nasal absorption dose not occur via passive mechanism. There are many other confounding factors which can influence the nasal membrane transport mechanism. Several studies have reported the effect of drug dose on nasal absorption, e.g. calcitonin (Thamsborg et al., 1990) and secretin (Ohwaki et al., 1985). In general, higher nasal absorption or therapeutic effect was observed with increasing dose. The volume that can be delivered to the nasal cavity is restricted to 0.05–0.15 mL with an upper limit of 0.20 mL. Different approaches have been explored to use this volume effectively including the use of solubilizers, gelling, or viscosity enhancing agents.

1.6.3 Dosage Form and Device-related Factors

There are several types of drug delivery systems which have long been used for the delivery of drugs to the nasal cavity, such as nasal spray, nose drops, saturated cotton pledget, aerosol spray, and insufflator (Proctor, 1985). Nasal drops are the simplest and most convenient dosage form but the exact amount that can be delivered cannot be easily quantified and often results in overdose. Moreover, rapid nasal drainage is a problem with drops. Solution and suspension sprays are preferred over powder sprays because powder results in mucosal irritation (Behl, 1998).

Recently, metered-dose devices have been developed that accurately deliver drug and used as the nasal delivery system for several topical drugs, such as corticosteroid, nasal decongestant and systemic delivery. They allow the application of a defined dose with a high dosing accuracy and a typical spray pattern. Dose volumes between 25 and 200 μL are available as standard. Spray characteristics vary according to the pre-compression mechanism, the type of selected pump and valve and the physical properties of the product (Kublik and Vidgren, 1998). Viscosity, thixotropic behavior, elasticity and surface tension of the liquid determine the spray pattern, the particle size of the drops, the dose and the dosing accuracy. The form of the actuator has to be adapted to the required use. For correct dosing the tip should avoid the collection of residual drops. The length of the actuator has an influence on deposition of the application into the nose. Special adaptors for children are available on the market. For safety reasons a captive insert is fitted into the actuator in order to avoid risks of liquid ejection without actuation, to decrease the dead volume in the actuator and to reduce contamination.

Particle size of the droplet

The particle size of the droplet produced depends on the shape and size of the device used. If the particle size produced is <10 μm, then particles will be deposited in the upper respiratory tract, whereas if particle size is <0.5 μm then it will be exhaled. Particles or droplets with size between 10-50 μm will be retained in the nasal cavity and subsequently permeated (Donovan and Huang, 1998). Numerous methods for sizing particles have been developed in the past. The most common ones are microscopy, light scattering, laser holography, and the cascade impactor method.

Site and pattern of deposition

The site and pattern of deposition is affected by formulation composition, the physical form of the formulation (liquid, viscous, semisolid, solid), the device used, the design of actuators and adapters, and administration technique (Kublik and Vidgren, 1998). The permeability of the site at which the formulation is deposited and the area of nasal cavity exposed affects the absorption of drugs. Dependent on the product delivered with respect to its rheological behavior, surface tension and required spray characteristics, the geometry and dimensions of the pump mechanism can be adapted to fulfill these demands. Special geometries of the orifice determine the spray pattern and distribution. The cone angle varies between 35° and 90°. A small cone angle

results in a more posterior deposition, while the wider angle shows a broad distribution in the anterior part of the nasal cavity (Newman et al., 1987).

2. Calcitonin

2.1 Biochemistry of Calcitonin

Calcitonin (CT) is a polypeptide hormone comprised of 32 amino acids. It is synthesized and secreted by the parafollicular cells (C cell) in the thyroid gland of mammals and by the ultimobranchial gland in other vertebrates like salmons and eels (Deftos, 1981; Stevenson and Evans, 1981). The basic structure of the CTs is characterized by a disulfide bridge between the cysteine residues at position 1 and 7 and a proline amide moiety at carboxyl terminal end.

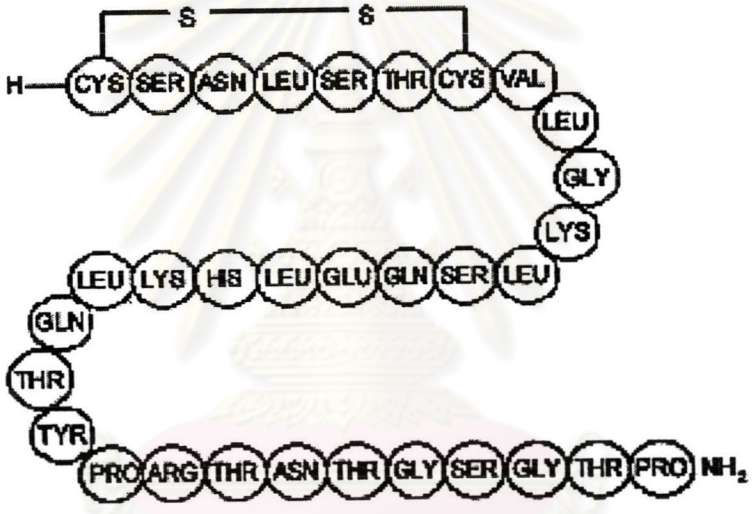


Figure 5 Amino acid sequence of salmon calcitonin

The amino acid sequence of CT has been determined for many species. Figure 5 shows the amino acid sequence of salmon calcitonin (salmon CT), one of the most potent and commonly used forms of CTs. However, all types of CTs have identical specific amino acid residues. Studies indicated that this similarity is vital for their biological activity rather than the species from which they are naturally produced (Stevenson and Evans, 1981; Singer et al., 1972; and Fischer et al., 1983).

CTs derived from the ultimobranchial bodies of salmons and eels are more potent than mammalian thyroidal CTs both *in vitro* and *in vivo*, and they differ from the human hormone by 13 and 16 amino acid residues, respectively (Figure 6). Therapeutically, salmon calcitonin appears to be more potent than other CTs, in part because it is cleared more slowly from the circulation (Stevenson and Evan, 1981).

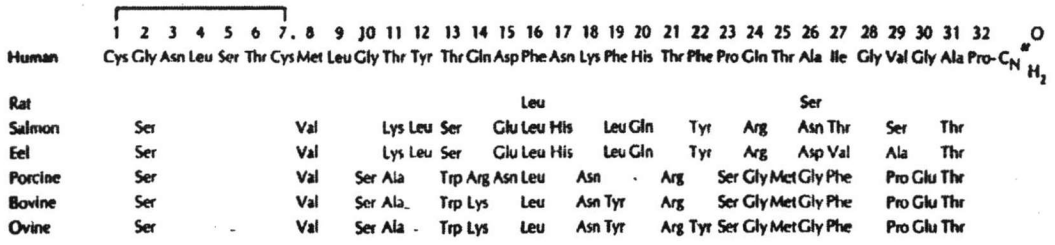
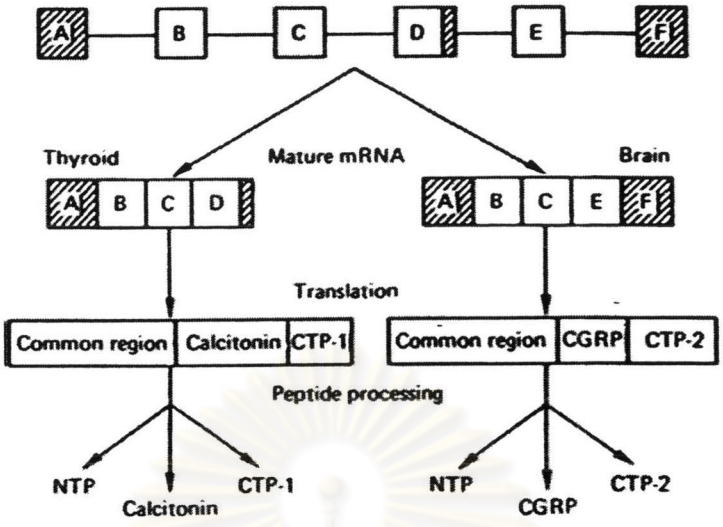


Figure 6 Comparison of primary structures of salmon and other CTs

2.2 Biosynthesis of Calcitonin

In vitro studies with fish and chicken ultimobronchial bodies suggested that CT was synthesized as a larger precurcor with a MW of ~15000. In mammals, information about the biosynthesis of calcitonin was derived from studies of CT mRNA isolated from rat thyroid tissue. CT has a particularly complex gene structure. The gene is composed of six exons and five introns, and there is evidence that different tissues which express this gene, there is differential splicing of the transcribed gene to produce different peptide (Figure 7). In the thyroid, the major product of the CT gene is CT, where as in the brain the major product is the CT gene related peptide (CGRP). CT is secreted as a larger precursor called procalcitonin. This is of 17,500 Da in man, and it contains a classical leader sequence. The 32 amino acid secreted peptide has a proline-amide group at C-terminus, which made the chemical synthesis of biological active CT extremely difficult. There is also a disulfide bridge at the N-terminal end of the molecules, between amino acids 1 and 7. Both the disulfide bridge and the proline-amide group are necessary for biological activity (Mundy, 1990).



A, B, C, D, E and F represent exons. Exons which are non-coding are hatched.
NTP = N-terminal peptide, CTP = C terminal peptide

Figure 7 Structure of the Calcitonin gene.

As shown in Figure 7, this gene can undergo alternative tissue-specific ribonucleic acid (mRNA) processing. In the thyroid, CT is preferentially expressed whereas in brain, CGRP is preferably expressed.

2.3 Regulation of Secretion

Calcium is a major physiologic regulator of CT secretion in normal man (Austin, 1979). The secretion of CT is regulated by the concentration of calcium in plasma. When plasma calcium is high, CT secretion increases; when plasma calcium is low, CT secretion low or undetectable. Normal circulating CT concentrations in human beings extremely low as 5 – 50 pg/mL (Heath and Sizemore, 1977; Pathermore and Deftos, 1978). Mean concentrations of CT in woman (25 -73pg/mL) are lower than those in men (25–51pg/mL). The circulating half-life of CT is about 10 minutes (Body and Heath, 1983). CT secretion can be stimulated by a number of agents, including catecholamines, glucagons, gastrin, and cholecystokinin, but there is little evidence about the physiological role of such secretion in response to these stimuli.

2.4 Metabolism and Excretion

The half-life of human and porcine CT has been determined to be less than 15 min. The Metabolic clearance of human CT in normal subjects is approximately 8 mL/kg per min, and is less in renal insufficiency. Following injection of a labeled hormone, the

two organs which concentrate to hormone to the greater extent are the liver and kidney. In the human, dog and rat, the kidney appears to be the principal site of binding and degradation of CT (Beaulieu and Kelly, 1990).

2.5 Mechanism of Action

CT acts by inhibiting bone resorption. A direct action of CT on bone was also demonstrated by perfusion studies in rats, in which there was a marked reduction in urinary hydroxyproline (Martin et al., 1966). These results indicated that CT acts by inhibiting bone resorption, thereby decreasing the circulation levels of the products of resorption, i.e., calcium, phosphate and hydroxyproline-containing peptide. It was subsequently established that this inhibition of bone resorption was brought about by an inhibitory effect of CT on osteoclast activity and, in long term, a reduction in osteoclast numbers.

Specific receptors for CT have been identified in plasma membrane of kidney and bone cells (Beaulieu and Kelly, 1990). Although contradictory results have been reported that CT resulted in a decrease in intracellular calcium concentrations, which may be responsible for different effects on bone and kidney. In these two organs CT increases cAMP levels (Figure 8). CT interacts with a plasma membrane receptor, activates adenylate cyclase, and decreases calcium concentration in the cytoplasm.

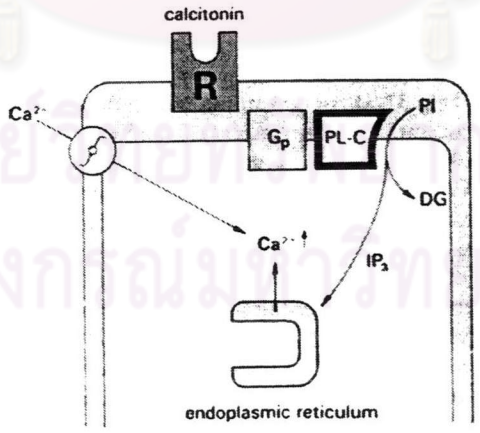


Figure 8. Mechanism of the cellular action of calcitonin.

2.6 Physiological Effects of CT

Effect on Bone

The calcium lowering effect of CT is not seen in normal adults because in this situation the rate of bone turnover is relatively slow. Even pharmacological doses of calcitonin cause only transient fall in serum calcium in normal adults. However, when bone turnover is high, such as Paget's disease of bone, CT administration is followed by sharp decrease in circulating calcium levels (Stevenson and Evans, 1981).

Effect on Gut and Kidney

CT also has effects on the gut and the kidney. It leads to the extraction of calcium and phosphate both in the urine and in the feces. It is unlikely that the effects on gut and kidney are of major importance in the maintenance of calcium homeostasis. However, it is possible that the initial calcium lowering effect of CT administration could be due to inhibition of renal tubular calcium reabsorption, working in combination with inhibition of bone resorption (Mundy, 1990).

2.7 Therapeutic Uses of CT

CT was introduced for the treatment of disorders of bone and mineral metabolism, principally owing to its inhibitory effect on osteoclastic bone resorption. Plasma calcium is reduced, and this is accompanied by an equally transient increase in plasma PTH (Burckhardt, 1973). CT administered intravenously, particularly synthetic salmon or human CT, can be used in the treatment of hypercalcemia. It has been shown to be useful in the treatment of hyperthyroidism, Paget's disease (González, 1987).

Salmon CT has been approved by the United States FDA for treatment of Paget's diseases of bone, hypocalcemia and post-menopausal osteoporotic syndromes. Since the discovery of this hormone in 1961, it has also been used to effectively reverse the bone loss observed in immobilized paraplegic patients as well as in patients treated with glucocorticoid medications and in malignant diseases such as multiple myeloma (Rico, 1992).

Salmon CT Therapy in Osteoporosis

CT has been evaluated in therapeutic trials in osteoporosis women in both the United States and abroad for more than 20 years. Reports of therapeutic responses vary from those demonstrating simple suppression of annual bone loss rates, as observed in the past with estrogen treatment, to others which reveal striking dose-related increments in bone mass of the vertebral and long bones (MacIntyre et al., 1988; Overgaard et al., 1989).

Several prospective, controlled trials have reported that calcitonin stabilizes and in some cases produces modest short-term increases in mineral density in trabecular bone, but not in cortical bone, even in osteoporotic patients treated for 5 years or less (Gruber, 1984; Mazzuoli, 1986; and Overgaard, 1995). Similar results were obtained with the use of salmon calcitonin to prevent menopausal trabecular bone loss (Reginster, 1993 and McDermott, 1987) as well as a relief of bone pain syndrome (Lyritis et al., 1991). Other reports of CT trials claim a decrease in vertebral fracture rates (Overgaard et al., 1992).

The skeletal response to CT therapy is related to the rate of bone turnover. There are at least two histological variants of postmenopausal osteoporosis, i.e., high bone turnover and low/normal turnover forms, which are also known as the active and inactive forms, respectively. In high turnover or active osteoporosis, a bone biopsy specimen will demonstrate an abundance of bone cells, i.e. osteoblasts and osteoclasts (Whyte et al., 1982). Patients with this form of postmenopausal osteoporosis respond rapidly and very well to CT treatment (Overgaard et al., 1989 and 1994).

Osteoporotic patients treated with CT give a variable response similar to those treated with either calcium or estrogens or both. Some osteoporotic patients on daily CT treatment regimens respond within one year with a substantial increase in bone mass and no progressive deteriorating anatomical vertebral changes (Mazzuoli et al., 1986; Alvioli, 1996).

Although the skeletal responses to either injectable or nasal spray form of salmon CT treatment is dose related (Overgaard and Christiansen, 1991; Thamsborg et al., 1993), osteoporotic patients with skeletal pain should be treated with 50-100 units of salmon CT subcutaneous daily. The greatest effect of nasal spray form is observed at a dose of 200 IU daily in women more than five years post-menopausal (Overgaard, 1994). Beneficial effects have also been obtained using 50 units of nasal spray on alternate days for two weeks of every month (Szucs et al., 1992) or 100 units of spray consecutive days of each month (Rico et al., 1990). Nasal spray doses of 200-400 IU in early postmenopausal period can decrease vertebral bone loss during the first year treatment, in addition to decreasing the bone turnover rate (Overgaard, 1994, 1995).

As a potent anti-osteoclastic drug, CT appears relatively innocuous as compared to the potential hazards of pharmacological doses of bisphosphanates, estrogens, androgens, sodium fluoride or non-steroid anti-inflammatory agents, especially in elderly postmenopausal osteoporotic women (Wimalawansa, 1993). Nasal spray forms

are also more appropriate for the elderly who, because of multi-drug regimens, are often unable to follow their prescribed dosages in rigid fashion, which is essential in some therapies, e.g., bisphosphanate and alendronate.

2.8 Permeation of CT Across Nasal Mucosa

In general, the permeability of the nasal mucosa to peptides falls off sharply with increasing molecular weight. For peptide with more than 10 amino acids bioavailabilities are in 1-3% range. Despite its 32 amino acid size, the bioavailability of intranasal salmon CT has been reported to be 40% relative to intramuscular administration. Other study by Lee et al. (1994) suggested that the average bioavailability of intranasal salmon CT was 1.6%.

Lang et al. (1998) studied the in vitro permeation of human CT in comparison with salmon CT and octreotide through excised bovine nasal mucosa applying confocal laser scanning microscopy. They reported the relatively high permeability of human CT and salmon CT, which was in contrast to their high molecular weight, and the apparent permeability coefficients (P_{eff}) of both CTs were found to be similar. The endocytic pathway is suggested to contribute to the transport of CT through the nasal epithelium.

2.9 Side Effects of Salmon CT

Nausea and mild gastric discomfort characteristically manifest at the initiation of subcutaneous therapy and either decrease in severity or disappear completely during the therapeutic interval (Gennari, 1983). Facial flushing, dermatologic hypersensitivity and local pruritic reaction at injection sites are usually mild. They also tend to disappear with continued administration of the drug. Since salmon CT is a potent inhibitor for free acid production, gastrointestinal side effects such as bloating or mild epigastric fullness can be minimized if the drug is administered 4-5 hours following the evening meal, i.e., preferably at bedtime (Banga and Chien,1988). Only rarely are urinary frequency and diarrhea encountered with the injectable forms of salmon CT.

As expected, the side effects of therapy are more severe when salmon CT is administered intramuscularly and minimized when given subcutaneously because of higher peak blood levels achieved following intramuscular injections. The side effects usually observed with the injectable forms of salmon CT are allayed considerably when it is administered in the nasal spray form (Overgaard et al., 1989; Wimalawansa, 1993). Considerable experience has also been accumulated for at least 20 years in treating patients with Paget's disease, which characteristically occurs in the elderly or osteoporotic disorders. Many of these patients are on multiple drug regimens for

cardiovascular diseases, gout, diabetes, rheumatoid arthritis and osteoarthritis. The absence of any documented adverse drug interactions reported for this relatively older population when treated with CT is also gratifying.

2.10 Physicochemical Characteristics of Salmon CT

The potency of salmon CT based on the hypocalcemic effect in rats, is expressed in International Unit (IU), as defined by the International Standard for Salmon CT established by the World Health Organization (WHO) in 1974 (Rafferty, Corran and Bristow, 2001). By convention, European Pharmacopoeia (2002) and British pharmacopoeia (2002) state that 1 mg of salmon CT is equivalent to 6000 IU of biological activity. A polypeptide with the same 32 amino acids sequence as salmon CT has been synthesized and is commercially available in a sterile and lyophilized form. Some physicochemical characteristics of the commercial synthetic salmon CT are as follows:

Amino acid sequence : H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-thr-Tyr-Pro-Arg-thr Asn-Thr-Asn_Thr-Gly_Ser-Gly-Thr-Pro-NH2, acetate salt

Description : White fluffy powder; lyophilized

Molecular formula : $C_{145}\ H_{240}N_{44}\ O_{48}\ S_2$

Molecular weight : 3431.9 g/mol

Solubility : very soluble in water (>100mg/mL); slightly soluble in alcohol and insoluble in chloroform, ether.

The salmon CT has an isoelectric point (PI) of 10.4, so that it would exist as a cation at lower pH. A pH range of 3.5 -3.9 was chosen to make a nasal formulation inorder to sufficiently apart from the PI.

2.11 Stability of Salmon Calcitonin and Degradation Mechanisms

Like many proteins and peptides, the therapeutic use of calcitonin is hampered by its physical instability (Cholewinski, Luckel and Horns, 1996). Extensive research has been performed on stabilization of calcitonin in aqueous solutions (Chien, 1991, Lee et al., 1992, and Windisch et al., 1997). The stability of salmon calcitonin in solution was evaluated by using HPLC (Lee et al., 1992). It is well-known that calcitonin is unstable upon incubation in aqueous solution. Cholewinsky (1996) have described several degradation pathways of calcitonin in aqueous solutions, the proteolytic degradation pathways as shown in Table 3.

The degradation rate profile for sCT in aqueous solution has previously been determined, and it was shown that sCT exhibits a first-order overall degradation in the pH range from 2 to 8 (Chien, 1991, Lee et al., 1992, Windisch et al., 1997). sCT shows greatest stability between pH 3 and 3.5. The main degradation products of sCT in the pH range from 2–6 has been identified (Lee et al., 1992, Windisch et al., 1997). At higher pH, degradation pathways mainly involve dimers and polymerisation (Cholewinski, 1996, Windisch et al., 1997).Possible degradation reactions are as follows:

- Fragmentation. At the C-terminal the molecule contains no crucial sequence such as Arg-Pro-NH_2, Asp-Pro-NH_2, Asp-Ser-NH_2 or Asp-Gly-NH_2, which are known to undergo fragmentation especially at basic pH. Therefore, no fragmentation is observed if the molecule is stored at pH 7 and at room temperature (Cholewinski, Luckel and Horns, 1996).

- Dimerization, polymerization, reduction. As already mentioned, CT contains a (S-S) disulfide bridge. At high pH values there is a β-elimination even at room temperature, causing the resulting free thiol groups to accomplish intermolecular arrangement at a fast reaction. As a consequence, the peptide forms dimers and polymers. Disulfides can be reduced to give the free thiols of the two cysteine residues. Concerning CTs this means that there is a linear molecule in which the two cysteine residues are bearing free thiols. According to an investigation carried out under stress condition (pH 2.2 for 3 hours at 70 °C), this reduced CT degradation product detected by HPLC was observed in considerable amount, up to 30 – 40% (Lee et al., 1992).

- Deamidation. Both salmon and human CTs contain two Asn and two Gln residues, which can be deaminated via a cyclic intermediate. Both peptides contain Asn in position 3 as well as in position 26 for salmon and 17 for human. The Asn residue in position 3 is sterically protected, because it is situated in the loop formed by the disulfide bridge of the two cysteines in positions 1 and 7. Should deamidation take place, which is unlikely, it would probably occur in position 26 for salmon CT or 17 for human CT (Lee et al., 1992; Windich et al., 1996).

- Aggregation. Wide range of proteins and peptides form fibrils in aqueous solutions, e.g., insulin, glucagons. The same phenomenon was observed for CTs. Salmon CT has a stronger tendency than human CT to aggregate in aqueous solution to form long, thin, fibrillar aggregates resulting in viscous and turbid dispersion (Cholewinski, Luckel and Horns, 1996).

Table 3 Degradation mechanisms for peptides and proteins in general and their relevance for CT (Cholewinski, Luckel and Horns, 1996).

Degradation route	Region of concern	Factors affecting the degradation
Deamination	Asn, Gln	pH, buffer species, ionic strength, temperature
Disulfide-scrambling/ oligomerization	Cys	pH (base), buffer species, metal ions, temperature, thiol scavengers
β-elimination	Cys	pH (base), buffer species, temperature, oxidizing agents
Physical instability	Whole molecule	pH, buffer species, ionic strength, temperature, cations, shaking, shear, hydrophobic surfaces

3. Possibility and Benefits of Local Manufacture of Salmon CT

At present, Thailand imports the preparations of synthetic salmon CT in two forms, i.e., injectable and nasal spray products. The former is available for subcutaneous or intramuscular administration. The latter or the nasal spray form is more convenient to use but is also more costly. Currently, the retail price for a bottle of 200 IU nasal spray costs about 3,400 baht whereas the injection (50 IU/mL) costs about 250 baht per vial. The exceedingly high cost of imported salmon CT nasal sprays resulted in a limited use of this product in many patients, especially those with post-menopausal osteoporosis, despite its many therapeutic benefits and ease of use. It is thus very important to encourage the local production of salmon CT preparations in Thailand, which is expected to help decrease the nation's import expenses as well as the cost of the healthcare treatment, particularly for patients under the 30 baht treatment program.

Consequently, the possibility of developing a salmon CT nasal spray for local manufacture has been explored in this thesis, with the objectives to evaluate the product's *in vitro* stability, both short and long terms, as well as its bioequivalence with respect to the innovator's product. The ultimate goal of the project is thus to develop a more economical, generic version of salmon CT nasal spray, which has acceptable stability and bioequivalence in order to promote its clinical use in Thai patients.

4. Assessment of Salmon CT Absorption after Nasal Administration

In order to evaluate the bioequivalence of the generic nasal spray of salmon CT, a sensitive technique to measure its concentration in plasma must be available. The early technique of assessing salmon CT systemic absorption relies on the hypocalcemic effect produced. Serum calcium was determined by atomic absorption spectroscopy and colorimetry, e.g. by forming a colored complex with O-phenolphthalein complexone. Newer techniques involve biological assays, which can directly measure the concentration of peptide in question with greater sensitivity and accuracy.

4.1 Radioimmunoassay

Radioimmunoassay (RIA) has brought great advance for the bioassay of CT with respect to ease, precision and sensitivity. The availability of synthetic salmon CT permitted the development of several homologous CT radioimmunoassay with high specificity and sensitivity, and this method has greatly advanced the knowledge of CT physiology and pathophysiology. The newer assays can detect CT in unextracted plasma at concentrations as low as 10 to 25 pg per mL, and clearly can measure the hormone in the blood of normal person (Austin and Heath, 1981).

4.2 Principles of Radioimmunoassay

Radioimmunoassay (RIA) is an extremely sensitive and quantitative, capable of measuring pictogram quantities or less, depending on the substance being assay. As a result, it is often used to measure the quantities of hormone or drugs resent in a patient's serum. The technique was introduced in 1960 by Berson and Yallow as an assay for the concentration of insulin in plasma. It represented the first time that hormone levels in the blood could be detected by an in vitro assay (Richard and Robert, 1987). This assay is based upon the ability of limited quantity of antibody to bind a fixed amount of radiolabeled e.g. Iodine 125 (^{125}I). It is assumed that both the labeled and unlabeled antigen have the same affinity for the antibody resulting in competitive binding between the two species. Therefore, the extent of the binding of radiolabeled antigen to antibody will depend on the amount of both label and unlabeled antigens. As the quantity of unlabeled antigen (standard or unknown sample) in reaction is increased, the amount of labeled antigen (^{125}I-peptide) able to bind to the antibody is decreased. The radioactivity is detected by a gamma counter.

Separation of the bound and free radiolabeled antigen is necessary in order to determine the quantity of the unlabeled antigen. This can be achieved by precipitating the antigen-antibody complexes by adding a second antibody directed toward the

immunoglobulin present in the original antiserum (Figure 9). The quantity of unlabeled antigen in an unknown sample is determined by comparing the radioactivity of precipitate, after centrifugation, with the value established using known standards in the same system (Ralph, 1988).

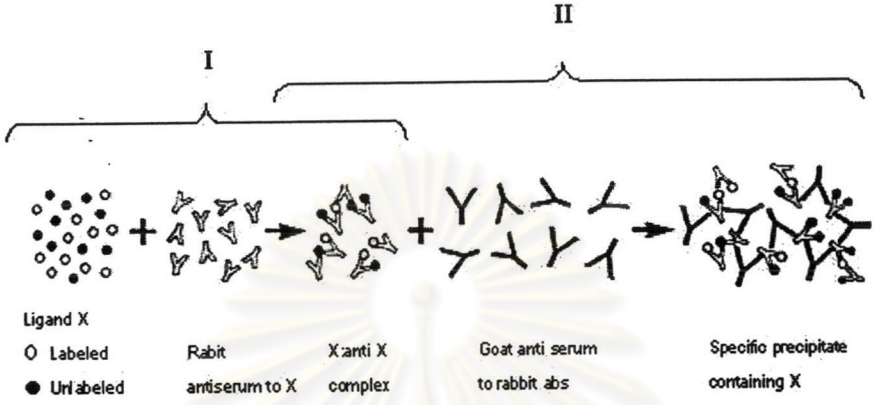


Figure 9 Diagram showing method of Radioimmunoassay. The Ab- Ligand complexes formed in step I are separated from unbound ligand by specific precipitation in step II with antiserum prepared against the Abs used in step I (Herman, 1980).

Terminology of Radioimmunology

1. Radioactivity is the property of unstable isotope throwing out or emitting energetic particles and rays from its nucleus. The amount of radioactive material is measured by how many nuclei decay each second. This is called the activity and is measured in units of curies, abbreviated Ci. One microcurie is equal to 1,000,000th of a Ci. The I^{125} used as a radioactive labeled antigen in this laboratory emits gamma rays (photons). These measurements from the gamma counter are called counts per minute (CPM).

2. Total count tubes are tubes that represent the total amount of radioactivity added in an RIA tube. These tubes are not decanted in the separation step. They represent the total amount of tracer aliquoted per tube. When the assay is counted, these tubes will have the highest CPM. These counts are not used as part of the dose estimate calculation for unknowns, but rather as a quality control comparison to the counts in the 100 % tubes.

3. Non-specific binding tubes (NSB) or backgrounds are tubes that contain labeled antigen, zero standard or 2nd antibody, but they never have any 1st antibody. When the assay is counted, these tubes will have the lowest CPM in a radioimmunoassay

system. These counts are considered to be error or background counts and during calculation should be subtracted from all other tubes to obtain a more accurate estimate of counts in the bound fraction.

4. B_o tubes (100% or maximum binding tubes) are tubes that contain labeled antigen and 1st antibody. It may contain assay buffer and 2nd antibody, but do not have any unlabeled antigen such as unknown samples or standards. After separating the free from the bound fraction, these tubes will have the highest CPM, other than the total count tubes.

5. The bound fraction of an assay tube is an assay tube that part of the labeled and unlabeled antigen immunology bound to the 1st antibody. This is the fraction that could count in gamma counter after incubation period. The free fraction is that part of labeled and unlabeled antigen that has not bound to the 1st antibody. After incubation, it is usually the fraction that is discarded and not counted.

6. 1st antibody is an antibody used in a radioimmunoassay system which will bind to the compound that wants to measure. It will also bind the labeled antigen and the standards

7. 2nd antibody is an antibody added at the end of a double antibody type radioimmunoassay which is directed at the immuno-globulin contained in the 1st antibody. It binds to the 1st antibody/antigen complex (bound fraction) and after centrifugation, is precipitated to the bottom of the assay tube. After the free liquid fraction, or supernatant, of the assay tube is decanted and throw away, the assay tube and the precipitates are counted in the gamma counter.