THE STUDY ON THE IMPROVING PERMEABILITY OF TRANSDERMAL CALCITONIN ENHANCING BY ULTRASOUND

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THE STUDY ON THE IMPROVING PERMEABILITY OF TRANSDERMAL CALCITONIN ENHANCING BY ULTRASOUND

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ABSTRACT

This research investigated the improving permeability of trandermal calcitonin enhancement by ultrasound. The influence of ultrasound on histological feature of ultrasound exposed skin membrane; stability of salmon calcitonin (sCT); and penetration enhancement of chemical enhancers of sCT, was evaluated. The histology of skin was examined after ultrasound for 10 min at high intensity (1 MHz, 3.2 watt (rms)) and low intensity (frequency 1 MHz, 2.3 watt (rms)). The histological change of specimens was tested by porcine ear skin. The specimens were evaluated under an electron microscope and most of the treated studies induced a significantly greater pore size of the skin when compared with the control. Moreover, the findings suggested that stability of sCT decreased after exposure to ultrasound. The effect of ultrasound on sCT was tested by *in vitro* release study with sCT in PBS (Phosphate Buffer Saline) and ultrasound applied at high intensity for 10 min. Cellulose acetate with a molecular weigh cut off point of 12,000-14,000 was used as a membrane and PBS pH 7.4 was used as a medium. The total of 2 ml of receiver phase was collected at 6, 12 and 24 hr and detected by HPLC. The result as expressed by the percent of cumulative release of sCT after application of ultrasound for 10 min was not significantly different from the control. However, the synergistic effect of chemical enhancers and ultrasound on the penetration of sCT was found; the amount of sCT release from chitosan HOBt solution and chitosan hydrogel was enhanced by ultrasound with low intensity for 20 minutes.

KEY WORDS: SALMON CALCITONIN/ ULTRASOUND/ IN VITRO RELEASE/CHIMICAL ENHANCER/CHITOSAN

87 pp.

การศึกษาผลการปรับปรุงการซึมผ่านของยาแคลซิโทนินทางผิวหนังโดยการประยุกต์ใช้คลื่นอัลตร้าซาวน์ (THE STUDY ON THE IMPROVING PERMEABILITY OF TRANSDERMAL CALCITONIN ENHANCING BY ULTRASOUND)

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บทคัดย่อ

การวิจัยนี้มีจุดประสงค์ในการศึกษาผลการปรับปรุงการซึมผ่านของยาแคลซิโทนินทางผิวหนังโดยการประยุกต์ใช้คลื่นอัลตร้าซาวน์ การศึกษาผลกระทบของอัลตร้าซาวน์ต่อจุลกายวิภาคของชิ้นเนื้อ, ความคงตัวของแซลมอนแคลซิโตนิน เมื่อสัมผัสกับอัลตร้าซาวน์ ความถี่ 1 เมกกะเฮิร์ต ที่ความแรง 2.3 และ 3.2 วัตต์ (รอบต่อวินาที) และผลของการเสริมกันของสารเพิ่มการแพร่ผ่านและการใช้อัลตร้าซาวน์ในการเพิ่มการแพร่ผ่านของแซลมอนแคลซิโตนินทางผิวหนัง ผลการศึกษาของการศึกษาทางจุลกายวิภาคของชิ้นเนื้อโดยใช้ผิวหนังของหูหมูเป็นตัวแทนของผิวหนังและใช้กล้องสแกนนิ่งไมโครสโคปในการตรวจสอบ ผลการศึกษาของพบว่าหลังจากผิวหนังหูหมูสัมผัสกับอัล-ตร้าซาวน์ทำให้รูขุมขนส่วนใหญ่มีขนาดใหญ่ขึ้นเมื่อเปรียบเทียบกับผิวหนังที่ควบคุม และความคงตัวของแซลมอนแคลซิโทนินลดลงเมื่อสัมผัสกับอัลตร้าซาวน์

ผลของการศึกษาในแบบจำลองทางผิวหนังในด้านการปลดปล่อยของตัวยาของแซลมอนแคลซิโทนินละลายด้วยฟอสเฟตบัฟเฟอร์กับการสัมผัสโดยอัลตร้าซาวน์พบว่าไม่มีการแพร่ผ่านของแซลมอนแคลซิโทนินอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับตัวควบคุม เมื่อใช้เซลลูโลสอะซิเตตรูขนาด 12,000-14,000 โมเลกุล และสารละลายฟอสเฟตบัฟเฟอร์เป็นตัวกลาง อย่างไรก็ตาม เมื่อการเสริมฤทธิ์กันในของสารเพิ่มการแพร่ผ่านและอัลตร้าซาวน์เกิดขึ้นในสารละลายไคโตซานและเจลไคโตซาน ผลการศึกษาพบว่าเมื่อไคโตซานจากทั้งสองรูปแบบสัมผัสกับอัลตร้าซาวน์สามารถเพิ่มเปอร์เซ็นต์การปลดปล่อยต่อพื้นที่ของแซลมอนแคลซิโทนิน โดยการเพิ่มนี้เป็นการเพิ่มขึ้นแบบเป็นเท่าตัว

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LIST OF ABBREVIATIONS

°C	degree celcius
cont.	continue
CV	coefficient of variance
HOBt	hydroxybenzotriazole
HPLC	high chromatographic column
hr	hour
kHz	kilohertz
MeOH	methanol
µg	microgram
MHz	megahertz
µl	microliter
µm	micrometer
PBS	phosphate buffer saline
Rpm	round per minute
SC	stratum corneum
sCT	salmon calcitonin
SD	standard deviation
W (rms)	watt per square centimeter (s)

CHAPTER I
INTRODUCTION

Calcitonin consists of 32 amino acids. It is a peptide hormone which inhibits bone resorption by inhibiting the activity of osteoclasts. Similar to activated vitamin D3, estrogen and bisphosphonate, calcitonin is used as a drug for the treatment of osteoporosis. When orally administered, calcitonin is easily degraded by the enzymes in the gastrointestinal tract. Due to its high hydrophilic property and high molecular weight, the absorption of calcitonin from the intestinal mucosa is poor. Calcitonin is clinically administered by intramuscular injection, even though side effects such as nausea and facial flushes are caused by a high blood concentration peak after injection. Nevertheless, intramuscular injection has problems such as infection at the site of injection, pain caused by needle insertion and poor patient compliance.

To avoid the above problems, transdermal administration was proposed to take the place of intramuscular injection. However, the stratum corneum acts as a barrier that limits the penetration of substances through the skin. Application of ultrasound to the skin increases its permeability (sonophoresis) and enables the delivery of various substances into and through the skin. Accordingly, this method is now attracting attention as a non-invasive administration method for many peptide drugs [69, 73].

In addition, the use of chemical penetration enhancers is a long-standing and widely used approach to increase transdermal and topical delivery. Some of its properties may be manipulated by application of a penetration enhancer to the skin. Thus, an effective penetration enhancer may increase the diffusion coefficient of the drug into the stratum corneum, or could improve partitioning between the formulation and the stratum corneum. On the other hand, a good enhancer may act by increasing the effective concentration of the drug in the vehicles [71].

Various absorption enhancers have been investigated, including surfactants, bile salts and cyclodextrins. However, most of these are associated with side effects, such

as irreversible changes in the histological properties. As a pharmaceutical excipient, chitosan has been added for enhanced drug release since effects of chitosan are concentration-dependent and reversible. In addition, chitosan has been noted for its application as a film-forming agent in cosmetics, a dye-binder for textiles, a strengthening additive in paper and a hypolipidic material in diets. It has been used extensively as a biomaterial, owing to its immuno-stimulatory activities, anticoagulant properties, antibacterial and antifungal action and for its action as a promoter of wound healing in the field of surgery. In addition, chitosan has a variety of promising pharmaceutical uses and is presently considered as a novel carrier material in drug delivery systems, as indicated by the large number of studies published over the last few years.

Therefore, a combination of ultrasound application and chemical enhancer may not only increase the total enhancement but can also increase the safety of enhancer.

In the present experiments, improvement on penetration of the calcitonin by ultrasound application was assessed. Additionally, stability of salmon calcitonin exposed with ultrasound was investigated to verify any unstable occurrences. As a result, the ultimate goals of this study were:

1. To investigate the influence of ultrasound on
   - Histological feature of ultrasound exposed skin membrane
   - Stability of salmon calcitonin
   - Penetration enhancement of salmon calcitonin
2. To investigate the influence of chemical enhancer on permeation of calcitonin.
3. To investigate the influence of the synergistic effect of chemical enhancers and ultrasound on the penetration of calcitonin.

CHAPTER II
LITERATURE REVIEW

1. Salmon calcitonin

1.1 structure and properties

Calcitonin was discovered in 1961 and it has been established, synthesized, and developed for use in treating disease. In 1983 it was demonstrated that calcitonin is an analgesic, which is also effective in the epidural and subarachnoid spaces [33, 55]. Salmon calcitonin (SCT) is a polypeptide hormone comprised of 32 amino acids. CT is a single-chain polypeptide hormone of 32 amino acids with an N-terminal disulfide bridge between positions 1 and 7 and a C-terminal amidated praline [48]. Calcitonin is a polypeptide hormone consisting of 32 amino acids, secreted by thyroid gland. The primary structure of salmon calcitonin is characterized by a disulfide bridge between the cysteine residues at positions 1 and 7 and a proline amide moiety at the C terminus. SCT is more potent than human or any other mammalian calcitonins. The major physiological role of calcitonin is control of calcium concentration and metabolism in the body in conjunction with parathyroid hormone. SCT primarily acts by inhibiting osteoclastic bone resorption and by stimulating osteoclastic bone formation. Clinically, SCT is used to treat hypercalcemia, Paget's disease and postmenopausal osteoporosis. The absolute bioavailability of sCT after subcutaneous injection has been reported as 11.2—23.1% in rats [50, 56, 57]. SCT is a hydrophilic and cationic (isoelectric point of 10.4) molecule with a relatively high molecular weight (3431.9 Da) that is soluble in water but only sparingly soluble in organic solvents. The polarity of sCT, which appears to be associated with basic amino acid residues (such as arginine, histidine and cysteine) in the molecule, might be reduced in the presence of appropriate counter anions if sCT interacts with the anions to form lipophilic ion-pair complexes [51].

The major physiological role of the sCT is to control the calcium concentration as well as its metabolism in the body. sCT, due to its ability to reduce osteoclast activity,

is commonly used in the treatment of bone diseases such as Paget's disease, hypercalcemia and osteoporosis. SCT is administered via parenteral and nasal routes since it is hydrolyzed by enzymes and acidic medium in the gastrointestinal tract. Because of its short half-life, current studies have been focused on delivery systems which serve to protect and stabilize the sCT and release it in a controlled way by using biodegradable and injectable systems [41].

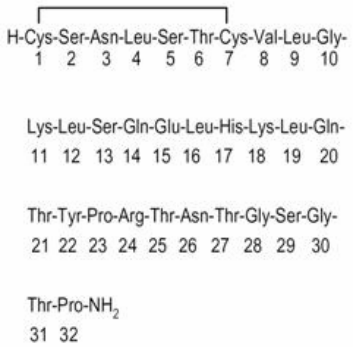


Figure 1. Structure of Calcitonin

1.2 common used indication

Calcitonin may effectively relieve chronic bone pain, due to malignancy or benign conditions, such as osteoporosis and Paget's disease. It appears, however, that the mechanism by which it exerts its analgesic action is independent from its peripheral actions on bone. Involvement of the opioid system, with the release of endorphin has been demonstrated, but several other factors, such as the serotoninergic and catecholaminergic terminals warrant further investigation [33].

1.2.1 Osteoporosis

Osteoporosis is a major public health issue. It is an age related disease that affects an estimated eighty percent of women. The risk for osteoporosis increases after menopause, with 1 in 5 white postmenopausal women affected. Whites are the racial group for which the most data on osteoporosis are available, but other racial and ethnic groups also are at risk. Osteoporosis is associated with an increased risk of fractures, which can lead to chronic pain, deformity, disability, and death. One of every 2 women and 1 of every 4 men over the age of 50 years will experience an osteoporotic fracture at some time in their remaining lifetime. Osteoporosis is responsible for more

than 1.5 million fractures annually, including more than 300,000 hip fractures, 700,000 vertebral fractures, 250,000 wrist fractures, and 300,000 fractures at other sites. Vertebral fractures, the most common type of fracture, often are accompanied by a loss of height, chronic pain, disfigurement (the characteristic stooped posture from kyphosis), and functional deficits. Hip fractures are less common than vertebral fractures, but they are more devastating. Up to 20% of patients with a hip fracture die within 1 year after the fracture.2 More than 30% of Patients with hip fractures are permanently disabled, and many patients require help with activities of daily living and require long term nursing care. The risk of another hip fracture is 4-fold higher in persons who already have suffered one hip fracture than in people without a history of hip fractures [29]. Conservative treatment regimens for osteoarthritis treat the symptoms but not the disease and are limited to control pain and inflammation and eliminate the risk factors. Furthermore, it is still debated if simple analgesics are effective as oral drugs of first choice. Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are associated with serious adverse events particularly gastrointestinal. Selective cyclo-oxygenase-2 (COX-2) inhibitors reduce the incidence of upper gastro-intestinal tract ulcerations, however, other toxicities such as fluid retention, hypertension, congestive heart failure, renal insufficiency and a risk for cardiovascular thrombosis may occur. The therapeutic role of high molecular weight hyaluronans, chondroitin sulfate and glucosamine in terms of pain relief and slowing the progression of osteoarthritis is also debated. Finally, arthroplasty for end stage disease process has a limited life span, relieves pain more predictably than it improves joint function and can be associated with local and systematic complications [32]. Calcitonin has potential efficacy in reducing pain and improving bone density but its current use for the relief of metastatic bone pain is not very common; characteristically, while it is mentioned in one recent review dealing with the issue of cancer pain, another makes no reference to it. Mostly low doses (100-200 IU) of SCT have been used in the management of metastatic bone pain. It is also noteworthy that the last clinical study assessing the efficacy of calcitonin in the management of cancer pain was published more than 5 years ago [40]. Previous data indicated calcitonin's safety and effectiveness on reduction of bone turnover and it is analgesic effect in relieving osteoarthritic (OA) pain. Furthermore, there is in vivo and in vitro

experimental evidence that calcitonin acts on both cartilage and subchondral bone by decreasing the enhanced turnover of the OA subchondral bone, reducing the severity of cartilage OA lesions and altering the biochemical composition and supramolecular organization of the OA cartilage matrix. The effective treatment of pain has been shown to modify the hormonal stress response to trauma and surgery and improve morbidity and mortality indices.

Opioids are very popular drugs, but most clinicians feel that their serious side effects may on many occasions outweigh their benefits. Recently, we have seen the use of new drugs administered epidurally in an effort to achieve anaesthesia without the unwanted side effects of opiates. Such drugs are: clonidine, Gaba-agonists, calcitonin, ketamine HCl, somatostatin, and adenosine.

1.2.2 Paget's disease

Paget's disease of bone is a chronic affliction of the adult skeleton featuring one or more areas of aggressive osteoclast-mediated bone resorption preceding imperfect osteoblast-mediated bone repair. This disturbance begins as a wedge of destructive osteoclasts at one end of a bone that slowly but relentlessly advances to disrupt the entire structure. The deranged skeletal remodeling that follows causes bone expansion and softening, sometimes with pain, fracture, or deformity, and rarely neoplastic transformation. Paget's disease typically manifests in middle or advanced age and is slightly more common in men. The prevalence is highest in Great Britain, Australia, New Zealand, North America, and Western Europe. 6 In the United States, approximately 1 percent of the population over 40 years of age is affected. So-called juvenile Paget's disease is an extremely rare autosomal recessive condition characterized by childhood deafness, fractures, and deformity resulting from generalized, lifelong acceleration of bone turnover, usually due to osteoprotegerin deficiency. Paget's disease typically involves just one bone (monostotic) or a few bones (polyostotic), primarily the skull or pelvis, or a vertebra, femur, or tibia. Osteolytic fronts progress approximately 1 cm yearly. Subsequent "mixed-stage" disease features cortical thickening (hyperostosis), disorganized coarse trabeculae (osteosclerosis), and bone expansion. In cases of advanced ("burnt out") disease, bones are widened and heterogeneously ossified. Remarkably, this pagetic process does not spread spontaneously to adjacent bones but there is no cure. Paget's disease is

usually asymptomatic and discovered incidentally. Most clinical manifestations are skeletal, but the prevalence of various signs and symptoms and complications is uncertain. Skeletal expansion or distortion may be obvious if the disease involves the skull, jaw, clavicle, or a long bone of the leg. Hypervascularity of affected bone may cause palpable warmth. Mild-to-moderate, deep, aching bone pain characteristically begins late in the clinical course, persists throughout the day and at rest, and seems worse at night. Achiness in a weakened femur or tibia often intensifies on weight bearing, especially if there are osteolytic lesions. Acute fractures through pagetic lesions can mend rapidly, although fracture malunion in the proximal femur is not uncommon. Chronic, sometimes painful, fissure fractures may occur along convex surfaces of curved bones. Adjacent joints will be damaged if there is deformity, and osteoarthritis can develop, especially in hips and knees. Osteosarcomas, or other skeletal sarcomas, develop in fewer than 1 percent of patients with Paget's disease but are more common and aggressive than in age-matched controls. Constant and worsening bone pain and sometimes a new mass or sudden fracture should raise concern about malignant transformation. The use of salmon calcitonin (administered by subcutaneous injection) for Paget's disease has been largely supplanted by the use of bisphosphonates, although treatment with salmon calcitonin remains an option if bisphosphonates are not tolerated or contraindicated. Salmon calcitonin typically decreases elevated markers of skeletal turnover by 50 percent, often decreases bone pain and warmth, sometimes improves neurologic complications (including "vascular steal" syndromes), and can heal osteolytic lesions.However, disease reactivation is likely soon after cessation of therapy, acquired resistance occurs in about 25 percent of patients, and bothersome side effects, including nausea and flushing, are common [43,49]

1.3 Route of administration

1.3.1 Intravascular injection

Despite rapid advances in peptide delivery technology, the administration of therapeutic peptides still relies on injections via the parenteral route, which are associated with poor patient acceptance due to pain, frequency and occasionally local adverse effects. Moreover, the metabolic diseases treated with peptide drugs require

long-term therapy [44]. Multiple injections are typically needed, and they usually have a low bioavailability and chemical stability problems which limits their use. Furthermore, these injections may result in large spikes in blood concentration with associated various side effects [50].

1.3.2 Nasal administration

Intranasal drug delivery has attracted a lot of interest as an alternative route for making drugs that are normally restricted to intravenous administration, such as peptide and protein drugs, systemically available. This is because of the unique anatomical structure and physiological functions of the nasal mucosa. The large mucosal surface, highly vascularized and porous endothelial membrane, and avoidance of first-pass metabolism contribute to the attainment of adequate bioavailability for medication and quick onset of action, comparable to that of injections. Moreover, the ready accessibility of intranasal administration makes it possible for patients on long term therapy to self-medicate. With these advantages, the nasal administration of drugs for systemic medication has been widely investigated in recent years, and many projects are now under clinical development. On the other hand, there are some disadvantages with intranasal drug delivery, including the low permeability of the mucosa to large molecules, enzymatic degradation, the very small absorption capability of the nasal cavity, limiting application to drugs that can be supplied in small doses, and the rapid mucociliary clearance, which shortens the period of time available for efficient absorption and causes low bioavailability [52].

Nasal delivery of calcitonin is viable and commercially available but it suffers the disadvantages of irritation of nasal mucosa and variable absorption in case of nasal disease conditions. Major side effects of nasal drug delivery route include ear, nose and throat disorders, such as rhinitis, rhinorrhea or hydrorhea and allergic rhinitis but are of minor intensity [50].

1.3.3 Oral administration

Hence, non-invasive alternative administrations are preferred and oral administration is considered one of the most attractive methods because of its convenience. To achieve successful peptide oral delivery, two important factors must be addressed: proteolytic degradation and intestine epithelial permeability . When delivered via the oral route, peptides are severely degraded by the gastrointestinal

enzyme pool, which includes a battery of pancreatic peptidases and brush-border peptidases. In addition, intestinal absorption militates against the use of peptides because of their large molecular sizes and hydrophilicities, which are viewed as being responsible for the disappointing therapeutic efficacies of orally delivered peptides. Conversely, the meeting of these dual requirements presents the possibility of successful oral delivery [44]. Oral administration of peptide and protein drugs is a challenge because of their large size, their susceptibility to luminal and brush border proteolysis, and their limited stability. Consequently, protein drugs exhibit poor oral bioavailability. To increase their availability into the body after oral administration, proteinaceous drugs require absorption enhancers, protease inhibitors, or both. In this context, the colon is an interesting organ in the gastrointestinal (GI) tract. In addition to its improved ability to respond to absorption enhancers, reports in the literature indicate that proteolysis occurs into a lesser extent in the human colon compared with the small intestine It was, therefore, suggested that colon-specific drug carriers could facilitate the oral absorption of proteinaceous drugs. Still, even the relatively lower proteolytic activity of the colon requires that protein drugs should be protected due to the longer residence time in the this organ. If protection is successful, the prolonged residence time could turn out to be advantageous because the drug would be exposed for extended periods of time to the absorptive epithelium [46, 58]. Therefore, safe and effective noninvasive administration routes are required as an alternative to injection. It is generally realized that pulmonary administration is superior to other noninvasive routes such as nasal, dermal, ocular or oral, owing to its relatively high bioavailability, limited side effects, rapid and sustained benefits, good long-term compliance, and ease of use.

1.3.4 Colonic adminstration

The colon is largely being investigated as a site for administration of protein and peptides, which are degraded by digestive enzymes in the upper gastrointestinal tract. In addition, drug administration to the site of action for local diseases of the colon does not only reduce the dose to be administered but also decreases the side effects [41].

1.3.5 Transdermal administration

Transdermal route is one alternative to invasive routes of drug administration for charged macromolecules. Advantages of this route include improved patient compliance, avoidance of first pass hepatic metabolism, controlled delivery and the possibility to modulate the rate of delivery [50].

2. Skin structure

Human skin is a uniquely engineered organ that permits terrestrial life by regulating heat and water loss from the body whilst preventing the ingress of noxious chemicals or microorganisms. It is also the largest organ of the human body, providing around 10% of the body mass of an average person, and it covers an average area of 1.7 m2. Whilst such a large and easily accessible organ apparently offers ideal and multiple sites to administer therapeutic agents for both local and systemic actions, human skin is a highly efficient self-repairing barrier designed to keep 'the insides in and the outside out'. Skin membranes can be examined at various levels of complexity. In some mathematical treatments of transdermal drug delivery the membrane can be regarded as a simple physical barrier; more complexity can be introduced by viewing skin as various barriers in series. We can then introduce barriers in parallel by considering drug transport through pores in the tissue. Degrees of complexity also exist when examining basic structures and functions of the membrane. In some extreme cases it may be that transdermal drug delivery is limited by metabolic activity within the membrane. Alternatively, immunological responses may prevent the clinical use of a formulation that has proven to be optimal during in-vitro studies. A further complication is introduced in clinical situations where topical delivery is intended to treat diseased skin states; here, the barrier nature of the membrane may be compromised and so data extrapolated from in-vitro experiments using healthy tissue may be inappropriate. Human skin is a highly complex organ though in many transdermal drug delivery studies it is often regarded somewhat simplistically as merely a physical barrier. The structure and function of human skin categorised into three main layers (Figure 2)

- The innermost subcutaneous fat layer (hypodermis)
- The overlying dermis
- The viable epidermis

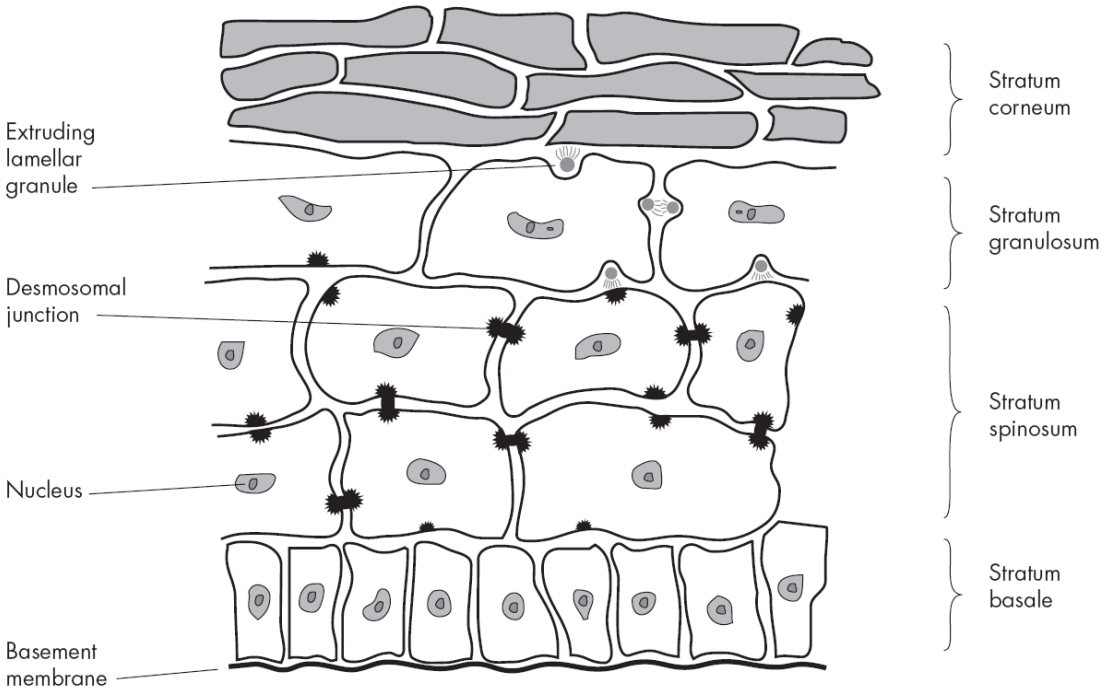


Figure 2 A structure of human skin

2.1 The subcutaneous fat layer

The subcutaneous fat layer, or hypodermis, bridges between the overlying dermis and the underlying body constituents. In most areas of the body this layer is relatively thick, typically in the order of several millimetres. However, there are areas of the body in which the subcutaneous fat layer is absent, such as the eyelids. This layer of adipose tissue principally serves to insulate the body and to provide mechanical protection against physical shock. The subcutaneous fatty layer can also provide a readily available supply of high-energy molecules, whilst the principal blood vessels and nerves are carried to the skin in this layer [68].

2.2 The dermis

The dermis (or corium) is typically 3–5 mm thick and is the major component of human skin. It is composed of a network of connective tissue, predominantly collagen fibrils providing support and elastic tissue providing flexibility, embedded in a mucopolysaccharide gel. In terms of transdermal drug delivery, this layer is often viewed as essentially gelled water, and thus provides a minimal barrier to the delivery of most polar drugs, although the dermal barrier may be significant when delivering

highly lipophilic molecules. The dermis has numerous structures embedded within it; blood and lymphatic vessels, nerve endings, pilosebaceous units (hair follicles and sebaceous glands), and sweat glands (eccrine and apocrine). The extensive vasculature of the skin is essential for regulation of body temperature whilst also delivering oxygen and nutrients to the tissue and removing toxins and waste products. The vasculature is also important in wound repair. The rich blood flow, around 0.05 mL/min per mg of skin, is very efficient for the removal of molecules that have traversed the outer skin layers. Capillaries reach to within 0.2 mm of the skin surface, and are found intertwined with the Malpighian layer of the viable epidermis. Molecules are thus removed, *in vivo*, from near the dermo-epidermal layer, ensuring that dermal concentrations of most permeants are very low. For transdermal delivery of most drugs, the blood supply thus maintains a concentration gradient between the applied formulation on the skin surface and the vasculature, across the skin membrane. It is this concentration gradient that provides the driving force for drug permeation. The lymphatic system also reaches to the dermo-epidermal layer and, whilst it is important in regulating interstitial pressure, facilitating immunological responses to microbial assault and for waste removal, the lymphatic vessels may also remove permeated molecules from the dermis hence maintaining a driving force for permeation [68].

2.3 The epidermis

The epidermis is itself a complex multiply layered membrane, yet varies in thickness from around 0.06 mm on the eyelids to around 0.8 mm on the load-bearing palms and soles of the feet. The epidermis contains no blood vessels and hence nutrients and waste products must diffuse across the dermo-epidermal layer in order to maintain tissue integrity. Likewise, molecules permeating across the epidermis must cross the dermo-epidermal layer in order to be cleared into the systemic circulation. The epidermis contains four histologically distinct layers which, from the inside to the outside, are the stratum germinativum, stratum spinosum, stratum granulosum and the stratum corneum. A fifth layer, the stratum lucidum, is sometimes described but is more usually considered to be the lower layers of the stratum corneum. The stratum corneum, comprising anucleate (dead) cells, provides the main barrier to transdermal delivery of drugs and hence is often treated as a separate membrane by workers within

the field. The term ‘viable epidermis’ is often used to describe the underlying layers, although the viability of cells within, for example, the stratum granulosum is questionable as the cell components degrade during differentiation.

2.3.1 The stratum basale

The stratum basale is also referred to as the stratum germinativum or, more commonly, the basal layer. The cells of the basal layer are similar to those of other tissues within the body; they contain the typical organelles such as mitochondria and ribosomes, and the cells are metabolically active. This layer thus contains the only cells (keratinocytes) within the epidermis that undergo cell division (via mitosis). On average, dividing basal cells replicate once every 200 to 400 h. After replication, one daughter cell remains in the basal layer whilst the other migrates upwards through the epidermis towards the skin surface. The keratinocytes of the stratum basale are attached to the basement membrane (dermo-epidermal membrane) by hemidesmosomes, which act rather like proteinaceous anchors for these lowest layer cells. Loss of adhesion between the basal cells and the basement membrane results in shedding of the skin, as found in some blistering conditions. Within the stratum basale and the adjacent cell layer, the stratum spinosum, keratinocytes are connected through desmosomes, again highly specialised proteinaceous cellular bridges.

2.3.2 The stratum spinosum

The stratum spinosum (also known as the spinous layer or prickle cell layer) is found on top of the basal layer, and together these two layers are termed the Malpighian layer. This spinous layer consists of two to six rows of keratinocytes that change morphology from columnar to polygonal cells. Within this layer the keratinocytes begin to differentiate and synthesise keratins that aggregate to form tonofilaments. Desmosomes connecting the cell membranes of adjacent keratinocytes are formed from condensations of the tonofilaments, and it is these desmosomes that maintain a distance of approximately 20 nm between the cells.

2.3.3 The stratum granulosum

As they pass from the stratum spinosum to the stratum granulosum (or granular layer), the keratinocytes continue to differentiate, synthesise keratin and start to flatten. Only one to three cell layers thick, the stratum granulosum contains enzymes that begin degradation of the viable cell components such as the nuclei and organelles. The

granular cells are so called because they acquire granular structures. Keratohyalin granules mature the keratins within the cell. Most importantly for topical and transdermal drug delivery, membrane-coating granules are also synthesised, probably in the endoplasmic reticulum and Golgi apparatus, and contain the precursors for the intercellular lipid lamellae seen in the stratum corneum. The lamellar granules are extruded from the cells into the intercellular spaces as the cells approach the upper layer of the stratum granulosum.

2.3.4 The stratum lucidum

The stratum lucidum is the layer in which the cell nucleus disintegrates and there is an increase in keratinisation of the cells concomitant with further morphological changes such as cell flattening. Occasionally, droplets of an oily substance may be seen in this cell layer, possibly arising from the disintegration of lysosomes. The stratum lucidum tends to be seen most clearly in relatively thick skin specimens, such as from the load-bearing areas of the body (soles of feet and palms). Indeed, some dermatologists question whether this layer is functionally distinct from the other epidermal layers, or if it is an artefact of tissue preparation. Most researchers tend to view the stratum lucidum as the lower portion of the stratum corneum and hence bracket these two layers together.

2.3.5 The stratum corneum

The stratum corneum (or horny layer) is the final product of epidermal cell differentiation, and though it is an epidermal layer it is often viewed as a separate membrane in topical and transdermal drug delivery studies. Typically, the stratum corneum comprises only 10 to 15 cell layers and is around 10 μm thick when dry, although it may swell to several times this thickness when wet. As with the viable epidermis, the stratum corneum is thickest on the palms and soles and is thinnest on the lips. This thin membrane, consisting of dead, anucleate, keratinized cells embedded in a lipid matrix, allows for survival of terrestrial animals without desiccation. The stratum corneum serves to regulate water loss from the body whilst preventing the entry of harmful materials, including microorganisms. The stratum corneum has been represented as a ‘brick and mortar’ model in which the keratinised cells are embedded in a mortar of lipid bilayers. However, it should be borne in mind that the keratinocytes are polygonal, elongated and relatively flat approximately 0.2 to

1.5 μm thick with a diameter of 34 to 46 μm (Figure 3). Typically, it takes 14 days for a daughter cell from the stratum basale to differentiate into a stratum corneum cell, and the stratum corneum cells are typically retained for a further 14 days prior to shedding. Since the keratinocytes of the stratum corneum are cornified, they are also termed ‘corneocytes’.The barrier nature of the stratum corneum depends critically on its unique constituents; 75–80% is protein, 5–15% is lipid with 5–10% unidentified on a dry weight basis. The protein is located primarily within the keratinocytes and is predominantly alphakeratin (around 70%) with some beta-keratin (approximately 10%) and a proteinaceous cell envelope (around 5%). Enzymes and other proteins account for approximately 15% of the protein component. The cell envelope protein is highly insoluble and is very resistant to chemical attack. This outer keratinocyte protein has a key role in structuring and ordering the intercellular lipid lamellae of the stratum corneum; the keratinocyte is bound to a lipid envelope through glutamate moieties of the protein envelope. The lipid envelope thus provides an anchor to the keratinocyte and links the proteinaceous domains of the keratinocytes to the intercellular lipid domains [62,68].

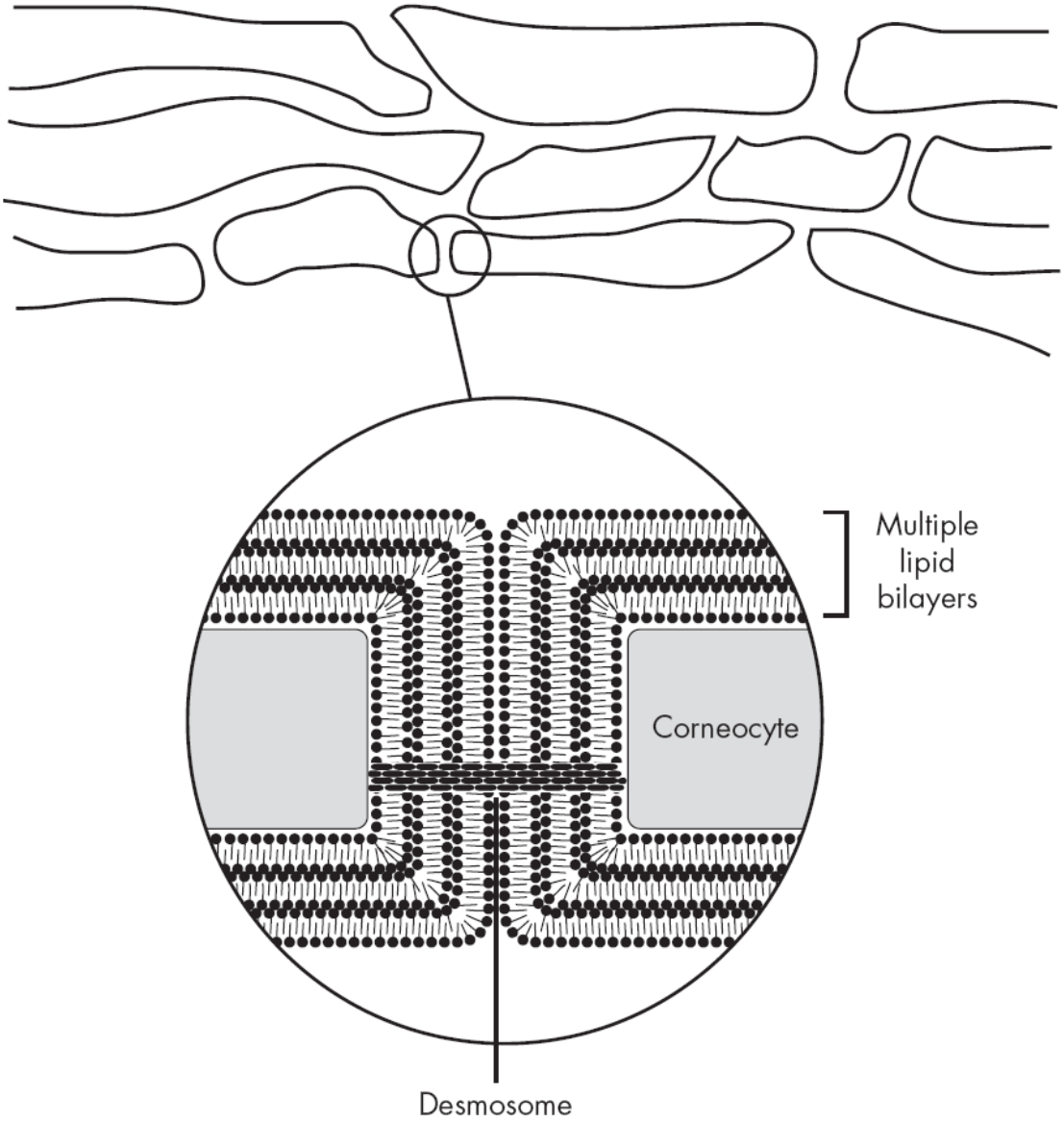


Figure 3 An illustration of the ‘brick and mortar’ model of human stratum corneum

3. Ultrasound: strategies for enhancement of transdermal drug delivery

Ultrasound is a sound wave possessing frequencies above 20 KHz. Ultrasound waves are characterize by two main parameters: frequency and amplitude. The amplitude of ultrasound waves can be represented in terms of the peak wave pressure, P, (in units of Pascal) or in terms of the intensity, I, (in units of W/cm^2). P and I are related by the equation $I = P^2/2\rho c$, where ρ is the water density (1000 kg/m^3), and c is the velocity of ultrasound in water (1500 m/s). Ultrasound can be applied either continuously or in a pulsed manner. In the latter case, an additional parameter, the duty cycle, is required to characterize the ultrasound application. Thus, ultrasound application for sonophoresis may be completely characterized by four mains parameter, frequency, intensity, duty cycle and exposure time. Ultrasound is generated using device referred to as a sonicator which consists of an electrical signal generator capable of generating an electrical AC signal at the desired frequency and amplitude. This signal is applied across a piezo-electric crystal (also known as the transducer) to generate ultrasound. The thickness of the piezo-electric crystal is selected so that it resonates at the operating frequency. Sonicators operating at various frequencies in the range of 20 KHz to 3 MHz are available commercially and can be used for sonophoresis. If a sonicator operating at the desired frequency is not available commercially, it is possible to assemble one using commercially available signal generators, amplifiers, and transducers.

For sonophoretic delivery, the desired drug is dissolved in a solvent and applied on the skin. Ultrasound is applied by contacting the ultrasound transducer with the skin through a coupling medium to ensure proper contact between the transducer and the skin

The coupling medium can be the same as the solvent used to dissolve the drug, or it can be commercially available ultrasound coupling gel. The coupling medium should result in proper transmission of ultrasound from the transducer to the skin. The transitive properties of a medium are indicated by its acoustic impedance, Z, is comparable to that of the skin (1.6×10^6 $kg/m^2/s$).

Table 1 Acoustic impedance of biological mediums

Biological Medium	**Acoustic Impedance, Z ($Kg/m^2/s$)**	**Absorption Coefficient, α at 1 MHz(cm^{-1})**
Air	$0.0004x10^6$	2.76
Blood	$1.6x10^6$	0.028
Bone	$6.3x10^6$	3.22
Fatty Tissue	$1.54x10^6$	0.14
Muscle	$1.6x10^6$	0.76
Skin	$1.6x10^6$	0.62
Water	$1.5x10^6$	0.0006

Every medium absorbs ultrasound to a certain extent, and its ability to do so is indicated by the absorption coefficient, α. The extent of absorption is given by the following equation:

$$f(\tau) = 1\text{-}\exp(-\alpha\tau)$$

Where $f(\tau)$ is the fraction of ultrasound intensity absorbed as the ultrasound beam propagates through a medium having absorption coefficient α and thickness τ. [69]

3.1 Biological effects of ultrasound [74-96]

Ultrasound over a wide frequency range has been used in medicine for the past century. For example, therapeutic ultrasound has been used for physical therapy, low-frequency ultrasound has been used in dentistry and high-frequency ultrasound has been used for diagnostic purposes. The utility of ultrasound is continuously expanding and new clinical applications are constantly being developed, including the use of high-intensity focused ultrasound for tumour therapy, lithotripsy, ultrasound-assisted lipoplasty and ultrasonic surgical instruments. Significant attention has thus been given to investigating the effects of ultrasound on biological tissues. Ultrasound affects biological tissues via three main effects: thermal, cavitational and acoustic streaming.

3.1.1 Thermal effects

Absorption of ultrasound increases temperature of the medium. Materials that possess higher ultrasound absorption coefficients, such as bone, experience severe thermal effects compared with muscle tissue, which has a lower absorption coefficient. The increase in the temperature of the medium upon ultrasound exposure at a given frequency varies directly with the ultrasound intensity and exposure time. The absorption coefficient of a medium increases directly with ultrasound frequency resulting in temperature increase. The use of a new safety parameter, time to threshold (TT). TT indicates the time after which a threshold temperature rise is exceeded, and how long a piece of tissue can be safely exposed to ultrasound, provided the safe threshold is known.

3.1.2 Cavitational effects

Cavitation is the formation of gaseous cavities in a medium upon ultrasound exposure. The primary cause of cavitation is ultrasound-induced pressure variation in the medium. Cavitation involves either the rapid growth and collapse of a bubble (inertial cavitation), or the slow oscillatory motion of a bubble in an ultrasound field (stable cavitation). Collapse of cavitation bubbles releases a shock wave that can cause structural alteration in the surrounding tissue. Tissues contain air pockets that are trapped in the fibrous structures that act as nuclei for cavitation upon ultrasound exposure. The cavitational effects vary inversely with ultrasound frequency and directly with ultrasound intensity. Cavitation might be important when low-frequency ultrasound is used, gassy fluids are exposed or when small gas-filled spaces are exposed.

3.1.3 Acoustic streaming effects

Acoustic streaming is the development of unidirectional flow currents in fluid that are the result of the presence of sound waves. The primary cause of acoustic streaming is ultrasound reflections and other distortions that occur during wave propagation. Oscillations of cavitation bubbles might also contribute to acoustic streaming. The shear stresses developed by streaming velocities might affect the neighbouring tissue structures. Acoustic streaming might be important when the medium has acoustic impedance that is different from that of its surroundings, the fluid in the biological medium is free to move or when continuous wave application is used.

3.2 Synergistic effects of ultrasound

Ultrasound might enhance transdermal transport by inducing skin alteration, as well as by inducing active transport (forced convection) in the skin. Various other means of transport enhancement, including chemicals, iontophoresis and electroporation, might enhance transport synergistically with ultrasound. Application of ultrasound alone as well as SLS alone, both for 90 min, increased skin permeability about threefold for SLS and eightfold for ultrasound. However, combined application of ultrasound and 1% SLS solution induced an increase in skin permeability to mannitol in the order of 200-fold. Ultrasound also exhibited a synergistic effect with electroporation. Ultrasound reduced the threshold voltage for electroporation as well as increasing transdermal transport at a given electroporation voltage. The enhancement of transdermal transport induced by the combination of ultrasound and electroporation was higher than the sum of the enhancement induced by each enhancer alone. Combined application of ultrasound and iontophoresis also has practical implications. The combination of ultrasound and electric current offers a higher enhancement than that offered by each of them individually under the same conditions. Since ultrasonic pretreatment reduces skin resistivity, a lower voltage is required to deliver a given current during iontophoresis compared to that in controls. This should result in lower power requirements as well as possibly less skin irritation [72-73].

4. *In vitro* study [65-69]

The tape-strip sampling technique has not been fully utilized partly due to inconsistent standardization. For example, normalization of the mass measured on tape strips is done by dividing the mass of chemicals on tape strips by the mass of stratum corneum removed or by the keratin content. A standard approach for reporting tape-strip data has not yet been determined. In addition, there is insufficient evidence that tape-strip measurements from different exposure groups (i.e., high-, medium-, or low-exposure) are representative of exposure based on internal dose metrics (e.g., blood and urine biological markers of exposure). A paucity of studies has demonstrated an association between dermal exposures measured using the tape-strip technique and urinary metabolite levels in humans under occupational-exposure conditions. Clearly, demonstrating that tape-strip measurements are predictive of internal dose can be of benefit to exposure assessment and epidemiological studies. Quantification of the absorption, distribution, metabolism, and elimination of chemicals that may come into contact with the skin is necessary for assessing exposures and human health risks associated with occupational and environmental exposures to chemicals. Membrane models have been used to quantify the behavior of chemicals in the skin.

4.1 Membranes for in vitro studies

A further concern with in vitro experiments arises from the appropriate membrane. From the historical findings, the most appropriate membrane to select for transdermal drug delivery studies is that for the species to be treated. However, there are considerable ethical and legal constraints on obtaining and using human material, and this has led to many investigators utilizing alternative models. The use of model membranes can be justified on many grounds, but the data obtained from these model membranes must be treated with caution in that results obtained from animal skins may not transfer directly to human skin either in vitro or in vivo. For formulation development or stability testing as a screening membrane, animal tissues can be of great value and are certainly more readily available and easier to store and handle. Moreover, data obtained from animal membranes can be more reproducible than that from donated human tissue, as the animal's history is usually controlled whereas that of surgically excised skin from a donor is less well controlled.

Optimally, the testing of topical delivery systems should be performed in vivo. This is often impossible, especially in the developmental stages when the toxicity or irritancy of new drugs, excipients or devices may not be documented. In vitro experimental procedures have, therefore, become increasingly important in this field because of the multitude of problems associated with in vivo protocols. Laboratory test systems require a membrane to mimic the barrier function of the stratum corneum. To this end, many different types of membrane, both biological and synthetic, have been investigated for potential usefulness in in vitro test systems. These membranes range in barrier function from negligible resistivity to permeabilities approximating that of human skin. Generally, membranes from animal sources tend to have higher resistivities to drug diffusion compared with synthetic membranes, probably because of the more complex biochemical composition of the former. The main objective of in vitro experimentation is to simulate diffusion conditions in man, thus obviating the requirement for in vivo research using humans or animals. Clearly, if an in vivo/in vitro correlation can be established then routine testing can be conducted in the laboratory. It should be emphasised, however, that the establishment of an in vivo / in vitro correlation is unique for a specific membrane, drug and delivery vehicle.

4.1.1 Natural membranes

It seems obvious that human tissue would be the most appropriate medium to use in a diffusion cell so that extrapolation of conclusions to the in vivo situation could be made. Excision of the skin does not alter its permeability properties significantly, provided the stratum corneum remains intact, and several studies have shown that the stratum corneum performs similarly in vitro and in vivo, even several days after harvesting. It should, therefore, be possible to design representative in vitro systems using excised human or animal tissue, since a similarity between laboratory animal and human skin has been observed.

- **Human skin**

The use of human skin for experimentation is not without problems: its availability is limited, in some countries the legal requirements for its procurement are particularly convoluted and permeability varies greatly between specimens taken from the same or different anatomical sites of the same donor (27% variance in vivo and 43% in vitro), and greater variations (45% in vivo and 66% in vitro) are noted between

specimens from different subjects or different age groups. Complicating these difficulties, metabolism and biotransformation of chemicals applied to the skin may continue after excision of the tissue from the donor, and may be extensive in some cases. Residual enzymes in the stratum corneum metabolized permeating testosterone molecules to the extent that only 5% of the applied dose penetrated as the parent compound [47]. This variance necessitates the use of a large number of skin samples to obtain representative, average results.

- Full thickness skin

Tissue sampling, handling and preparation procedures will vary dependent on the study being undertaken and composition of the membrane required. The most satisfactory source of human tissue is that obtained from cadavers or amputations. This tissue may be stored in the frozen state for prolonged periods without any significant alteration in its barrier potential. The processing of full thickness skin (stratum corneum, epidermis and dermis) is fairly simple but requires a dermatome. A number of techniques for the preparation of this membrane have been reported. Typically excess fat is trimmed away from the dermal side of the excised skin and the tissue layer is clamped between metal plates and frozen at approximately -24°C. The plate in contact with the stratum corneum is warmed slightly so that it may be removed while ensuring that the dermal side of the tissue adheres to the lower plate. A dermatome may then be used to excise a strip of tissue of desired thickness from the thawing surface. Specimens containing a relatively high density of hair follicles may pose a problem in this cutting procedure. Alternatively, a dermatome may be used to excise strips of tissue of the required thickness directly from the cadaver or amputated limbs. These strips may then be frozen and stored until required without any further manipulation.

Other sources of full thickness skin that have been used in laboratory diffusion experiments are neonate foreskin, callus, and scalp tissue; however, these membranes are atypical in that they have a greater permeability than skin from other anatomical sites, or have been formed in response to pressure abrasion. If full thickness skin is used it must be borne in mind that in vivo the permeating molecule would not have to traverse the dermal layer; the vasculature at the dermo-epidermal junction would rapidly clear any diffusant reaching this interface. This is an additional resistive

pathway to diffusion introduced by in vitro methodology. It has been suggested that the presence of the dermis in excised diffusion membranes may present a significant, additional, artificial barrier to the diffusion of lipophilic moieties

- Stratum corneum plus epidermis

The relatively thick aqueous environment of the dermis may present an unfavourable partitioning environment for lipophilic diffusants and, therefore, in vitro experiments are best conducted, for certain diffusants, without the dermis in place. To overcome these problems, simply sectioned samples of skin with a dermatome set at a depth of 200 μm, the approximate thickness of the stratum corneum plus epidermis. This appears to be the easiest method of sample preparation and has the added advantage of not exposing the tissue to possibly deleterious chemicals. Conversely, several chemical techniques have been investigated for the isolation of the stratum corneum and epidermis from dermis and subdermal tissue. The most facile appears to be the heat separation technique and modifications which requires the skin to be heated to 60°C for 2 min (by simple immersion in water or clamping between previously warmed metal plates) after which the stratum corneum-epidermis may be lifted from the dermis using blunt dissection [48].

- Stratum corneum

In many cases only the stratum corneum is required for experimental work. Proteolytic enzymes may be employed to degrade the viable cells of the epidermis which are then easily removed from the dead horny layer. The stratum corneum plus epidermis, previously separated from the dermis, is placed epidermal side down on filter paper soaked in 0.0001% trypsin and 0.5% sodium bicarbonate or buffer solution (pH between 8.0 and 8.6) and incubated at 37°C for 24 h after which the digested epidermis may be removed. Other proteolytic enzymes such as pepsin, papaln, ficin, elastase or pronase may be used but these are less efficient and require higher concentrations for equivalent effect. Bacterial metabolites such as purified fractions of staphylococcal exfoliation may also be used to degrade the epidermal cells. These enzymes may also affect the biochemical composition of the stratum corneum and thereby possibly alter its permeability properties. Stretching of the skin sample causes splitting of the strata and may also be used as a method of stratum corneum preparation

- **Animal models**

Given the limited availability of human tissue and the fact that a number of percutaneous investigations may be too toxic to be carried out on living subjects, a number of animal models have been investigated for their usefulness in predicting percutaneous absorption kinetics. The skin of experimental animals differs markedly from that of humans in features such as thickness and biochemical composition of the stratum corneum, and especially in the density of hair follicles and glands. Furthermore, the lipid content of the skin is a major determinant in its barrier potential and that differences between species or between sites are due to varying lipid composition [48]. Even in situations where animal membranes may resemble human tissue (e.g., "hairless" animal species) direct extrapolation of permeation results to the human situation is not implied. Generally, in vitro data agree closely with in vivo observations in that common laboratory animal skin is more permeable than human tissue, while pig and monkey skin give permeability results that are most comparable.

- Mouse

Laboratory rodents are a convenient source of animal skin for research purposes because these animals are fairly easily handled and are relatively inexpensive when compared with larger species. However, it has generally been reported that murine skin is more permeable than human tissue, probably due to the thinner stratum corneum, and the medium is, therefore, of limited value.

- Hairless mouse

These laboratory animals have been used extensively in transdermal absorption research, probably because of the perceived similarity between their skin structure and that of humans. Homozygous animals carrying the hr hairless, recessive genes develop a normal coat of hair up to the age of about 10 days after which the complete hair shaft is lost from the follicle. Sparse, thin hairs grow at intervals during the life of the animal but are soon lost. Hyperkeratosis of the stratum corneum and upper part of the hair canals begins about 2 weeks post partum and cysts may develop in the hair follicles or sebaceous glands with subsequent keratinization. While the number and diameter of the hair follicles in hairless mouse skin approaches that of human skin more closely than most other laboratory animals, the stratum corneum of these animals is less than half as thick as that of human tissue with commensurately lower barrier

properties. The integrity of the horny layer must be assured if hairless mouse skin is to be used in diffusion experiments. In this regard, the curved toenails of these animals grow excessively long and may inflict substantial injury when males fight.

- Rat

The rat is a common laboratory animal which has been used extensively in the study of transdermal drug absorption. Excised rat skin has generally been considered more permeable than human or pig skin; however, in the diffusion of certain compounds rat skin appears to be as good as pig skin in modelling absorption through human tissue. Studies using the hairless rat and the fuzzy rat have also demonstrated these animals to be useful in certain circumstances.

- Guinea pig

Relatively little use has been made of the guinea pig as a model for transdermal research. Histological studies have shown that guinea pig skin is fairly similar in appearance to human skin and one would therefore assume that their in vitro permeabilities would be similar. Generally, the clipped, full thickness skin of these animals has been used and this membrane has demonstrated greater permeability to steroidal passage than human tissue.

- Rabbit

Although rabbit skin have been shown good agreement between the in vivo and in vitro permeation results of water through rabbit skin, this membrane is generally recognized as the most permeable of the common laboratory animals [50]. These findings would suggest that the rabbit is a poor predictive model for transdermal absorption studies. However, rabbit skin is a good indicator of dermal toxicity, because of its rapid absorptive characteristics, and has been used for this purpose

- Monkey

One may assume that the skin characteristics of primate animals should be very similar to those of humans, making them valuable models in absorption research. Close correlations of human and primate permeation data have been demonstrated by several studies. However, these animals require additional holding and laboratory facilities as well as extra care in experimentation which makes their use undesirable

- Pig

In several in vitro studies the skin of the pig or miniature pig has proved to be a good animal model for human skin. The permeation rate of tritiated water through dermatomed, full thickness pig skin was reported to be very slightly greater than that through human tissue [51]. Moreover, there was a statistically significant correlation between the percutaneous penetration of 10 compounds through whole pig skin in vitro and values reported for human skin in vivo [51]. They further report that better agreement between in vitro and in vivo results is obtained if sectioned pig skin is used instead of full thickness membrane, presumably because the barrier thickness of the pig skin would then approach that of human tissue. Although the stratum corneum of the pig is almost twice the thickness of the human layer, pig skin is very similar to human tissue in the density of hair follicles (both models average 11 follicles cm $^{-2}$) and that this is the lowest density found in any laboratory animal. However, pig skin follicles, incorporating coarse hair shafts, are almost twice the diameter of their human counterparts. The thickness of the porcine dermis makes the use of full thickness skin impractical; however, it was unable to be separated the epidermis from full skin samples by the methods of heat, trypsin or ammonia because of the presence of the coarse hair follicles. From above results, it can be assumed that the best method of pig skin preparation is to section the sample using a dermatome [101-103].

- Shed snake skin

Although shed snake skin is not a mammalian integument, many compounds penetrate snake skin and human stratum corneum at similar rates. Since snakes moult periodically, a single animal can provide repeated sheds, thus eliminating inter-individual variability. Skins can be obtained without injury to the animal and do not have to be subjected to chemical or heat stress prior to use. The epidermis is shed as a large, intact sheet; thus a single snake skin can provide multiple samples. Shed snake skin is not a living tissue, can be stored at room temperature for relatively long periods and is easily transported. Stored or fresh skins appear to show no differences in permeability. Since snake skin lacks hair follicles, the problems associated with transfollicular routes of penetration, which may be significant in mammalian skins, can be avoided.

- Egg-shell membrane

An interesting investigation used egg-shell membrane which, like human stratum corneum, consists mainly of keratin. The membrane is prepared by immersing the whole egg in 0.5 M hydrochloric acid which dissolves the outer shell. Thereafter, the contents of the egg may be removed and the membrane washed and refrigerated or soaked in isopropyl myristate under vacuum to impregnate the keratin matrix. The replacement of water in the membrane with this lipid is assumed to increase its likeness to stratum corneum biochemistry.

- Synthetic stratum corneum

The use of cultured cells as permeation barriers has recently received some attention. Permeation through cultured hamster buccal epithelium, mouse keratinocytes, epithelial and endothelial cells and newborn rat skin epidermal cell monolayers has been investigated. A more recent development is the commercial availability of model human skin produced by in vitro culture of dermal and epidermal cells. A recent evaluation indicates that although it is considerably more permeable to a number of permeating moieties than human skin, it has the same differentiating capacity as human skin. It appears that it may be possible to improve the barrier function of this membrane by utilisation of different culture conditions. If a membrane of this type can be manufactured which mimics human skin, it will, obviously, become one of the preferred percutaneous penetration membranes of the future.

4.1.2 Synthetic membranes

It may, theoretically, be possible to adequately simulate the in vivo permeation of a drug using a specific diffusion system and synthetic membrane. The commercial availability, stability, interbatch uniformity and ease of usage make the use of synthetic media highly desirable. The barrier potential of porous membranes is dictated by the probability of a diffusant molecule entering and diffusing through the pores, and the factors governing selectivity to diffusion would be the relative molecular size, molecular shape and its electrostatic interactions with the membrane. Conversely, aporous media appear to offer some rate-limiting factor to permeation and may, therefore, more closely simulate diffusion through biological tissue. The barrier properties here generally relate to the solubility of the diffusant in the polymer matrix

(partition coefficient between donor vehicle and membrane) and the ease of diffusant passage through the polymer.

– Cellulose media

Cellulose is a relatively rigid structure consisting of glucopyranose rings joined by ,B-1,4-linkages. This conformation allows only two types of movement in the chains: inversion of the pyranose ring (chair to boat forms) or rotation around the glycosidic linkage. In addition, the cellulose chains exist in a partially crystallized form due to interchain hydrogen bonding. Commercial cellulose membranes have a cut off of 8000-15000 daltons for molecular dialysis and on delivery formulations from receptor media in a simple drug release apparatus. Interestingly, they state that this membrane was chosen for investigation in preference to cellulose or silicone media because hydrocortisone acetate was not found to diffuse through the latter two membranes into propylene glycol receptor phase using their particular diffusion cell.

– Synthetic polymers

Diffusion of a molecule through continuous synthetic polymer is analogous in many ways to diffusion through unstirred liquids. Mass transfer through the matrix is dependent on the frequency of void formation of sufficient size to accommodate the diffusant. Voids are formed by the random oscillation of polymer chains and the larger the diffusant species the greater the number of neighbouring polymer units which would have to move in a specific manner in order to generate a void of sufficient volume to accommodate the diffusant. The degree of bonding interaction between the polymer chains will determine the rigidity of the matrix and, thus, the propensity for hole formation and resultant permeability. Furthermore, crystallinity within the matrix generates regions of low diffusivity and the presence of solvents facilitates the oscillation of polymeric segments, both situations altering the overall permeability of the membrane [97].

4.2 *In vitro* model

Drug permeation across the skin is evaluated using different *in vitro* models. These include horizontal-type skin permeation system, Franz diffusion cell and the flow-through diffusion cell [102].

4.2.1 Horizontal-type skin permeation system

This has been widely used for the evaluation of drug permeation across skin. The cell is divided into receptor and donor compartments with a low solution volume (3.5 ml) for each compartment and a small membrane area (0.64 cm^2).They are continuously stirred by a matched set of star-head magnets, which are rotated at a speed of 600 rpm. The system is controlled by circulated thermostated water through a water jacket surrounding the two compartments [101].

4.2.2 Franz diffusion cell

The cell is composed of two compartments: donor and receptor. The receptor compartment has a volume of 5–12 ml and an effective surface area of 1.0–5.0 cm^2. The diffusion buffer is continuously stirred at 600 rpm by a magnetic bar. The temperature in the bulk of the solution is maintained by circulating thermostated water through a water jacket that surrounds the receptor compartment [101].

4.2.3 Flow-through diffusion cells

Flow-through diffusion cells have the advantage that they can be used when the drug has lower solubility in the receptor compartment. In addition, these cells can be fully automated and connected directly to HPLC. They have a large-capacity donor chamber to allow appropriate loading of the applied compound and a low volume (0.3 ml) receiving chamber that ensures rapid removal of penetrant at relatively low (1.5 ml or less) pumping rates. Furthermore, various sized support disks allow skin pieces as small as 4 mm in diameter to be use [101].

4.3 Considerable receptor solution for in vitro study

Ideally, the receptor solution used for in vitro permeation experiments should mimic the in vivo situation. The selection of an appropriate receptor solution to mimic the real condition is thus essential for good in vitro experimental design. The selection of a receptor solution largely depends on the nature of the permeant and on diffusion cell design. Aqueous receptor solutions are the most commonly used media for hydrophilic and moderately lipophilic permeants. Buffered solutions such as phosphate, around pH 7.4, are often used where species are ionisable. For unionisable species, water may be used and an antimicrobial agent may be added to the receptor solutions. Typically, 25% ethanol/water receptor phases have been shown to provide reasonable sink conditions for many lipophilic molecules traversing the skin [96].

The percutaneous permeation of a drug across the skin is measured by collecting receptor fluid using either static or continuous flow-through collection. As mentioned previously, the solubility of the drug in the receptor fluid determines the diffusion-cell apparatus to be used. A static diffusion cell such as the Franz diffusion cell or horizontal-type skin permeation system can be used if the permeation of the drug across the skin will not result in a concentration of .10% of the maximal solubility in the receptor fluid. By contrast, if the drug has a low solubility in the receptor fluid, a flow-through apparatus is recommended. A further consideration is that the activity of hydrolytic enzymes released during tissue isolation might be higher with the static apparatus than the flow-through apparatus [97].

CHAPTER III
MATERIALS AND METHODS

Materials and Methods

1. Material

1.1 Chemical

The reagent and substances used in the present study were listed as the following:

1. Miacalcic (Salmon Calcitonin nasal Spay 2200 IU/mL; Novastis)
2. Calco (Salmon Calcitonin Injection 100 IU/mL; LisaPharma, Italy)
3. Acetonitrile (Lab scan, Thailand)
4. Sterile water (Thai Nakorn Pattana CO., LTD, Thailand)
5. Tetramethylammonium hydroxide (Merck, German)
6. Disodium Hydrogen orthophosphate
7. Potassium dihydrogen orthophosphate
8. Sodium chloride
9. Methanol (Lab scan, Thailand)
10. Orthophosphoric acid
11. 1- Methyl-2- pyrrolidone(Fluka, German)
12. Sodium Lauryl sulphate (Ajax Fineochem)
13. Ethyl oleate (Fluka, German)
14. Chitosan (Seafresh)
15. Oleic acid (Pamreac)
16. Isopropyl myristate (Fluka, German)
17. Acetone (Lab scan, Thailand)

1.2 Instrument

1. High performance liquid chromatography analyzed by Water
    a. Separation module (Water 2690)
    b. UV spectrophotometer (Water 2485)

2. High performance liquid chromatography analyzed by Shimadzu Corporation, Japan
    a. Column (Thermosil®, BDS C18 5µm, 250 × 4.6 mm) USA
    b. Model LC 10AD pump (Shimadzu Corporation, Japan)
    c. SPD 10AV detector model (Shimadzu Corporation, Japan)
    d. SIL 10AV auto injection model (Shimadzu Corporation, Japan)
    e. Computer integrator, RF 10A version 1.1 software LC program (Shimadzu Corporation, Japan)
3. Franz diffusion cells (NK supplies, Thailand)
    a. Water bath (Thermomix®, Braun Melsungen AG, West Germany
    b. Magnetic stirring stand (Gallenkamp, England)
4. Liquid Chromatography Mass spectrophotometer
5. Scanning Electron Microscope (SEM)
6. Franz cell diffusion
7. Fourier transform infrared spectrum (GX PERKIN ELMER)
8. Analytical balance (Model 2482, Sartorius, Germany)
9. pH meter (SA 520 pH meter, Orion, USA)
10. Ultrasonicator (Cavitator® Mettler Electronics®, USA)
11. Centrifuge machine (Model 30 F, Hettich Universal Dupont, Germany)
12. Vortex mixer
13. Micropipette (5-50 µl: Axygen, USA)
14. Micropipette (20-200 µl: Axygen, USA)
15. Micropipette (100-1000 µl: Axygen, USA)
16. Membrane filter (cellulose acetate membrane, 0.45 µm, Sartorius, Germany)
17. Pipette tips (20-200 µl: Axygen, Inc., CA., USA)
18. Centrifuge tube (2 ml: Axygen, Inc., CA., USA)

2. A validated assay for Salmon Calcitonin analysis

2.1 High performance liquid chromatography made by Water

2.1.1 Method I The method uses a 5-µm octadecasilyl silica column (100 × 4.6 mm) at 65°C A: 1 M tetramethylammonium hydroxide—water—acetonitrile, 20:880:100 B: 1 M tetramethylammonium hydroxide—water—acetonitrile, 8:392:600

Solutions A and B are adjusted to pH 2.5 with phosphoric acid. Detection was by UV spectrophotometry at 210 nm; Flow rate: 1 ml/min Injection volume 100 µl

Table 2 Linear gradient of HPLC method for Salmon Calcitonin of Method I

	Time	Flow	%A	%B
1		1	72	28
2	30	1	48	52
3	32	1	72	28
4	35	1	72	28

2.1.2 Method II The method uses a 5-µm octadecasilyl silica column (100 × 4.6 mm) at 50°C A: 1 M tetramethylammonium hydroxide—water—acetonitrile, 20:880:100 B: 1 M tetramethylammonium hydroxide—water—acetonitrile, 8:392:600 Solutions A and B are adjusted to pH 2.5 with phosphoric acid. Detection was by UV spectrophotometry at 210 nm; Flow rate: 1 ml/min Injection volume 100 µl

Table 3 Linear gradient of HPLC method for Salmon Calcitonin of Method II

	time	Flow	%A	%B
1		1	65	35
2	30	1	43	57
3	32	1	65	35
4	35	1	65	57

2.2 High performance liquid chromatography made by Shimadzu Corporation, Japan

The analytical method consists of a 5-µm octadecasilyl silica column (100 × 4.6 mm) at 50°C. The gradient system of biphasic mobile phase is A: 1 M tetramethylammonium hydroxide:water:acetonitrile, 20:880:100 and B: 1 M tetramethylammonium hydroxide:water:acetonitrile, 8:392:600. Phase A and B are adjusted to pH 2.5 with phosphoric acid and detected by UV spectrophotometry at 210 nm; flow rate: 1 ml/min; injection volume 20 µl and linear gradient shown in Table 4.

Table 4 Linear gradient of HPLC method analyzed by Shimadzu Corporation

	Time	Flow (ml/min)	%A	%B
1		1	65	35
2	30	1	43	57
3	32	1	65	35

2.3 Determination of salmon calcitonin by Liquid Chromatography Mass Spectrophotometer

The LCMS system consist of a binary pump, and an autosampler equipped with a 10 µl. Mobile phase were A: 5% acetonitrile in water with 0.1% formic acid and B: 80% acetonitrile in water with 0.1% formic acid.

Table 5 Linear gradient of LCMS method for Salmin Calcitonin

	Time (min)	Flow (ml/min)	%A	%B
1		1	100	0
2	30	1	50	50
3	32	1	100	0

3. Preparation Salmon Calcitonin in Chitosan formulation

3.1 Preparation Synthesis of PEG-Crosslinked Chitosan (Chitosan Hydrogel)

According to Fangkanwanwong J. [100], they were demonstrated PEG-Crosslinked Chitosan preparation. For preparation Chitosan HOBt aqueous solution, Chitosan (0.1 g, 0.61 mmol) was vigorously stirred with two molar equivalents of HOBt H_2O (0.188 g, 1.22 mmol) in 10 mL of deionized water at ambient temperature until the clear solution.

PEG (Molecule 1,450, 20.00 g, 13.8 mmol) was reacted with succinic anhydride (2.762 g, 27.6 mmol) in the presence of a catalytic amount of pyridine at 60 ºC for 24 h. The crude product was purified by reprecipitating in diethyl ether,

washing several times, and drying under vacuum to obtain carboxy-terminated poly(ethylene glycol) (COOH-PEG-COOH).

To the Chitosan HOBt aqueous solution of 1 (10 mL), 5 (0.479 g, 0.305 mmol, 10 mL) and WSC HCl (0.235 g, 1.22 mmol, 10 mL) were added with distilled water. The reaction was carried out at ambient temperature to obtain a gel.

3.2 Preparation salmon calcitonin Combine with Chitosan HOBt Solution Formulation

Miacalcic 3.6 ml (approximately amount salmon calcitonin 2.36 mg) was evaporated to dryness by speed vacuum then was mixed and stirred with 1 ml chitosan HOBt solution. Final weight of formulation as 576.7 mg

3.3 Preparation salmon calcitonin combince with Chitosan Hydrogel

Miacalcic 3.6 ml (approximately amount salmon calcitonin 2.36 mg) was evaporated to dryness by speed vacuum then was mixed with 1 ml chitosan solution. After that, diPEG 9 mg and WSC 23.5 mg was added and stirred in chitosan solution formulation. Final weight of formulation as 847.6 mg

3.4 An analysis FTIR Chitosan Hydrogel and Salmon Calcitonin

Chitosan hydrogel, salmon calcitonin injection and salmon calcitonin in chitosan hydrogel formulation were dried approximately 0.2 mg and mixed with KBr. These were scanned by FTIR spectrum (GX PERKIN ELMER)

4. Ultrasound application

Ultrasound (Faculty of Engineering, Mahidol University, Thailand) is applied at frequency 1 MHz can be adjusted to low intensity; 2.3 Watt (rms) and high intensity; 3.2 Watt (rms) with various applied duration of 0 and 10 min.

4.1 Skin toxicity studies, histopathology of skin

4.1.1 Preparing 2.5% Glutaraldehyde in 0.1 M CaCo

2.5% Glutaraldehyde in 0.1 M CaCo consist of 5% Glutaraldehyde: 0.2 M Sodium Cacodylate: 0.2 M Calcium acetate in 5:4:1.(0.2 M Sodium Cacodyte buffer contained Sodiun Cacodyte 4.28 g, Single distilled water 100 ml and 0.2 M Calcium acetate made of Calcium acetate 3.16 g, Single distilled water 100 ml)

4.1.2 Sample preparation for scanning electron microscopy

The surface appearance and pore size were analyzed by Scanning electron microscope to evaluate the morphological variations of the specimens comparing among untreated (control), immediately after treated and after treated for 24 hours (reversibility). Samples were prepared by primary fixation with 2.5% glutaraldehyde and by drying them under critical point to preserve membrane integrity. The samples were then coated in a cathodic evaporator with a platinum/palladium alloy and observed by a Hitachi scanning electron microscope S-2500 (Japan). The sample preparation is shown in Table 6**.**

Table 6 Process of preparing sample before for scanning electron microscopy

Activity	**Chemical**	**Temperature**	**Time**	**Repetition**
Primary fixation	2.5% Glutaraldehyde in 0.1 M buffer pH 7.2	4 ºC	2 hr	1
Wash	0.1 M buffer pH 7.2	4 ºC	20 min	3
Secondary fixation	1% osmium tetroxide in 0.1 M buffer pH 7.2	4 ºC	1 hr	1
Wash	0.1 M buffer pH 7.2	4 ºC	20 min	3
Dehydration	30% ethanol 50% ethanol 70% ethanol 85% ethanol 90% ethanol 95% ethanol 100% ethanol 100% ethanol	4 ºC 4 ºC 4 ºC 4 ºC 4 ºC 4 ºC 4 ºC 4 ºC	15 min 15 min 15 min 15 min 15 min 15 min 15 min 15 min	2 2 2 2 2 2 2 2
Critical point dry				
Mount on specimen stub with graphite tape				
Coat with platinium/palladium alloy				
Store stubs in Desiccator				

4.1.3 Evaluation by Scanning Electron Microscope (SEM)

Histological changes in the porcine ear skin are examined after ultrasound, immediately after pretreatment of ultrasound or combination with chemical enhancer. A specimen of the exposed area is taken for histological examination. The adjacent untreated skin area is also assessed as the control. The specimens are evaluated under a Scanning Electron Microscope (SEM)

4.2 Stability of salmon calcitonin

4.2.1 Temperature

Miacalcic 1 ml was diluted in 8 mL of 0.1 M phosphate buffer saline (PBS), pH 7.4. 1 ml of solutions is used immediately upon preparation. Salmon calcitonin stability was determined in Saline phosphate buffer solution at 25ºC for 24 hour. Approximately 200 µl of fresh stock solutions are assayed by HPLC.

4.2.2 Ultrasound

Miacalcic 1 ml was diluted in 8 mL of 0.1 M phosphate buffer saline (PBS), pH 7.4. 1.6 ml of solutions was used immediately upon preparation. Salmon calcitonin stability was determined in Saline phosphate buffer solution at 10 min in high and low intensity. Approximately 200 µL was assayed by HPLC.

5. In vitro release study: Franz diffusion cells

For this investigation, static Franz glass diffusion cells are used. These cells are consisted of donor and receptor chambers between which a membrane is positioned. The diffusion area is 7.07 cm^2 and the receptor chamber volume is10 ml.

The receptor chamber is maintained at 37 ºC. A solution of PBS composing of Disodium Hydrogen orthophosphate: Potassium dihydrogen orthophosphate: Sodium chloride, 2.38 g: 0.19 g: 8 g in 1000 ml sterile water for injection, adjusted pH to 7.4 by Orthophosphoric acid is used as the receptor medium. Each cell is homogeneous by a magnetic bar and continuously stirred during the experiment. All diffusion cells are prepared and allowed to equilibrate for 1 hour before applied the samples

5.1. Membrane preparation

5.1.1 Regenerated cellulose tubular membrane preparation

Regenerated cellulose tubular membrane (Cellu-sep) with an average pore size of 12,000-14,000 molecular weight cut off. Regenerated cellulose tubular membranes were soaked in PBS for 15 min prior to use.

5.1.2 Skin preparation

Porcine ears were obtained from a local market. Full thickness membranes were prepared by the heat-separation technique by being soaked in 60ºC water for 45 seconds, followed by careful removal of the lipid layers. The epidermis was then

washed with water and kept at -20 ºC. The membrane was thawed for 30 minutes prior to use.

5.2 In vitro release study of salmon calcitonin in PBS

Miacalcic 1 ml (approximately amount salmon calcitonin 0.66 mg) was evaporated to dryness then the residue was reconstituted by PBS 500 µl. Calcitonin solution at a concentration of 1.21 mg/ml in 500 µl has been added to the donor compartment; 6 ml sample has been taken at 48 hours and evaporated to dryness by speed vacuum. The residue has been reconstituted in 300 µl of sterile water and 100 µl has been injected onto the chromatographic column. Regenerated cellulose tubular membrane was used as membrane.

5.3 In vitro release study of salmon calcitonin in PBS and the applied ultrasound

Miacalcic 1 ml (approximately amount salmon calcitonin 0.66 mg) was evaporated to dryness then the residue was reconstituted by PBS 1,600 µl. Calcitonin solution at a concentration of 0.52 mg/ml in 1,600 µl was added to the donor compartment; 6 ml sample has been taken at 24 hours and evaporated to dryness by speed vacuum. The residue has been reconstituted in 200 µl of sterile water and 100 µl was injected onto the chromatographic column. Regenerated cellulose tubular membrane was used as membrane.

5.4. In vitro release study of salmon calcitonin in chitosan HOBt solution and the applied ultrasound

Each cell is homogeneous by a magnetic bar and continuously stirred during the experiment. All diffusion cells are prepared and allowed to equilibrate for 1 hour before applied the samples. Chitosan HOBt solution was separated to control and treat by low intensity (2.3 walt (rms) ultrasound for 20 min each approximately 0.3 mg. Regenerated cellulose tubular membrane was as membrane.

Table 7 Process of *In vitro* penetration study of salmon calcitonin in chitosan HOBt solution.

Formulation / Condition	Chitosan solution
Added in donor chamber	0.3 mg
Duration time	12 hr.
Receiver	10 ml

Receiver phase was collected 10 ml. Each 10 ml-sample was withdrawn within 12 hours and evaporated to dryness by speed vacuum. The residue was reconstituted in 400 μl of mobile phase A and 20 μl has been injected onto the chromatographic column.

5.3. In vitro release study of salmon calcitonin in Chitosan Hydrogel and the applied ultrasound

Each cell is homogeneous by a magnetic bar and continuously stirred during the experiment. All diffusion cells are prepared and allowed to equilibrate for 1 hour before applied the samples. Salmon Calcitonin in chitosan hydrogel was added follow to table 7 in the donor compartment. Chitosan HOBt solution was separated to control and treat by low intensity (2.3 watt (rms) ultrasound for 20 min each approximately 0.3 mg. Regenerated cellulose tubular membrane was used as membrane.

Table 8 Process of *In vitro* release study of salmon calcitonin in Chitosan Hydrogel

Formulation / Condition	Chitosan Hydrogel
Added in donor chamber	0.3 mg
Duration time	12 hr.
Receiver	10 ml

Receiver phase was collected 10 ml. Each 10 ml-sample was withdrawn within 12 hours and evaporated to dryness by speed vacuum. The residue was reconstituted in 400 µl of mobile phase A and 20 µl has been injected onto the chromatographic column.

5.4 In vitro permeation experiments of salmon calcitonin

Each cell is homogeneous by a magnetic bar and continuously stirred during the experiment**.** Miacalcic 0.5 ml was added in donor phase and porcine skin was used as membrane. Each 6 ml sample was taken at 48 hours and centrifuged at 5000 rpm for 10 minutes and evaporated to dryness by speed vacuum. The residue has been reconstituted in 350 µl of PBS and 100 µl has been injected onto the chromatographic column. The result implied that there was no detectable concentration of calcitonin solution analyzed by RP-HPLC method. Porcine ear skin was used as membrane.

6. Statistical analysis

All skin permeation experiments have been repeated at least three times and data are expressed as the mean value ±S.D. Statistical data are analyzed by one-way analysis of variance (ANOVA). A multiple comparison test is used to compare different control, ultrasound, and enhancers at *P*-value of 0.05 is considered to be significant.

CHAPTER IV
RESULTS AND DISCUSSION

1. A validated assay for Salmon Calcitonin analysis

1.1 High performance liquid chromatography analyzed by Water

1.1.1 Method I

- **Method development**

RP-HPLC-UV was developed and validated as a sensitive and specific method in order to apply as a suitable analytical process for in vitro permeability studies. pH of mobile phase was adjusted and the optimal pH was at 2.5. Moreover, the fixed wavelength was detected and confirmed to be 210 nm.

- **Specificity**

Specificity experiment was carried out using blank samples from drug-free phosphate buffer solution and water comparing with the samples from drug solutions. Chromatogram obtained from blank sample is represented in Figure 4-5. The retention time of calcitonin was 25.7 min (Figure 6). There were no any interfering peaks observed over concerned retention time.

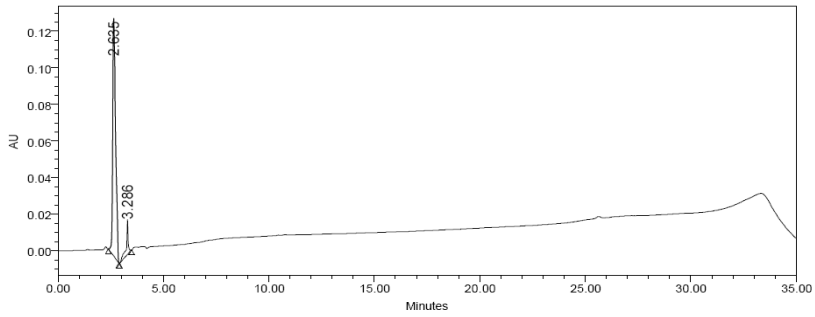


Figure 4 Chromatogram of PBS solution

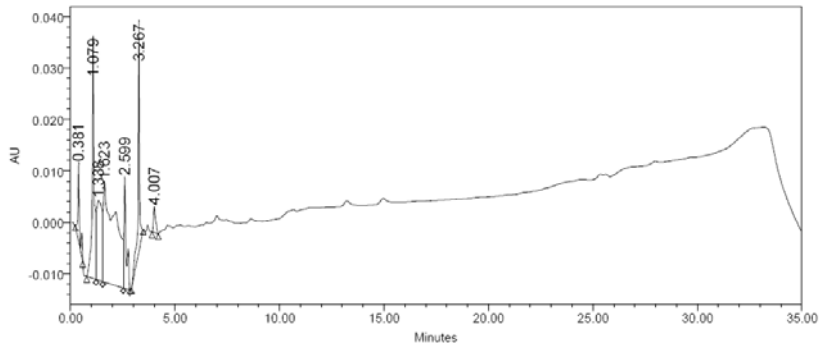


Figure 5 Chromatogram of Water

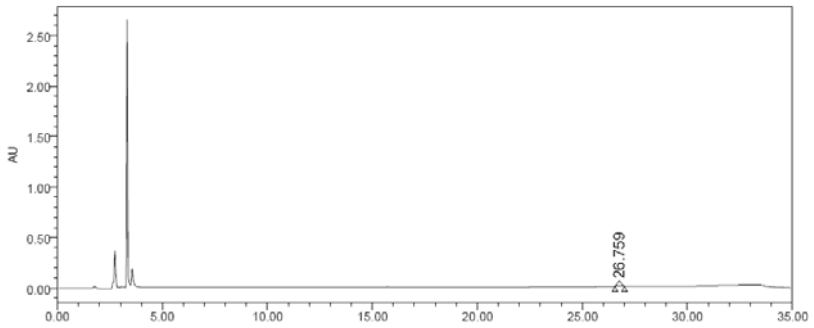


Figure 6 Chromatogram of Calcitonin injection

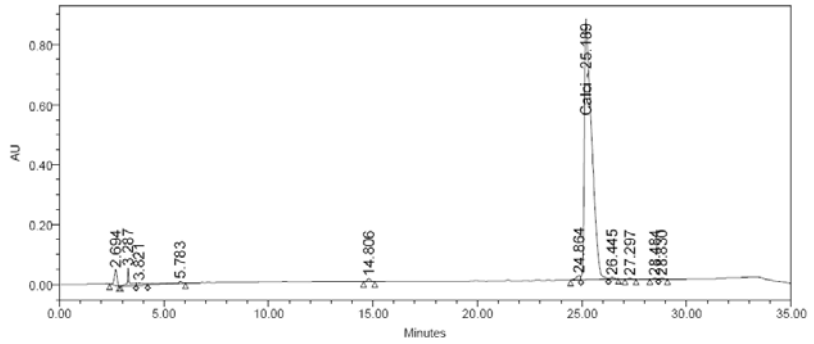


Figure 7 Chromatogram of salmon calcitonin nasal spay 2200 IU/ml

- **Linearity**

A three-point linearity curve was constructed for three consecutive days. Samples were quantified using the concentration–peak area relationships and were calculated by the simple regression analysis y = mx + c. The developed method was found to be linear over concentration ranges of 25-100 μg ml^{-1}. The determination was successfully applied to detect salmon calcitonin concentration.

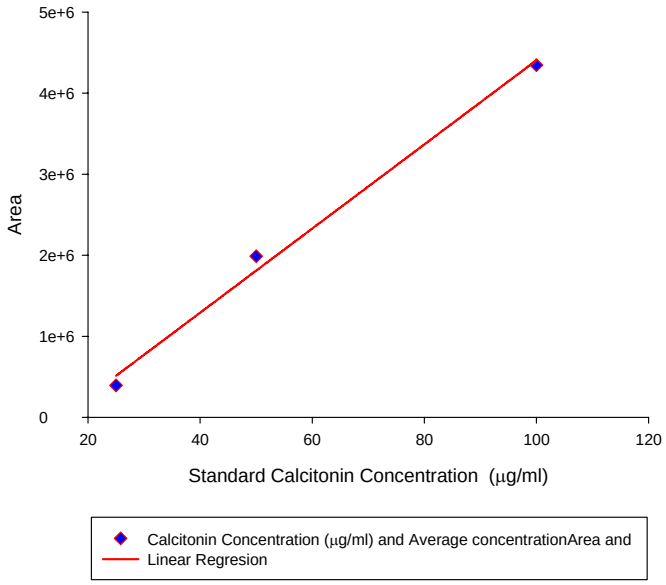


Figure 8 Linearity of Calcitonin concentration in Method I

According to the validation of calcitonin, linear equation was y = 41447x: y = area, x= conc (μg/ml), r^2=0.9419.

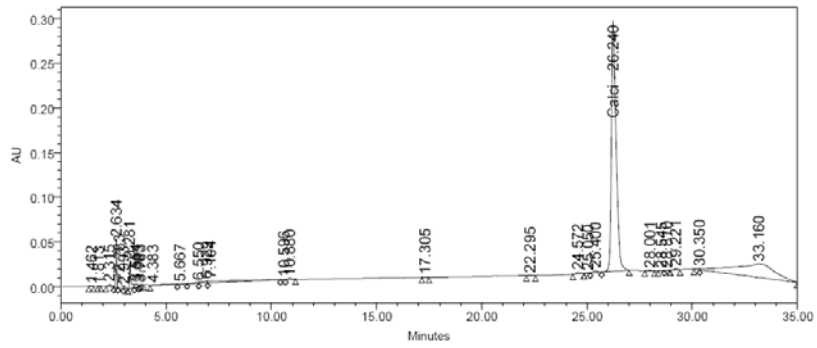


Figure 9 Chromatogram of standard salmon calcitonin 100 μg/ml

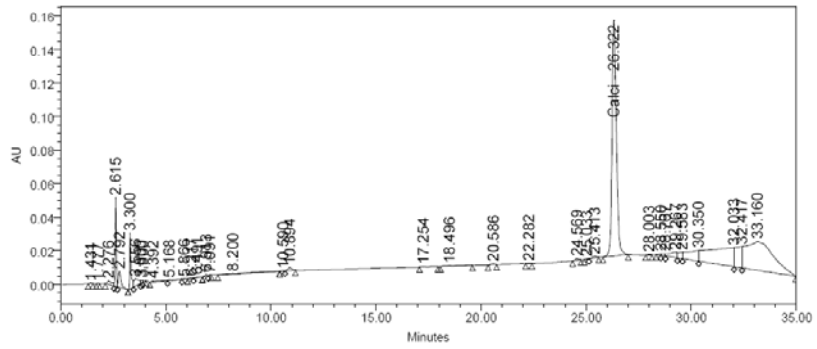


Figure 10 Chromatogram of standard salmon calcitonin 50 μg/ml

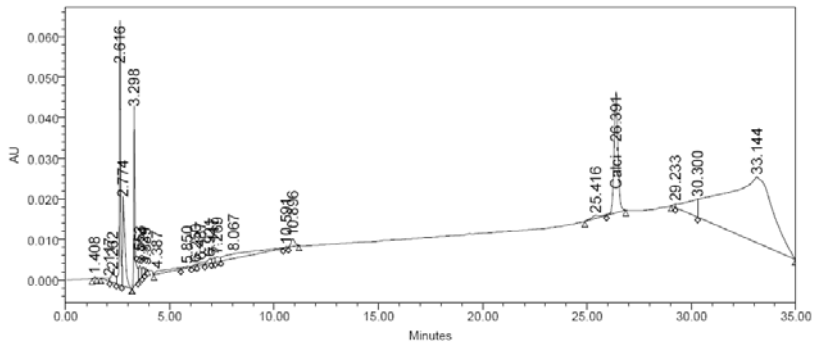


Figure 11 Chromatogram of standard salmon calcitonin 25 μg/ml

1.1.2 Method II

- **Method development**

RP-HPLC-UV was developed and validated as a sensitive and specific method in order to apply as a suitable analytical process for in vitro permeability studies. pH of mobile phase was adjusted and the optimal pH was at 2.5. Moreover, the fixed wavelength was detected and confirmed to be 210 nm.

- **Specificity**

Specificity experiment was carried out using blank samples from drug-free phosphate buffer solution and water comparing with the samples from drug solutions. Chromatogram obtained from blank sample is represented in Figure 12-13. The retention time of calcitonin was 20.9 min (Figure 14). There were no any interfering peaks observed over concerned retention time.

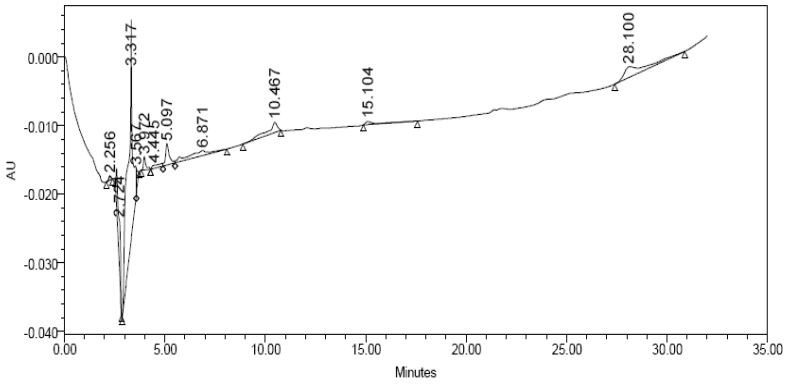


Figure 12 Chromatogram of water (Method II)

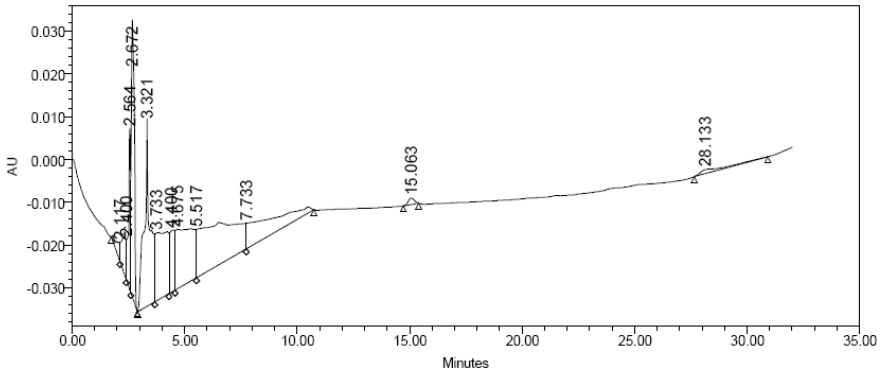


Figure 13 Chromatogram of PBS (Method II)

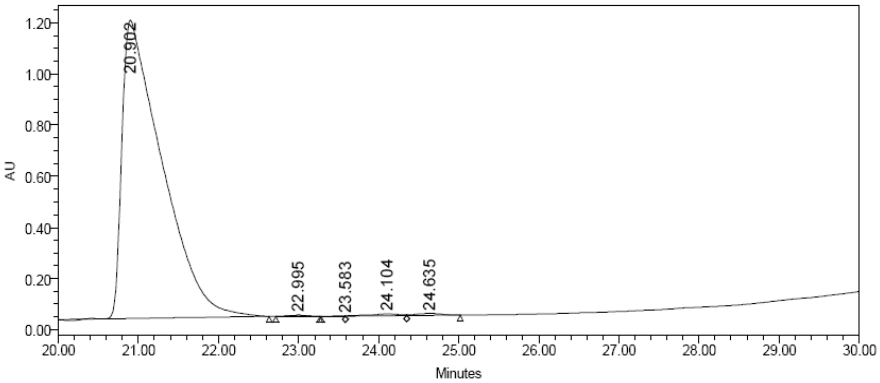


Figure 14 Chromatogram of Nasal spay (Miacalcic®) (Method II)

- **1.1.2.3 Linearity**

A four-point linearity curve was constructed for three consecutive days. Samples were quantified using the concentration–peak area relationships and were calculated by the simple regression analysis: y = mx + c. The minimum correlation coefficient of the calibration curves was 0.9537. Linearity curve is shown in Figure 15. The developed method was found to be linear over concentration ranges of 12.5-100 µg ml^{-1}. The determination was successfully applied to detect salmon calcitonin concentration and showed higher sensitivity of quantitation limit over Method I.

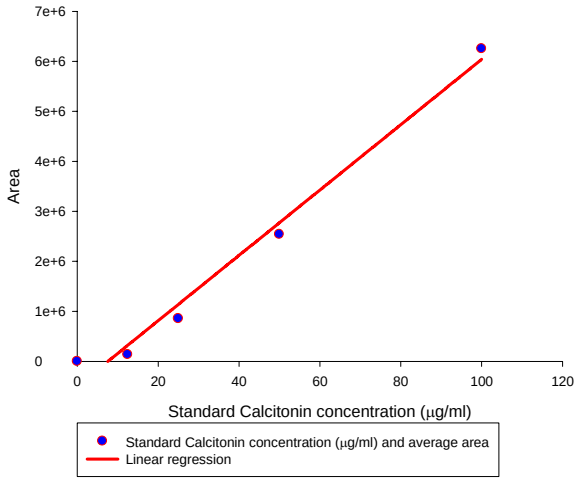


Figure 15 Linearity of Calcitonin concentration (Method II)

According to calibration curve of calcitonin, linear equation was y = 58374x: y = area, x= conc (µg/ml), r^2= 0. 0.9537.

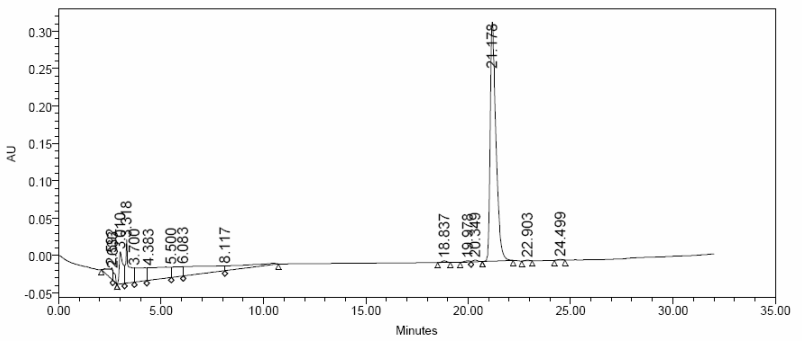


Figure 16 Chromatogram of standard salmon calcitonin 100 µg/ml (Method II)

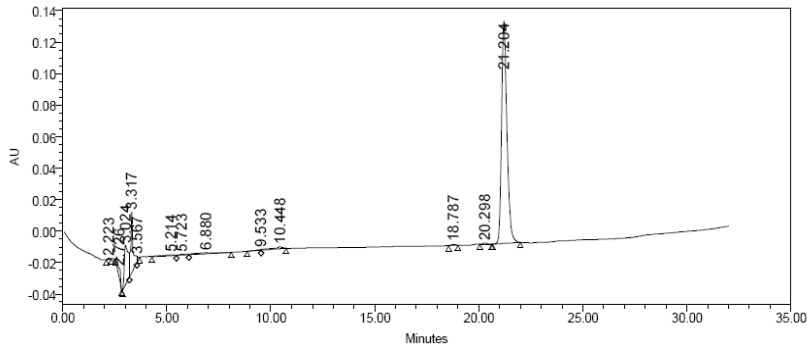


Figure 17 Chromatogram of standard salmon calcitonin 50 μg/ml (Method II)

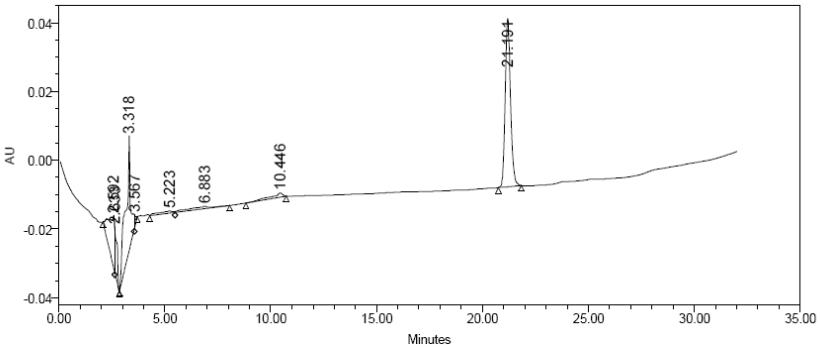


Figure 18 Chromatogram of standard salmon calcitonin 25 μg/ml (Method II)

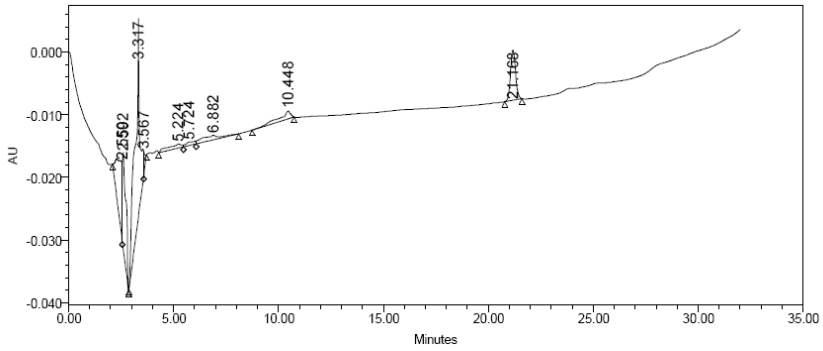


Figure 19 Chromatogram of standard salmon calcitonin 12 μg/ml (Method II)

Both methods of High performance liquid chromatography analyzed by Water can detect salmon calcitonin with good specificity and linearity. The method II probably was better than the method I because method II showed higher sensitivity with shorter retention time. From the result, quantitation limit of salmon calcitonin was observed at 12.5 μg/ml within 32 min (Figure 16-19). In contrast with Method II, the higher quantitation limit of salmon calcitonin was monitored at 25 μg/ml within 35

min (Figure 9-11). The result met with requirement that the most practical method should be rapid and sensitive to perform the appropriate analytical system.

1.2 High performance liquid chromatography analyzed by Shimadzu Corporation, Japan

1.2.1 Method development

RP-HPLC-UV was developed and validated as a sensitive and specific method in order to apply as a suitable analytical process for in vitro permeability studies. pH of mobile phase was adjusted and the optimal pH was at 2.5. Moreover, the fixed wavelength was detected and confirmed to be 210 nm.

1.2.2 Specificity

Specificity experiment was carried out using blank samples from drug-free phosphate buffer solution and water comparing with the samples from drug solutions. Chromatogram obtained from blank sample is represented in Figure 20-21. The retention time of calcitonin is 7.9 minutes (Figure 22). There were no any interfering peaks observed over concerned retention time.

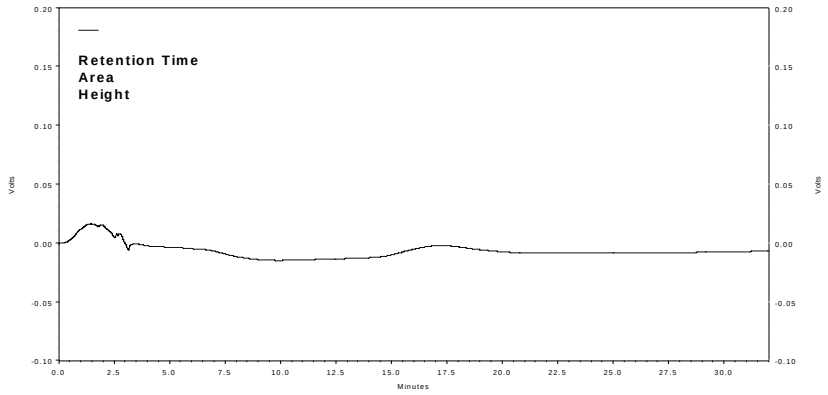


Figure 20 Chromatogram of PBS (Shimadzu Corporation)

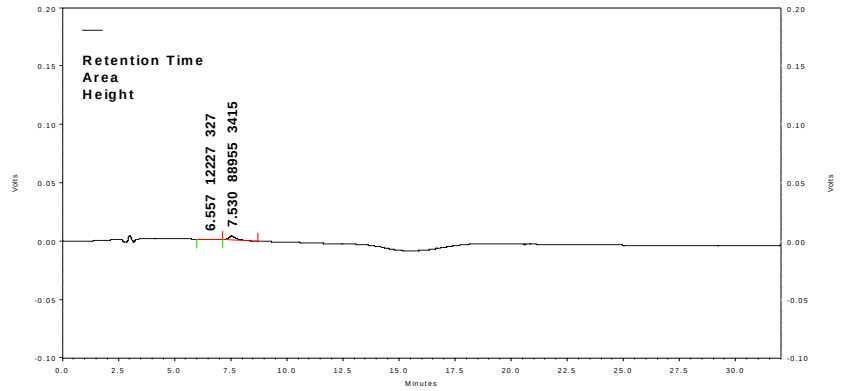


Figure 21 Chromatogram of salmon calcitonin injection (Shimadzu Corporation)

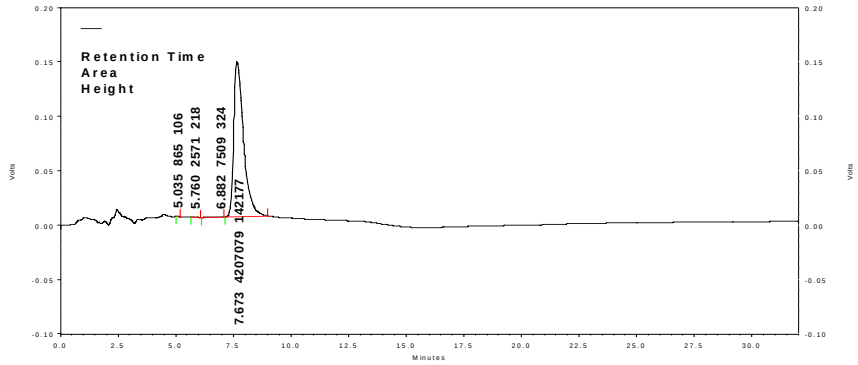


Figure 22 Chromatogram of salmon calcitonin nasal spray (Shimadzu Corporation)

1.2.3 Linearity

A 50 μg standard USP salmon calcitonin was diluted at 100, 25, 12.5, 6.25 and 3.125 μg/ml by sterile water. A 20 μl diluted standard salmon calcitonin was injected onto HPLC-UV. No interference of other components in chromatogram is observed in Figure 23. The developed method was found to be linear over concentration ranges of 6.25-100 μg/ml. The determination was successfully applied to detect salmon calcitonin concentration and showed higher sensitivity of quantitation limit over HPLC of Water.

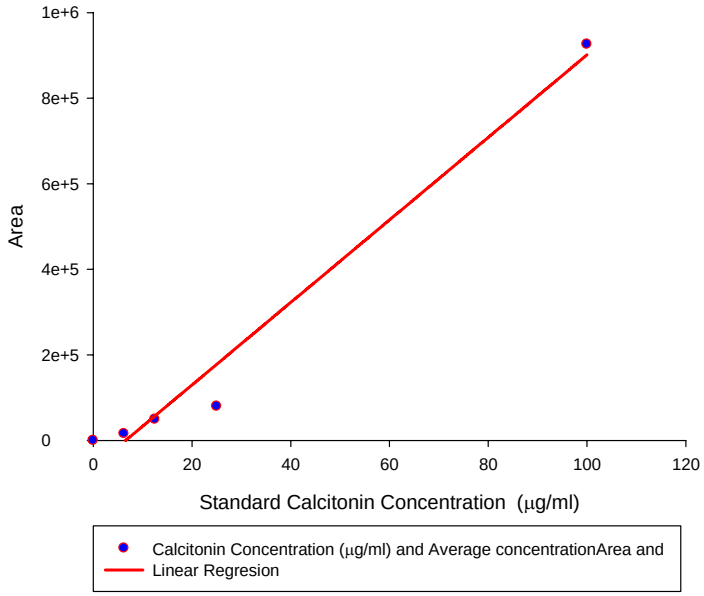


Figure 23 Linearity standard calcitonin (Shimadzu Corporation)

According to the calibration curve of salmon calcitonin, linear equation obtained from calibration curve was y = 9151.3x: r^2 = 0.9886.

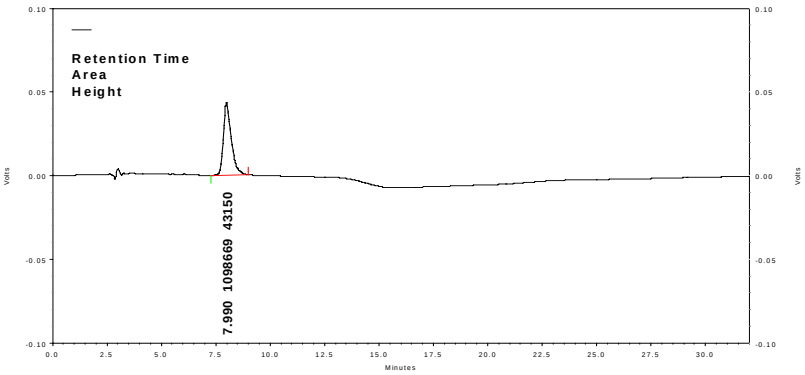


Figure 24 Chromatogram of standard salmon calcitonin 100.0 µg/ml (Shimadzu Corporation)

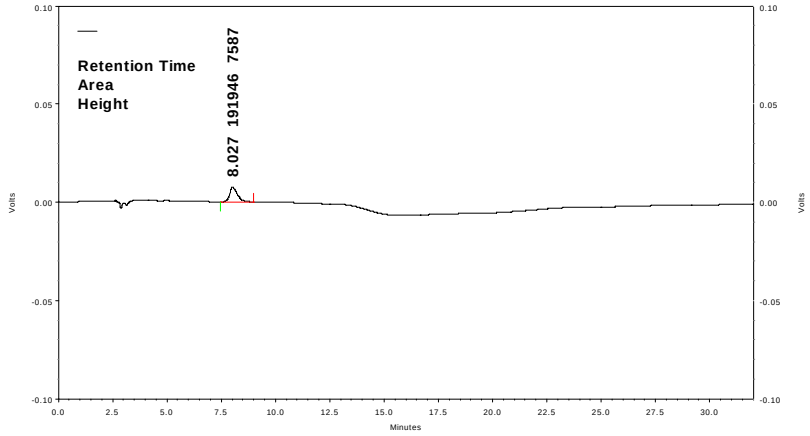


Figure 25 Chromatogram of standard salmon calcitonin 25.0 µg/ml (Shimadzu Corporation)

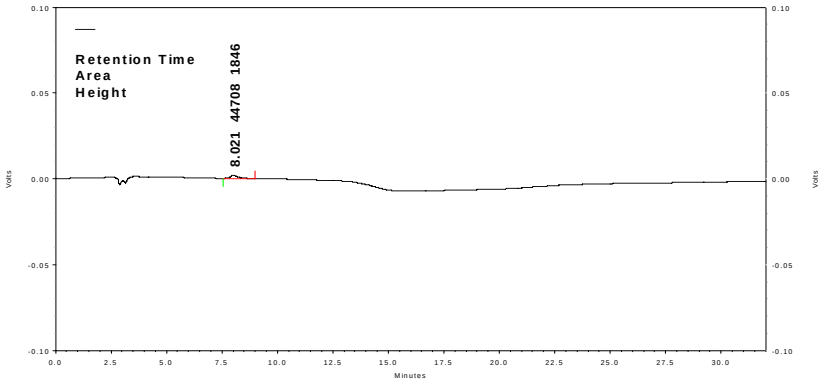


Figure 26 Chromatogram of standard salmon calcitonin 12.5 µg/ml (Shimadzu Corporation)

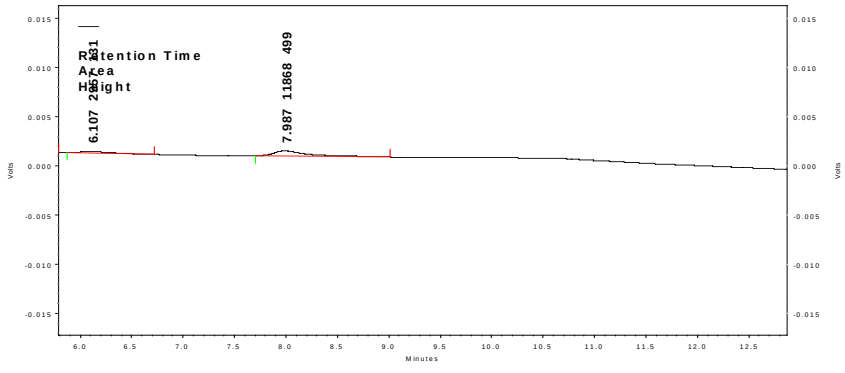


Figure 27 Chromatogram of standard salmon calcitonin 6.25 µg/ml (Shimadzu Corporation)

In the same way, high performance liquid chromatography analyzed by Shimadzu Corporation can detect salmon calcitonin with good specificity and linearity. The limit of quantitation of salmon calcitonin was detected at 6.25 μg/ml within 32 min (Figure 24-27). This method showed higher sensitivity than the methods analyzed by Water. Therefore, salmon calcitonin analysis was carried out on HPLC of Shimadzu Corporation in order to investigate on permeation of calcitonin throughout the experimentation.

2. A validated LCMS assay for Salmon Calcitonin analysis

2.1 Method development

A standard calcitonin of 50 μg/ml was tested by LCMS. The result of experimentation showed that the overall retention time was 15 min by UV detector and MS. Five percentage of formic acid was used as mobile phase by binary gradient LCMS (Figure 28-29). Although, this study was not completed due to the inoperative machine, it anticipated that LCMS analytical method can perform as a sensitive and functional for further *in vivo* salmon calcitonin study.

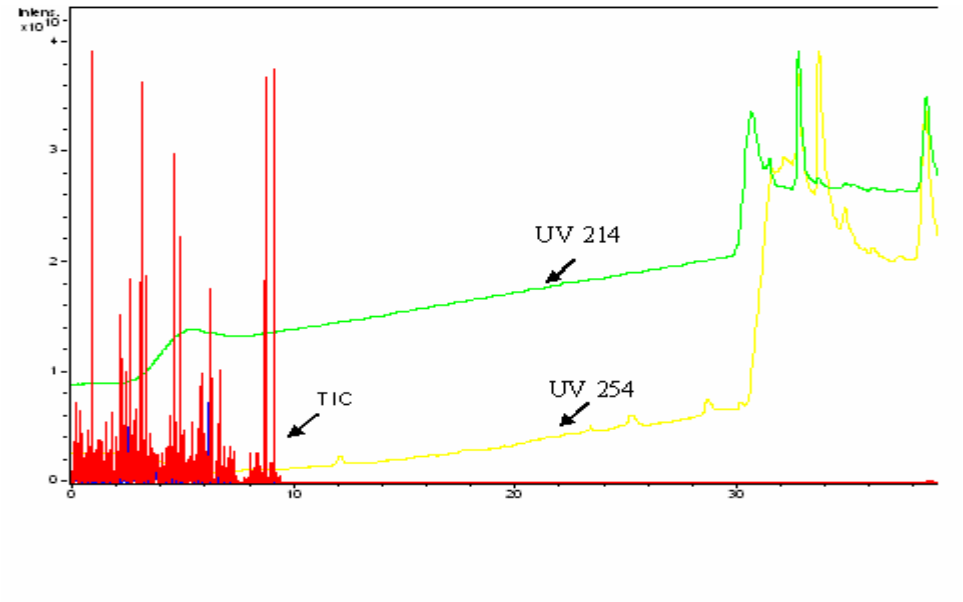


Figure 28 Chromatogram of sterile water for injection; Yellow= UV 214, Green= UV254, Red= TIC, Blue = BPC

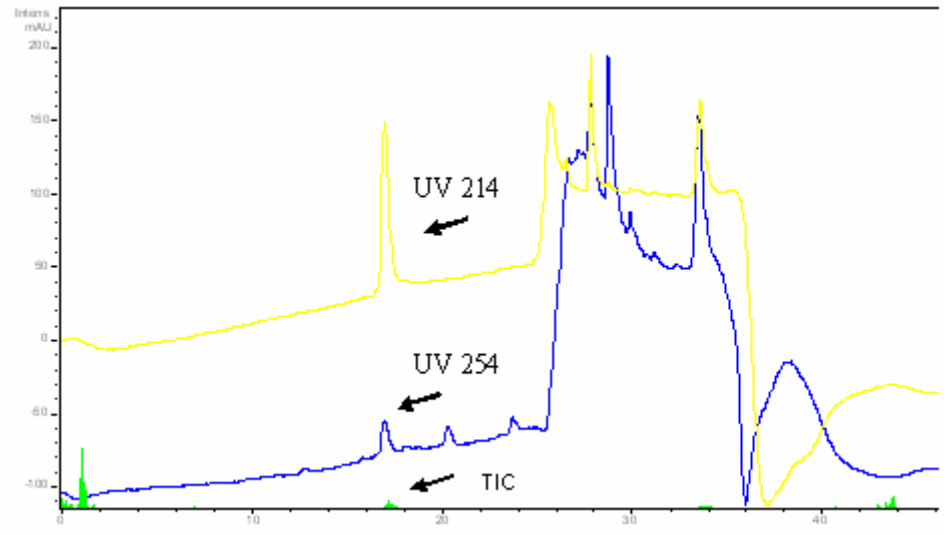


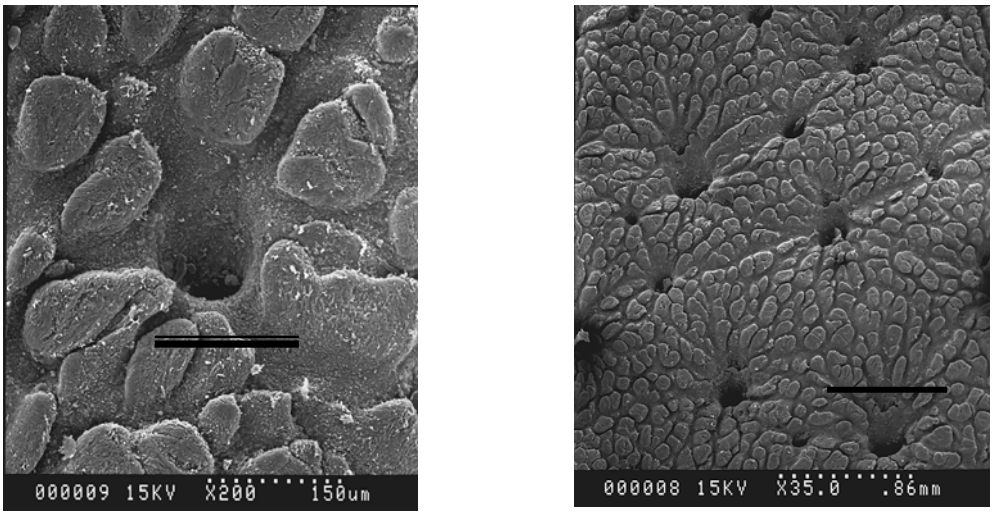
Figure 29 Chromatogram of standard salmon calcitonin 100 μg/ml; Yellow= UV 214, Blue= UV254, Green= TIC, Magenta = BPC

3. Ultrasound

3.1. Histology and electron microscopy of pretreated skin

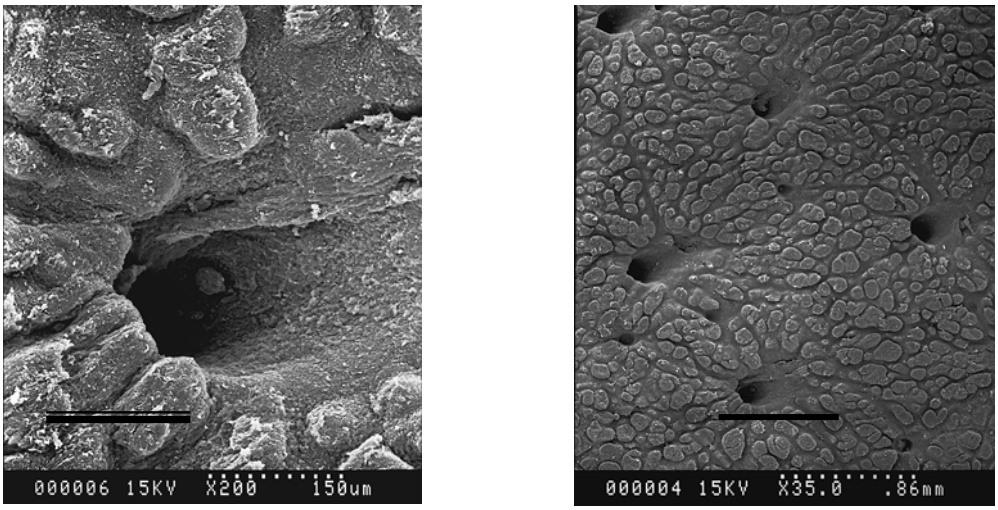
Histological changes in the full thickness pig ear skin were examined after each treatment. Immediately after pretreatment of each enhancer or combination enhancements, a specimen of the exposed area was taken for histological examination. The untreated skin area was also assessed as the control (Figure 30). The specimens were evaluated under an electron microscopy and the images showed that most of the treated studies induced the pore size of the skin significantly. ($p>0.05$)

The average pore sizes of control skin were 70-80 µm. The average pore sizes of treated skin at 2.3 W (rms), 1.0 MHz for 10 minutes were in the ranges of 100-150 µm. The average pore sizes of treated skin at 3.2 W (rms), 1.0 MHz for 10 minutes were in the ranges of 150-200 µm. The average pore size of treated skin at 3.2 W (rms), 1.0 MHz for 20 minutes were in the ranges of 200-300 µm.



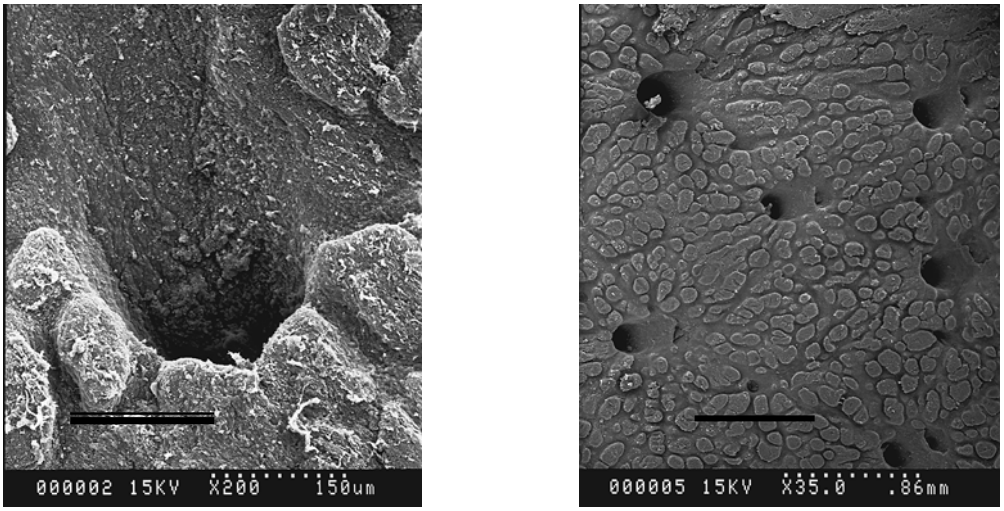
A) B)

Figure 30 Surface membrane of pig ear skin A is the pore size of pig skin aperture. B is the overviewed of whole skin. A Bar, 150 µm: B Bar , 860 µm.



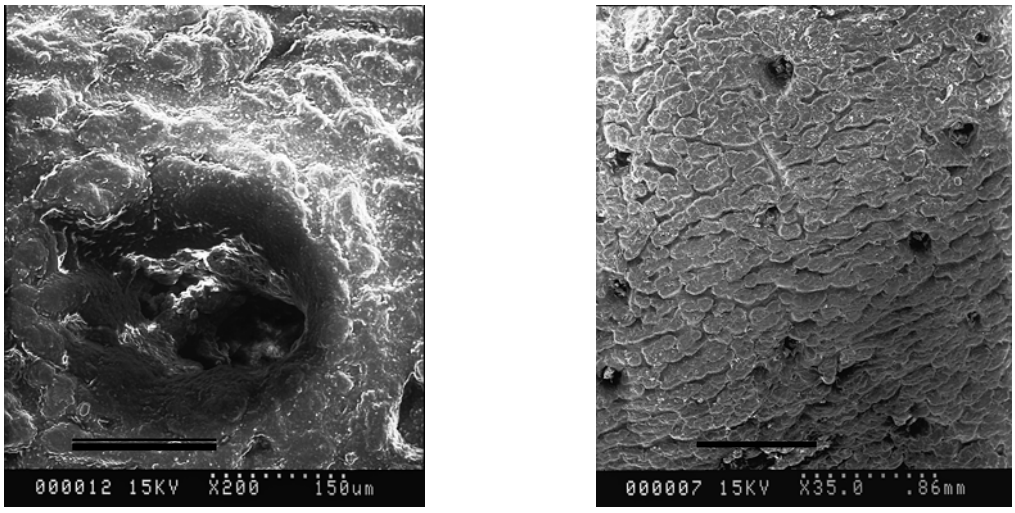
A) B)

Figure 31 Surface membrane of treated pig ear skin with low intensity ultrasound for 10 minutes (2.3 Walt (rms), 1 MHz 10 minutes) A is the pore size of pig skin aperture. B is the overviewed of whole skin. A Bar, 150 µm: B Bar , 860 µm.



A B

Figure 32 Surface membrane of treated pig ear skin with high intensity ultrasound for 10 minutes (3.2 walt (rms), 1 MHz 10 minutes). A is the pore size of pig skin aperture. B is the overviewed of whole skin. A Bar, 150 µm: B Bar , 860 µm.



A B

Figure 33 Surface membrane of treated pig ear skin with high intensity ultrasound for 20 minutes (3.2 walt (rms), 1 MHz 20 minutes). A is the pore size of pig skin aperture. B is the overviewed of whole skin. A Bar, 150 µm: B Bar , 860 µm.

3.2 Stability of salmon calcitonin

Evaluation on stability of salmon calcitonin was evaluated in order to investigate the effect of temperature and ultrasound exposure.

3.2.1 Temperature

Degradation of salmon calcitonin in PBS was investigated at 25 ºC. Final residual ratio was approximately 0.65 between salmon calcitonin incubated over 24 hr compared with freshly calcitonin injected at 0 minute. Although, many studies claimed that vials of calcitonin salmon nasal spray (2200 IU/mL) were stored for three days at 25, 40, or 60 degrees ºC which was stable under all study conditions except initial storage at 60 degrees. On the other hand, amount of salmon calcitonin kept at 25ºC showed sign of low stability over 24 hr. This phenomenon can be explained by the aggregation of the salmon calcitonin induced by metal ion presenting in the solution.

Table 9 Stability of salmon calcitonin at 25 ºC compared with control

Temperature	**Control**	**25 ºC**
Salmon Calcitonin Concentration (µg/ml)	81.92	53.73

3.2.2 Ultrasound

When Salmon Calcitonin in PBS was exposed by ultrasound for 10 min with both low and high intensity, the residual ratio compared to the untreated of Salmon Calcitonin was approximately 0.42 and 0.54, relatively. It was shown from both results that ultrasound application significantly affected stability of salmon calcitonin. Many researches have studied on the possibility of deaggregated effect of ultrasound on peptide drug which was believed to involve in the peptide stabilization. The contrary result suggested that additional sonication parameters should be concerned: application mode between pulse and continuous, frequency set.

Table 10 Stability of salmon calcitonin exposed with high and low intensity

		Intensity of ultrasound	
Applied by Ultrasound 10 min	**Control**	**Low**	**High**
Salmon Calcitonin Concentration (μg/ml)	81.92	34.97	44.50

4. In vitro release study of salmon calcitonin: Franz diffusion cells

4.1 In vitro release of Salmon calcitonin solution

Each 6 ml sample was taken at 48 hours and evaporated to dryness by speed vacuum. The residue has been reconstituted in 300 µl of PBS and 100 µl has been injected onto the chromatographic column. The Calcitonin Concentration filled in donor phase. (µg/ml) was 1209.94 µg/ml. The result implied that the average minimum concentration of Calcitonin solution that can be detected by RP-HPLC method was equivalence 0.36 µg/ml. The result of percent cumulative release of calcitonin per area was 5.11± 2.96 %/cm^2 $x10^{-2}$

4.2 In vitro release study of salmon calcitonin in PBS with ultrasound application

The result of this study showed that percent cumulative release of salmon calcitonin after ultrasound application with high intensity for 10 min was not significantly different from control. It was not in agreement to the assumption that molecules of salmon calcitonin may not be fragmented by ultrasound. Therefore, the molecules were not small enough to passage through a pore size of a membrane which was less than 12,000-14,000 molecular weight cut off.

The aggregation process may be anticipated since percent cumulative release of salmon calcitonin longer than 6 hr produced the steady quantity. Further observation is proposed by TEM (Transmission Electron Microscopy) to confirm any aggregation incident.

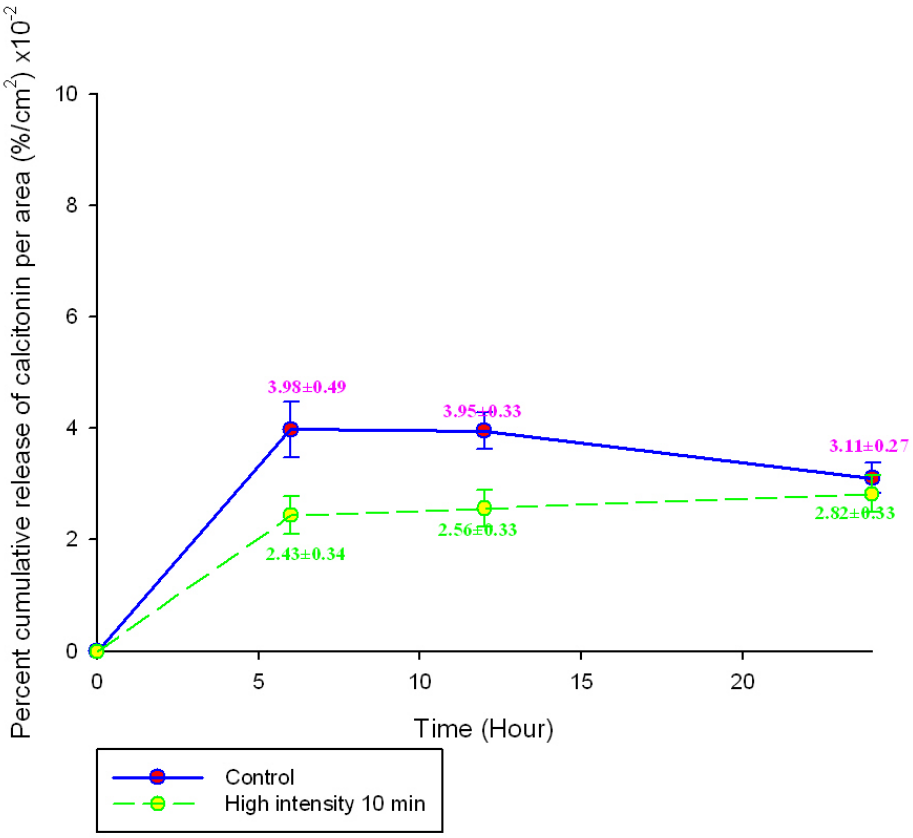


Figure 34 Comparison Percent Release salmon calcitonin per area between control (no applied ultrasound) and applied ultrasound high intensity for 10 min at 6, 12, 24 hr

4.3 In vitro release study of salmon calcitonin chitosan HOBt solution and the applied ultrasound

The release calcitonin amount of chitosan solution formulation in receiver phase was as well as0.024 $x10^{-2}$ $\%/cm^2$. On the other hand, chitosan solution was applied by ultrasound 1 MHz in low intensity; 2.3 walt (rms) for 20 minutes. The release profile of chitosan solution formulation was higher as well as 0.042 $x10^{-2}$ $\%/cm^2$. According to this result, the effect of ultrasound can be concluded that the applied ultrasound can increase the release calcitonin amount of chitosan solution.

4.4 In vitro release study of salmon calcitonin chitosan Hydrogel with ultrasound application

Consistent with chitosan HOBt solution, Chitosan hydrogel formulation was applied by ultrasound at 1 MHz with low intensity; 2.3 Watt (rms) for 20 minutes. The result implied that ultrasound can increase released amount of calcitonin as well as $0.024x10^{-2}\%/cm^2$. In contrast with chitosan hydrogel formulation without ultrasound application, released amount of salmon calcitonin was below quantitation limit. The comparison is shown in Figure 35.

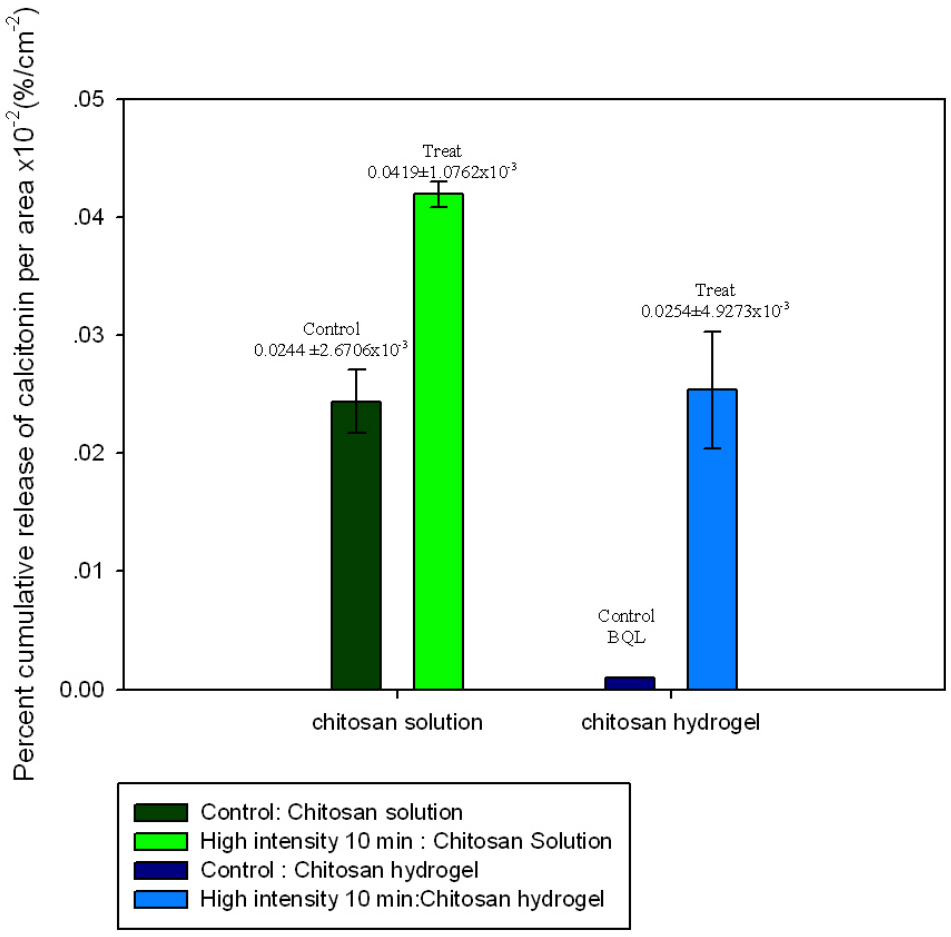


Figure 35 Comparison of chitosan formulations, ultrasound application in vitro franz cell diffusion

5. In vitro permeation experiments

It was clearly understandable that calcitonin alone can not passage through the physiological membrane to give required systemic effect and additionally, extra penetration enhancements may improve the overall outcome.

In further experimentation, chemical enhancers were added as pretreatment at 1 and 2 hours prior to the calcitonin, the result was shown that calcitonin concentration can not be detected. On the contrary, calcitonin solution along with chemical enhancers over 48 hr expressed the detectable outcome. The result relating with Isopropyl myristate as chemical enhancer is shown in Table 11. Isopropyl myristate was filled for 0.5 ml in donor phase for 48 hour. The result of percent release salmon calcitonin per area produced the percent release in term of 238.58 $x10^{-2}$ $\%/cm^2$

Table 11 Concentration of drug permeation from solution

Calcitonin Concentration filled in donor phase. (µg/ml)	**Percent Release salmon calcitonin per area(%/cm^2 x10^{-2})**
604.95	0.023± 0.0025

6. An analysis FTIR Chitosan Hydrogel and Salmon Calcitonin

The FTIR of chitosan hydrogel, pure salmon calcitonin solution and salmon calcitonin in chitosan hydrogel are shown in figure 36-38 respectively. For chitosan hydrogel, the important peaks were at 2,973, 2730, 1,630 and 1,566 cm^{-1} correspond to C-H stretching, the ester C=O, amide I and amide II, respectively. For calcitonin, the important peaks were 1,637 and 1,540 cm^{-1} correspond to amide I and amide II, respectively. Finally, the important peaks calcitonin in chitosan hydrogel were 1,639 and 1,566 cm^{-1} corresponded to amide I and amide II similar to chitosan hydrogel and salmon calcitonin.

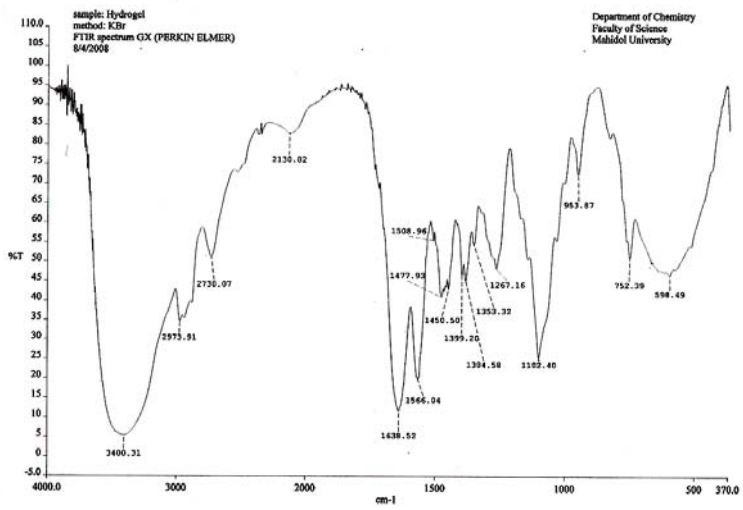


Figure 36 FTIR of chitosan hydrogel formulation

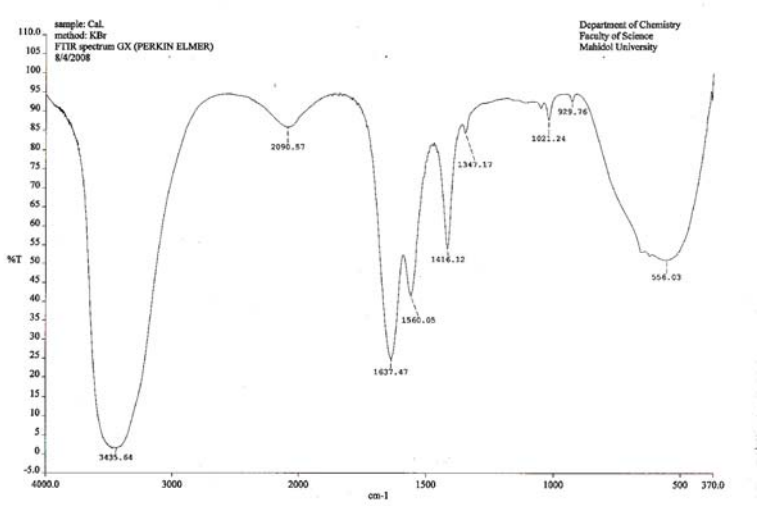


Figure 37 FTIR of pure salmon calcitonin

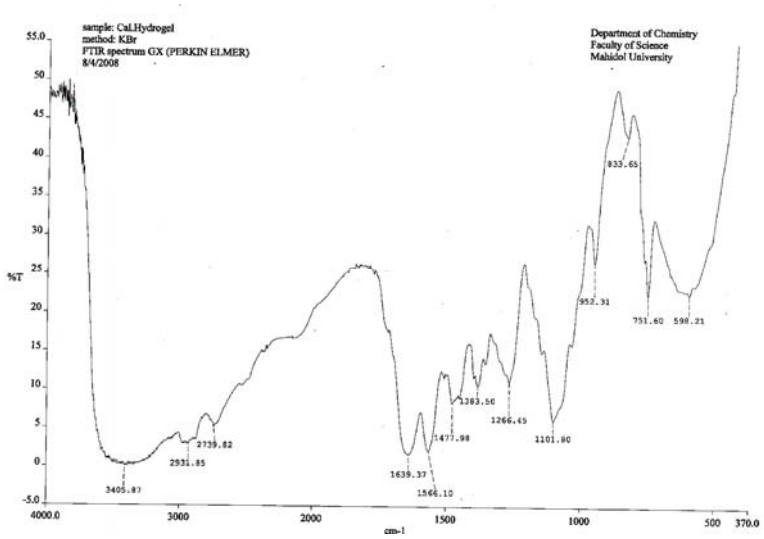


Figure 38 FTIR of salmon calcitonin with chitosan hydrogel formulation

CHAPTER V
CONCLUSION

The high performance liquid chromatographic method with reverse phase 5-μm octadecasilyl silica column (100 × 4.6 mm) can detect salmon calcitonin by mobile phase as A: 1 M tetramethylammonium hydroxide:water: acetonitrile, 20:880:100, B: 1 M tetramethylammonium hydroxide:water:acetonitrile, 8:392:600. Solutions A and B were adjusted to pH 2.5 with phosphoric acid. UV spectrophotometry was as detector by at 210 nm; flow rate: 1 ml/min at temperature 50-60 ºC. This validation method displayed good specificity, linearity and precision. Both of high performance liquid chromatographic analyzed by Water and Shimadsu Corporation can sensitively detect salmon calcitonin.

The stability of salmon calcitonin was investigated by temperature and the sonophoresis. Salmon calcitonin was degraded at room temperature for 24 hr and the residual ratio was approximately 0.65. The stability salmon calcitonin with ultrasound was evaluated by exposed with ultrasound at 1 MHz and varied intensity: low (2.3 watt (rms)) and high intensity (3.2 watt (rms)) for 10 min. The result of sonophoresis affected salmon calcitonin concentration was decreased. The ratio of high and low intensity when.compared with control was 0.42 and 0.54, relatively

The histology of skin was examined after ultrasound for 10 min at high and low intensity. The histological change of specimens was tested by porcine ear pig. The specimens were evaluated under scanning electron microscopy and the illustrations showed that treated condition induced the pore size of the skin significantly. ($p>0.05$)
In vitro release study of salmon calcitonin in PBS pH 7.4 was evaluated by cellulose acetate as a membrane having molecular weigh cut off 12,000-14,000 and PBS pH 7.4 as a medium. The result of percent cumulative release calcitonin per area was 5.11± 2.96 $x10^{-2}$ %/cm^2 at 48 hr. Percent cumulative release calcitonin per area was quite low.

It is probably because of physical instability of salmon calcitonin in aqueous solution. Molecule of salmon calcitonin (molecular weight 3431.85) may aggregate and formed become larger particles than the cellulose acetate membrane having pore size 12,000-14,000 molecular weigh cut off, so molecule of salmon calcitonin can not be passed cellulose acetate membrane.

The effect of ultrasound on the stability of salmon calcitonin was tested by *in vitro* release study of salmon calcitonin in PBS and the applied ultrasound at frequency 1 MHz, high intensity 3.2 watt (rms) for 10 min, the cellulose acetate was used as a membrane having molecular weigh cut off 12,000-14,000 and PBS pH 7.4 was used as a medium. Receiver phase 1 ml was collected at 6, 12 and 24 hr and detected by HPLC. The result of this study showed that the percent cumulative release of salmon calcitonin after applied ultrasound high intensity for 10 min was not significantly different from control. It was in agreement to the assumption that molecules of salmon calcitonin may not be enough fragmented by ultrasound. Therefore, the molecules were not small enough to passage through a pore size of a membrane which was less than 12,000-14,000 molecular weight cut off.

The aggregation process may be predicted due to percent cumulative release of salmon calcitonin increased within 6 hr, it may mean that molecular weight of salmon calcitonin occurred to aggregation within 6 hr.

In vitro release study of salmon calcitonin chemical enhancer and the applied ultrasound was tested. Chitosan HOBt solution and chitosan hydrogel were as chemical enhancer. It was believed that chemical enhancer plus with ultrasound low intensity (2.3 watt (rms) for 20 min) increase percent release of salmon calcitonin than chemical enhancer only. The percent cumulative release of salmon calcitonin in Chitosan HOBt solution only was 0.024×10^{-2} %/cm^2 and then ultrasound was added for 20 minutes the percent cumulative of release salmon calcitonin increased to 0.042×10^{-2} %/cm^2. The percent cumulative release of salmon calcitonin in Chitosan hydrogel only was not detectabel and then ultrasound was added for 20 min the percent cumulative release of release salmon calcitonin increased to 0.025×10^{-2} %/cm^2.

In vitro permeation experiments in porcine skin with chemical enhancer were examined. Isopropyl myristate was used as chemical enhancer and 0.5 ml of Isopropyl myristate was filled in donor phase until 48 hour. The enhancer solution was incubated

for 48 hr. then Miacalcic 2200 IU/ml 500 µl was added. The result of percent release of salmon calcitonin per area produced 238.58 $x10^{-2}$ %/cm^2.

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APPENDIX

APPENDIX A
SPECIFICATION OF CALCITONIN

Figure 39. Structure of Calcitonin

APPENDIX B
LINEARITY OF SALMON CALCITONIN

Table 12 Demonstration linearity of Salmon Calcitonin concentration in condition 1 analyzed by Water

Salmon Calcitonin Concentration. (μg/ml)	Area
100	4347521
50	1987357
25	394633

Table 13 Demonstration of linearity of Salmon Calcitonin concentration in Condition 2 analyzed Water

Salmon Calcitonin Concentration. (g/ml)	Area
100	6252801
50	2538577
25	855141
12.5	134995

Table 14 Demonstration of linearity of Salmon Calcitonin concentration analyzed by Shimadzu Corporation, Japan

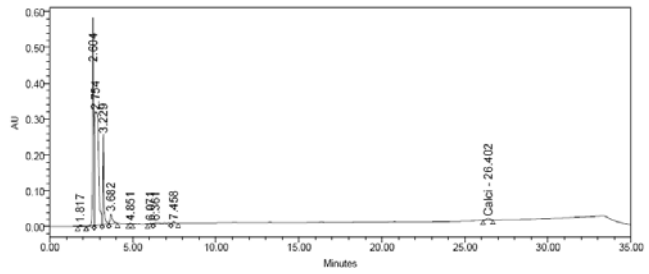
Concentration (μg/ml)	Average Area
100	925848
25	79724
12.5	49368
6.25	15760

APPENDIX C

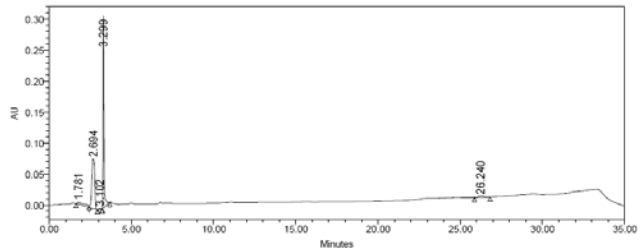
IN VITRO RELEASE STUDY OF SALMON CALCITONIN

Table 15 Percent cumulative release of calcitonin per area (%/cm^2 x10^{-2})

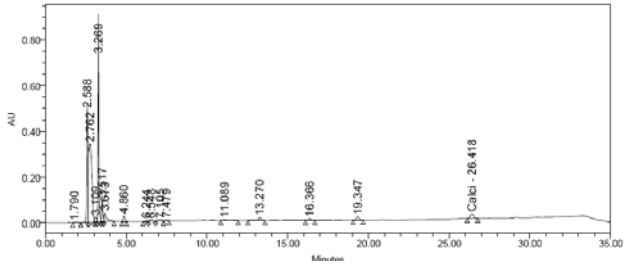
Number	Cumulative amount Calcitonin concentration after 48 hr. in receiver phase. (µg/ml)	Percent cumulative calcitonin per area (%/cm^2 x10^{-2})
1	1.03	2.42
2	3.54	8.28
3	1.98	4.62
Average	2.18±1.27	5.11±2.96



Chromatogram of drug release from solution Number 1



Chromatogram of drug release from solution Number 2



Chromatogram of drug release from solution Number 3

Figure 40 Chromatogram of the concentration of drug release from solution; (n=3**)**

Table 16 Comparison Percent Release salmon calcitonin per area between control (no applied ultrasound) and applied ultrasound high intensity for 10 min at 6, 12, 24 hr

Condition	Time (hr)	Cumulative amount (μg/ml)	Percent Release salmon calcitonin per area(%/cm^2 x10^{-2})
Control	6	1.15	3.11
	12	1.14	3.09
	24	0.90	2.43
High intensity	6	0.70	2.29
	12	0.77	2.09
	24	0.82	2.22

Table 17 comparison percent cumulative salmon calcitonin per area (%/cm^2) ; chitosan formulation and ultrasound application in vitro franz cell diffusion

Formulation	Cumulative salmon calcitonin x10^{-2} (%/cm^2)	
	No applied by Ultrasound	Applied by Ultrasound
	Receiver	Receiver
Chitosan Solution	0.024	0.042
Chitosan Hydrogel	BLQ	0.025

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Tinlaganan S, Iemsam-arng J, , Sathirakul K, Study in vitro skin penetration and drug release to optimize dermatological formulation strategy of Calcitonin. Poster presented at the 9 th National Graduate Conferences, 14-15 March 2008, Chonburi, Thailand.

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