จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัยปีที่สอง
เรื่อง

การศึกษาประสิทธิภาพและกลไกของไคโตแซน ในการเป็นสารเพิ่มการดูดซึมทางจมูกของยาเป็ปไทด์

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ชื่อโครงการวิจัย การศึกษาประสิทธิภาพและกลไกของไคโตแซนในการเป็นสารเพิ่มการดูดซึมทางจมูกของยาเป็ปไทด์

ชื่อผู้วิจัย ภาคภูมิ เต็งอำนวย

เดือนและปีที่ทำวิจัยเสร็จ กันยายน 2542

บทคัดย่อ

วัตถุประสงค์ ศึกษาประสิทธิภาพของไคโตแซนสองชนิดคือ ไคโตแซนในรูปเบสอิสระ (CSJ) และรูปเกลือกลูตาเมต (CSG) ในการเป็นสารเพิ่มการดูดซึมทางจมูกของยาเป็ปไทด์แซลมอนคัลซิโทนิน (sCT) ในหนูขาว และเปรียบเทียบกับผลของฮัยดร็อกซีพรอพิลเบต้าซัยโคลเด็กซตริน (HPβCD) และไดเมธิลเบต้าซัยโคลเด็กซตริน (DMβCD) **วิธีการ** หนูทดลองจะได้รับสารละลายของ sCT (ขนาดยา 10 IU ต่อกิโลกรัม) ที่มีไคโตแซน CSJ หรือ CSG อยู่ด้วยในช่วงความเป็นกรด-ด่าง (pH) และที่ความเข้มข้นต่างๆ จากนั้นจะดูผลการดูดซึมของ sCT โดยการวัดร้อยละของการลดระดับแคลเซียมในเลือด (%D) รวมถึงวัดระดับของตัวยา sCT ในเลือดด้วยวิธีเรดิโออิมมูโนแอสเสย์ **ผลการทดลอง** พบว่า CSJ จะให้ค่า %D ที่สูงขึ้นถ้า pH ของสารละลายมีค่าลดลง ซึ่งจะสอดคล้องกับการที่ไคโตแซนในรูปเบสอิสระจะมีการไอโอไนซ์และดูดน้ำมากขึ้นใน pH ที่เป็นกรด ขณะที่ CSG ซึ่งเป็นเกลือที่ละลายน้ำได้กลับจะให้ค่า %D ที่สูงขึ้นตาม pH ของสารละลายและสูงที่สุดที่ pH 6.0 จึงได้เลือกค่า pH ที่ 4.0 เป็นค่า pH ที่เหมาะสมสำหรับ CSJ และ pH ที่ 6.0 สำหรับ CSG มาทำการศึกษาต่อไป พบว่าประสิทธิภาพในการเพิ่มการดูดซึม sCT ของทั้ง CSJ และCSG จะเพิ่มขึ้นตามความเข้มข้นของพอลิเมอร์ในช่วง 0.25 ถึง 1.0 เปอร์เซ็นต์และมีค่าคงที่ที่ 1.25 เปอร์เซ็นต์ เมื่อทำการวัดระดับ sCT ในพลาสมา พบว่าค่าการเอื้อประโยชน์สัมบูรณ์เมื่อเทียบกับการฉีดยาเข้าหลอดเลือดดำมีค่าตามลำดับเท่ากับ 2.45, 1.91 และ 1.22 เปอร์เซ็นต์ สำหรับ 1 % CSJ, 5 % DMβCD และกลุ่มควบคุมซึ่งได้รับแต่ sCT ทางจมูกโดยไม่มีสารช่วย พบว่าสารช่วยทั้งหมดที่ศึกษาต่างก็มีประสิทธิภาพในการเพิ่มการดูดซึม sCT แต่CSJ และ DMβCD จะมีประสิทธิภาพสูงที่สุด ขณะที่ HPβCD จะมีประสิทธิภาพน้อยที่สุด นอกจากนี้จากการศึกษาในหลอดทดลอง ยังพบว่าสารไคโตแซนทั้งสองชนิดไม่สามารถยับยั้งการทำงานของเอนไซม์ทริปซินและลิวซีนอะมิโนเป็ปทิเดสซึ่งเป็นเอนไซม์ที่สำคัญในการสลาย sCT ในเนื้อเยื่อบุโพรงจมูกได้ ดังนั้น ฤทธิ์ในการเพิ่มการดูดซึม sCT ทางจมูกของไคโตแซนทั้งสองตัว จึงไม่ใช่เนื่องมาจากการป้องกันการถูกสลายตัวโดยเอนไซม์เหล่านี้ **สรุป** ผลการทดลองบ่งชี้ว่า สารพอลิเมอร์ที่มีประจุบวกเช่นไคโตแซน มีศักยภาพสูงที่จะนำมาใช้เป็นสารเพิ่มการดูดซึมทางจมูกของ sCT ได้อย่างมีประสิทธิภาพ

Project Title Efficacy and Mechanistic Studies of Chitosan as Nasal Absorption Enhancer of Peptide Drugs

Investigator Parkpoom Tengamnuay

Year September, 1999

Abstract

Objective. To evaluate the *in vivo* efficacy of chitosan as nasal absorption enhancers of peptides in rats and compare the results with that of hydroxypropyl- and dimethyl-β-cyclodextrins (HPβCD and DMβCD). **Methods.** Two types of chitosans, i.e., the free base (CSJ) and the glutamate salt form (CSG) were evaluated for their nasal absorption enhancing effect on salmon calcitonin (sCT) using an *in vivo* rat absorption technique. Solutions containing sCT and chitosans (0 to 1.25 % w/v) in isotonic phosphate buffers (pH 3.0 to 6.0) were nasally administered at the dose of 10 IU/kg. The plasma calcium lowering effect in each sCT-treated rat was determined by calculating the total percent decrease in plasma calcium (%D). **Results.** CSJ showed an increase in %D as the solution pH was decreased in accordance with the increased ionization and hydration of the free base chitosan at the more acidic pH. However, CSG showed an increase in %D with increasing pH, with maximum calcium lowereing effect observed at pH 6.0. At their optimal pH (4.0 for CSJ and 6.0 for CSG), the absorption enhancing effect of both chitosans was concentration dependent from 0.25 to 1.0 % w/v and leveled off at 1.25 % w/v. Using specific RIA, the absolute bioavailability of plasma sCT was determined to be 2.45, 1.91, and 1.22 % for 1 % CSJ, 5 % DMβCD, and control group (intranasal sCT alone), respectively. All the enhancers showed significant absorption enhancement with the highest effect observed with CSJ and DMβCD whereas the effect of HPβCD was the smallest. Also, the two chitosans did not possess any inhibitory effect on the *in vitro* activities of trypsin and leucine aminopeptidase, two major nasal proteolytic enzymes responsible for the degradation of sCT in the nasal cavity. Thus, the nasal absorption enhancement of chitosans may not involve protection of the peptide against proteolytic degradation in the nasal cavity. In conclusion, cationic polymer chitosans may have promising potential as an effective nasal absorption enhancer of sCT.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS ii

ABSTRACT (THAI) iii

ABSTRACT (ENGLISH) iv

TABLE OF CONTENTS v

LIST OF TABLES vi

LIST OF FIGURES viii

LIST OF ABBREVIATIONS xi

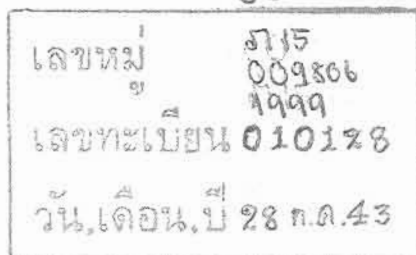
CHAPTER

- I. INTRODUCTION 1
- II. LITERATURE REVIEW 5
- III. MATERIALS AND METHODS 17
- IV. RESULTS AND DISCUSSION 42
  - Part I. Determination of Optimal Enhancing Conditions for Chitosans 42
    - 1.1 Effect of Varying pH 45
    - 1.2 Determination of Optimal Enhancing Concentration 75
    - 1.3 Plasma sCT Determination 88
  - Part II. Effects of Chitosans on Nasal Proteolytic Enzymes Activities 98
    - 2.1 Degradation Studies with Trypsin 98
    - 2.2 Degradation Studies with Leucine Aminopeptidase 105
- V. CONCLUSIONS 113

REFERENCES 116

APPENDICES 124

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย



LIST OF TABLES

Table		Page
1.	Summary of sample preparations for the *in vivo* absorption experiments	25
2.	Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of 0.15 M isotonic phosphate buffer at various pH (Baseline groups)	48
3.	Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of sCT alone (10 IU/kg) at various pH (Control groups)	49
4.	Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of sCT (10 IU/kg) with 1 % CSJ at various pH	50
5.	Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of sCT (10 IU/kg) with 1 % CSG at various pH	51
6.	Comparison of the total percent decrease in plasma calcium level following nasal administration of different baseline and control groups at various pH	52
7.	Comparison of the total percent decrease in plasma calcium level following nasal administration of sCT with 1 % chitosans at various pH	53
8.	Plasma calcium level (percent of initial value) in rats following nasal administration of sCT (10 IU/kg) with various concentrations of CSJ (pH 4.0)	77
9.	Plasma calcium level (percent of initial value) in rats following nasal administration of sCT (10 IU/kg) with various concentrations of CSG (pH 6.0)	78
10.	Comparison of plasma calcium pharmacokinetic parameters and the percent total decrease in plasma calcium (%D or hypocalcemic extent) following nasal administration of sCT (10 IU/kg) with various concentrations of CSJ and CSG at their respective optimal pH	79

11. Changes in plasma calcium level (percent of initial value) following nasal administration of sCT (10 IU/kg) in rats with 5 % DMβCD and 5 % HPβCD as absorption enhancers 84

12. Plasma sCT concentrations in rats following intravenous and intranasal administration of sCT with or without enhancers 91

13. Pharmacokinetic and bioavailability parameters of sCT following intranasal (with and without enhancers) and intravenous administration in rats 93

14. Effects of various concentrations of CSJ on trypsin activity as determined from the formation p-nitroaniline 101

15. Effects of various concentrations of CSG on trypsin activity as determined from the formation p-nitroaniline 102

16. Effects of various concentrations of CSJ on LAP activity as determined from the formation of β-naphthylamine 108

17. Effects of various concentrations of CSG on LAP activity as determined from the formation of β-naphthylamine 109

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure	Page
1. Chemical structures of chitin and chitosan	13
1/1 Diagrams of the surgical procedure of the *in vivo* nasal absorption experiment	22
2. Absorption spectra of orthocresolphthalein complexone (OCPC) in the presence of calcium (2.5 x 10^{-5} M) at pH 10.0.	43
3. Representative calibration curve of orthocresolphthalein complexone (OCPC) color reagent containing calcium at different concentrations	44
4. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 4.0. Each point represents mean ± SD (n = 5 rats/group)	54
5. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 5.0. Each point represents mean ± SD (n = 5 rats/group)	55
6. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 6.0. Each point represents mean ± SD (n = 5 rats/group)	56
7. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 3.0. Each point represents mean ± SD (n = 5 rats/group)	57
8. Comparison of percent plasma calcium following nasal administration of isotonic phosphate buffers (baseline groups) at different pH's. Each point represents mean ± SD (n = 5 rats/group)	65

9. Comparison of area under plasma calcium vs time curve (AUC_{0-240}) after nasal administration to baseline and control groups at various pH's (4.0-7.4). Data = mean ± SD (n = 5 rats/group) 66

10. Percent of plasma calcium vs time after nasal administration of sCT without enhancers (control groups) at various pH (3.0-7.4). Each point represents mean ± SD (n = 5 rats/group) 67

11. Comparison of the total percent decrease in plasma calcium (%D) after nasal administration of sCT with or without 1 % w/v chitosans (CSJ and CSG) at various pH's. Data = mean ± SD (n = 5 rats/group) 71

12. Percent plasma calcium after nasal administration of sCT with 1 % w/v CSJ at various pH's (3.0-6.0). Each point represents mean ± SD (n = 5 rats/group) 72

13. Percent plasma calcium after nasal administration of sCT with 1 % w/v CSG at various pH's (3.0-6.0). Each point represents mean ± SD (n = 5 rats/group) 73

14. Percent of plasma calcium versus time following nasal administration of sCT (10 IU/kg) with various concentrations of CSJ (pH 4.0) to rats. Each point represents mean ± SD (n = 5 rats/group) 80

15. Percent of plasma calcium versus time following nasal administration of sCT (10 IU/kg) with various concentrations of CSG (pH 6.0) to rats. Each point represents mean ± SD (n = 5 rats/group) 81

16. Percent of plasma calcium versus time following nasal administration of sCT with 5 % DMβCD and 5 % HPβCD as absorption enhancers in comparison with the control and baseline groups. Each point represents mean ± SD (n = 5 rats/group) 85

17. Histogram comparing the average total percent decrease in plasma calcium (% D) following nasal administration of sCT with different enhancers under

their corresponding optimal pH and concentration 86

18. Representative standard binding curve for the determination of sCT in plasma 90

19. Plasma sCT concentration-time profiles following intranasal administration of sCT (10 IU/kg) with or without enhancers in comparison with the intravenous injection (0.15 IU/kg) 92

20. Representative calibration curve of p-nitroaniline in 0.15 M phosphate buffer (pH 6.0) 100

21. Formation of p-nitroaniline following incubation of BAPA with trypsin in the presence of varying concentrations of CSJ. The reactions were carried out in 0.15 M phosphate buffer pH 6.0 103

22. Formation of p-nitroaniline following incubation of BAPA with trypsin in the presence of varying concentrations of CSG. The reactions were carried out in 0.15 M phosphate buffer pH 6.0 104

23. Representative calibration curve of β-naphthylamine in 0.15 M phosphate buffer (pH 6.0) 107

24. Formation of β-naphthylamine following incubation of L-Leu-β-NA with LAP in the presence of varying concentrations of CSJ. The reactions were carried out in 0.15 M phosphate buffer pH 6.0 110

25. Formation of β-naphthylamine following incubation of L-Leu-β-NA with LAP in the presence of varying concentrations of CSG. The reactions were carried out in 0.15 M phosphate buffer pH 6.0 111

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
AUC	=	Area under the curve
BAEE	=	N-α-Benzoyl-L-arginine-ethylester
BAPA	=	N-α-Benzoyl-DL-arginine-p-nitroanilide
^{o}C	=	Degree Celcius
Ca	=	Calcium
cm	=	Centimeter
CSJ	=	Chitosan free amine
CSG	=	Chitosan glutamate
CT	=	Calcitonin
% D	=	total percent decrease in plasma calcium
DMβCD	=	Dimethyl-β-cyclodextrin
% F_{abs}	=	Absolute bioavailability
% F_{rel}	=	Relative bioavailability
Fig.	=	Figure
gm	=	Gram
HPLC	=	High pressure liquid chromatography
HPβCD	=	Hydroxypropyl-β-cyclodextrin
hr	=	Hour
i.n.	=	Intranasal
i.p.	=	Intraperitoneal
i.v.	=	Intravenous
IPB	=	Isotonic phosphate buffer
IU	=	International Unit
kg	=	Kilogram

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L-Leu-β-NA	=	L-Leucyl-β-naphthylamine
LAP	=	Leucine aminopeptidase
M	=	Molar (mole/liter)
mM	=	Millimolar (mmole/liter)
ml	=	Milliliter
μg	=	Microgram
μl	=	Microliter
mg	=	Milligram
min	=	Minute
mosmole	=	Milliosmole
N	=	Normality
OCPC	=	Orthocresolphthalein complexone
rpm	=	Revolutions per minute
r^2	=	Correlation coefficient
RIA	=	Radioimmunoassay
sCT	=	Salmon calcitonin
SD	=	Standard deviation
SE	=	Standard error
sec	=	Second
UV	=	Ultraviolet
U/ml	=	Units/ml

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จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I
INTRODUCTION

The nasal cavity has long been recognized as a site of drug administration. However, its use has been limited to local treatments of the nose such as allergic rhinitis, or to produce systemic psychotropic effects with the abuses of certain narcotics (Chien and Chang, 1985). Recently, greater emphasis has been placed on the nasal cavity as a potential route for the systemic administration of peptide drugs. Transnasal systemic delivery of peptides and proteins offers several advantages over the more conventional approaches like oral and parenteral drug administrations. Since these compounds are extensively destroyed by numerous enzymes of the GI-tract, their ability to reach the systemic circulation and exert their pharmacological activity is greatly compromised when administered orally. On the other hand, parenteral drug therapy suffers from the standpoint of patient compliance, especially when the administration needs to be performed frequently. This has necessitated the search for alternative routes of peptide delivery, among which the nasal route appears to be very promising.

The nasal mucosa is rich in blood supply and its capillary permeability is greater than the intestinal mucosa (Watanabe et al., 1980). Drug which is absorbed across the nasal epithelium enters the systemic circulation directly without hepatic first pass metabolism (Colaizzi, 1985). In addition, there are no pancreatic enzymes and strongly acidic conditions in the nasal cavity, thus rendering its environment less rigorous for peptide drugs than in the GI-tract. The volume of the nasal secretion is also much less than that of the GI-tract, leading to a smaller dilution effect and better contact of the drug with the absorptive epithelium (Stratford and Lee, 1986). Nasal administration is also considered to be non-invasive as opposed to the parenteral route, thereby encouraging self-administration and increasing patient compliance.

Also, drugs can be administered in a pattern which simulates the release cycle of the endogenous peptides, thereby preserving their biological rhythms and reducing unwanted side effects (Banga and Chien, 1988).

Although the nasal bioavailability of peptides is much higher than the oral bioavailability, it still is much less than 100% when compared to intravenous administration. This is due to the poor membrane penetration of peptides and their degradation by numerous peptidases in the nasal mucosa (Hussain et al., 1992). To improve the nasal bioavailability of peptide drugs, various groups have explored the possibility of using chemical substances to enhance drug permeation across the epithelial membrane (Illum et al., 1989). However, many effective absorption enhancers like surfactants, bile salts and fatty acids increase the nasal membrane permeability by aggressive mechanisms which often result in severe or permanent damages to the nasal mucosa. Consequently, these materials are unacceptable for chronic use in humans and novel classes of safer nasal absorption enhancers need to be discovered and evaluated.

Chitosan is a cationic polysaccharide derived from naturally occurring chitin in crab and shrimp shells by deacetylation. Chitosan has been shown to have mucoadhesive properties, a mechanism believed to be important for nasal absorption enhancement. Mucoadhesion of the drug dispersed or dissolved in chitosan solution can decrease the rate of drug clearance from the nasal cavity and results in a longer contact time with the absorptive epithelium (Illum et al., 1994). Also, it has been suggested that chitosan may widen the tight junctions which results in the increased transport of hydrophilic molecules via a paracellular pathway (Artursson et al, 1994). However, the safety and effectiveness of various chitosans have not been extensively investigated and thus have become the topic of current research project. Previous studies in this laboratory using an *in situ* rat nasal perfusion technique have found that both the free amine and salt forms of chitosan can enhance the nasal absorption of

model dipeptide L-Tyr-D-Arg in a concentration-dependent manner. The absorption enhancing effect of the free amine chitosan was dependent on pH, with the greater effect observed at a lower pH in accordance with the nature of the free amine polymer to swell and dissolve better in the more acidic environments. On the other hand, the enhancing effect of chitosan glutamate, which is a soluble salt form, appears to be less pH-dependent.

At only 0.02% w/v, both forms of chitosan exhibit the enhancing effect equivalent to that produced by 5% w/v hydroxypropyl-β-cyclodextrin (HPβCD), a commonly studied nasal absorption enhancer with a very mild effect on the nasal mucosa. Determination of the extent of mucosal protein and phosphorus release in the nasal perfusates revealed that the two chitosans are as safe as HPβCD, which caused a minimal release of these susbstances. Furthermore, morphological evaluation of the rat nasal mucosa following daily administration of 1% solutions of both chitosans and 5% HPβCD for 14 days indicated that the three enhancers produced only mild to moderate irritation, the most common signs being mucus hypersecretion and goblet cell distention. Their effects on the rat nasal epithelial integrity are also reversible as judged from the reduction in the extent of lactate dehydrogenase release, a cytosolic enzyme marker, following removal of chitosans from the nasal mucosa. Therefore, the data previously obtained strongly support the promising potential of chitosan as a novel class of safe and effective nasal absorption enhancers (Tengamnuay et al., 1998).

The primary purpose of this research is to continue the studies on chitosan in order to obtain more information on its safety and efficacy, particularly after *in vivo* application. Salmon calcitonin, a polypeptide hormone, which plays a key role in calcium metabolism, was selected as a model peptide for these *in vivo* absorption experiments using rat as a model animal. Since very few information is available regarding its efficacy relative to other enhancers, the *in vivo* absorption enhancing

effect of chitosan was also compared with two most commonly studied cyclodextrin-type enhancers, i.e., dimethyl-β-cyclodextrin (DMβCD) and hydroxypropyl-β-cyclodextrin (HPβCD). Furthermore, this research also investigated the possible inhibitory effects of chitosan on two nasal proteolytic enzymes, i.e., leucine aminopeptidase and trypsin, which are responsible for degradation of salmon calcitonin in the nasal mucosa (Morimoto et al., 1995). Results from these studies would give a better understanding on the relative adjuvant activities of chitosans and their mechanisms of absorption enhancement.

The aims of this research were as follows:

1. To evaluate the efficacy of chitosan as nasal absorption enhancer of salmon calcitonin using *in vivo* nasal absorption technique in rat
2. To determine the optimal pH and concentration of chitosan for nasal absorption enhancement of salmon calcitonin
3. To compare the efficacy of two different types of chitosan (free amine versus glutamate salt form) with that of cyclodextrin derivatives in improving the nasal absorption of salmon calcitonin
4. To study the inhibitory effect of chitosan on the proteolytic enzyme activity of leucine aminopeptidase and trypsin using *in vitro* technique

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CHAPTER II
LITERATURE REVIEW

Recently, biologically active macromolecules have become increasingly important as a new class of therapeutic agents. Most of them are peptides and their derivatives such as insulin, calcitonin, growth hormone and its analogs, adrenocorticotropic hormone (ACTH) and its analogs, interferons, vasopressin, luteinizing hormone releasing hormone (LHRH) and its analogs etc. These peptides have clinical implications in the treatment of several chronic, hormone-deficiency diseases such as diabetes, paget's disease, dwarfism, cancer etc. (Chien and Chang, 1985). Since these peptides are mostly destroyed by the acids and enzymes in the gastrointestinal tract, they cannot be administered orally. Currently, the only effective route of administration of these drugs is by parenteral administration, e.g. intravenous, subcutaneous or intradermal injection. However, the parenteral route suffers several serious drawbacks such as patient compliance, high risk of overdose, infections and local thrombophlebitis as a result of its invasive nature of administration.

Non-oral, non-parenteral routes of administration such as nasal, sublingual, rectal, pulmonary, and transdermal routes have received greater attention as alternative means for systemic delivery of peptide drugs. Among these routes, nasal administration offers several promising advantages. Although limited in the surface area, the nasal mucosa is rich in blood supply and its capillary permeability is greater than the intestinal mucosa (Watanabe et al., 1980). Drug which is absorbed across the nasal epithelium enters the systemic circulation directly without hepatic first pass metabolism (Colaizzi, 1985). In addition, there are no pancreatic enzymes and strongly acidic conditions in the nasal cavity, rendering the environment of the nasal cavity less rigorous for peptide drugs than in the GI-tract. The volume of the nasal secretion is also much less than the GI-secretion, leading to smaller dilution of the

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administered drug and better contact with the absorptive epithelium (Stratford and Lee, 1986). Nasal administration is also considered to be non-invasive as opposed to the parenteral route, thereby encouraging self-administration and increasing patient compliance. Another advantage of nasal administration of peptides is that the drugs can be given in a pattern which simulates the release cycle of the endogenous peptides, thereby preserving their biological rhythms and reducing unwanted side effects (Banga and Chien, 1988).

The mucosal membranes of the mammalian nasal cavity are the moist lining epithelium, including several types of epithelia. A small portion extending into the nasal cavity from the nares is a stratified squamous epithelium. The remainder of the nasal membrane is made up of respiratory epithelium, which is composed of goblet cells, ciliated cuboidal and columnar cells, and the olfactory epithelium, which is a pseudostratified neuroepithelium. Another connective tissue layer called the submucosa usually connects the mucosa to the underlying structures. On the surface of the mucosal epithelium, there exists a layer of mucus composed of muco-polysaccharides secreted from the goblet cells of the mucosa (Hsieh, 1994).

The nasal vasculature consists of a rich capillary network found in the subepithelium and around the nasal glands and cavernous plexuses. It is characterized by fenestrated endothelium. Thus, the nasal mucosa is obviously well suited for heat exchange and for potential drug absorption. In general, drugs absorbed via the nasal mucosa enter the right side of the heart for direct distribution to the systemic arterial circulation prior to traversing the liver (Su, 1991).

Barriers to nasal delivery of protein/peptide drugs

Since protein and peptide drugs are relatively large hydrophilic molecules, the mammalian mucosa possesses several extremely efficient barriers to restrict their entry to the systemic circulation. Although nasal administration of peptide drugs

generally results in much greater absorption than the oral administration, the absolute bioavailability as compared to the intravenous injection is still low. For example, nasal administration of insulin (Tengamnuay and Mitra, 1990b) and calcitonin (O'hagan and Illum, 1990) resulted in only 1% absorption. It thus appears that several absorption barriers may be present in the nasal mucosa which are responsible for their lower-than-expected nasal bioavailabilities. These barriers include the presence of many mucosal proteolytic enzymes which are responsible for biochemical degradation of peptides before and during absorption as well as the epithelium itself which acts as an efficient barrier against membrane penetration.

The proteolytic enzyme barrier is perhaps the most important barrier against the absorption of peptide and protein drugs since these molecules are highly susceptible to hydrolysis by many enzymes present in the nasal cavity. The enzymes that may be present in the mucosal tissues include exopeptidases (aminopeptidase and carboxypeptidase), endopeptidases, angiotensin-converting enzymes, dipeptidases, aminotripeptidases, prolidases, prolinases and carnosinases (Zhou,1994). Furthermore, these enzymes are widely distributed in the mammalian body, especially between the entry point into the systemic circulation and the target site, thereby making them a very efficient system to decrease the systemic bioavailability of many peptide drugs.

Other types of barriers include various clearance systems at the administration sites and the structural barriers of the epithelium which lines the mucosal surface. In order to penetrate the epithelium, a drug molecule may have to diffuse either through the epithelial cell membrane (transcellular transport), or alternatively, through the tight junctions between cells (paracellular pathway). Thus, diffusion of a drug molecule across an epithelial barrier is dependent on several factors such as pKa, molecular weight, structural conformation and hydrophilic characteristics of the drug as well as pH of the environment (Zhou, 1994).

In general, only the relatively small compounds with adequate lipophilicity can partition into the membrane lipids of the epithelial cells to be absorbed by the transcellular route. The absorption of large, hydrophilic drugs like peptides, on the other hand, appears to be limited to the paracellular pathway (McMartin et al., 1987). However, the paracellular pathway occupies only a small portion of the total surface area of the nasal epithelium. This, together with the molecular weight-restricted permeation through the tight junctions, further limits the nasal absorption of most peptide drugs (Anderberg et al., 1993).

General approaches to bypassing enzymatic and epithelial barriers

In order to bypass the enzymatic and epithelial barriers for the purpose of increasing the nasal bioavailability of high molecular weight protein and peptide drugs, several approaches are available: (i) inhibition of their enzymatic degradation; (ii) improving their resistance to breakdown or their permeability across the membrane by structural modification; (iii) by special pharmaceutical formulations which prolong their retention time with mucus at the administration site; and (iv) increasing their permeability across the relevant membrane by using chemical absorption enhancers (Zhou, 1994).

Inhibition of proteolytic enzymes

For several years it has been known that protease inhibitors increase the absorption of protein drugs. Compounds such as boroleucine and phosphinic acid dipeptide have been suggested as having good potential in enhancing the bioavailability of peptide and protein drugs due to their appropriate molecular sizes and potent inhibitory effects on various proteolytic enzymes (Hussain et al., 1989). However, the safety of these inhibitors after nasal administration is doubtful and must be further tested (Hussain et al., 1992). Bestatin and puromycin are another class of

inhibitors that have been studied. Nevertheless, their effects on peptidases were much less effective than boroleucine (Hussain et al., 1989).

Chemical modification

Hydrogen bonding potential or lipophilicity of peptides also can be altered by chemical modification. This often leads to conformational changes of the peptides and thereby may increase their permeability and/or stability during transport across the cell membrane. For example, when four methyl groups were added to the peptide, acetamido-D-Phe-D-Phe-D-Phe-carboxamide by methylation, it was found that the penetration rate of this peptide through Caco-2 cell membrane was significantly enhanced (Conradi et al., 1992). Tengamnuay and Mitra (1990a) also reported that by substituting L-Arg with D-Arg in the L-Tyr-L-Arg structure resulted in a dipeptide analogue which was highly resistant to hydrolysis by the nasal mucosal enzymes.

Formulation approach

The formulation approach has been employed to develop an effective nasal delivery system for peptide and protein drugs for many years. The most popular dosage form is a powder or microspheres system since the nasal solutions and sprays tend to provide lower peptide drug availability. In a recent study by Bjork and Edman (1988), insulin (0.75 and 1.7 IU/kg) which was dispersed in degradable starch microspheres (DSM) and administered nasally as a drug powder resulted in a dose-dependent decrease in blood glucose in rats. The bioavailability of the nasal insulin was found to be 30 %, whereas the administration of DSM alone or soluble insulin alone produced no effect. The effectiveness of the nasally delivered peptide in this dosage form was due to the uptake of water by DSM and subsequent swelling which produced dehydration of the epithelial cells, leading to a widening of the tight junctions and thereby facilitating paracellular transport of large hydrophilic molecules (Bjork et al., 1990). When DSM was combined with an enhancer, lysophosphatidylcholine (LPC), the extent of insulin nasal absorption was improved

even further (Farraj et al., 1992). This improved formulation also has been used for enhanced nasal absorption of human growth hormone (Illum et al., 1990).

Use of nasal absorption enhancers

Many researchers in nasal drug delivery have included certain adjuvants in the nasal peptide formulations in an attempt to increase their systemic bioavailability. The compounds that have been studied as nasal absorption enhancers are, for example, anionic and cationic surfactants (Hirai et al., 1981a), bile salts (Gordon et al., 1985; Pontiroli, 1985), bile salt-fatty acid mixed micelles (Tengamnuay and Mitra, 1990a and 1990b), fusidic acid derivatives (Longenecker et al., 1987; Baldwin et al., 1990), medium chain fatty acids (Mishima et al., 1987) and cyclodextrins (Merkus et al., 1991).

The mechanisms of action of these absorption enhancers are not clearly known, but several possibilities have been postulated. The first is the increased solubility of the drugs brought about by the surfactant-type enhancers. Because proteins and peptides usually form aggregates in aqueous solutions, their dissociation into more readily soluble monomers by these enhancers may facilitate their paracellular transport (Brange et al., 1992). Secondly, surfactant-type enhancers like hydrophobic bile salts may also facilitate transcellular transport of insulin probably by reverse micelle formation (Gordon et al., 1985). A third mechanism of the enhancer is to inhibit the activity of proteolytic enzymes. Some enhancers such as bile salts, fusidic acid derivatives and cyclodextrins have been shown to inhibit mucosal proteolytic activity. However, these compounds are not specific inhibitors or substrate analogues of the proteinases or peptidases (Zhou et al., 1990a). It is postulated that binding of the peptide drug with the enhancer may prevent the formation of the enzyme-substrate (enzyme-protein drug) complexes which are intermediates of the degradation process. Fourthly, the positively charged enhancers may react with the negatively charged membrane surface, and thereby reduce the

peptide drug-mucus interactions (Chandler et al., 1991a) resulting in an increase of the drug bioavailability. The fifth possibility is that the enhancers can lower the barrier function of the mucosal membrane and associated mucus layer. For example, bile salts, including sodium taurodihydrofusidate (STDHF), have been shown to reduce the viscosity of the mucus layer adhering to all mucosal surfaces and increase the pore size within the cell membrane, thereby allowing diffusion of insulin through the cells (Longenecker et al., 1987). The barrier function of the mucosal membrane could also be lowered by a more severe mechanism, e.g., the removal of certain membrane components like membrane proteins and phospholipids. Several enhancers, especially the surfactant-type compounds, have strong solubilizing capacity which may not only dissolve or dissociate the peptide aggregates but also may effectively cause leaching of these membrane components, leading to a substantial increase in membrane permeability (Tengamnuay, 1989).

Although these enhancers are able to significantly increase nasal absorption of peptides, many studies have later revealed that their use can cause damages to the membrane. This has caused great concerns and prevented them from potential application in clinical setting, particularly during chronic administration. For example, many enhancers can cause cellular changes in the mucosa which include loss of nasal membrane components (Shao et al., 1992: Shao and Mitra, 1992), ciliotoxicity (Hermens et al., 1990), and severe alterations in the morphology of the nasal mucosa such as necrosis and even complete loss of epithelium (Tengamnuay and Mitra, 1990b; Donovan et al., 1990; Ennis et al., 1990). The membrane damaging properties of these enhancers, especially the surfactant type, pose problems in their prospective clinical applications, especially in long term therapy of many hormone-deficient diseases. As a result, more efforts have been pushed toward finding novel absorption enhancers which can give better safety and efficacy.

Recently, cyclodextrins, one class of cyclic polysaccharides, have been suggested as potential enhancers for non-parenteral peptide absorption. Merkus et al. (1991) reported that 109 % of nasal bioavailability (2.0 IU/ kg) could be obtained when insulin was co-administered with 5 % dimethyl-β-cyclodextrin (DM-β-CD), a highly soluble cyclodextrin derivative. A recent report by Irie et al. (1992) also provided similarly promising results for this compound. However, it was found later that DM-β-CD caused the release of many membrane and cellular components such as phospholipids, enzymes and proteins (Shao et al., 1992). These researchers also found that hydroxypropyl-β-cyclodextrin (HP-β-CD), another derivative, was the least membrane damaging. However, its absorption enhancing effect was also minimal (Verhoef et al., 1994). DM-β-CD also shows, to some extent depending on concentration (1-5%), damages to the ciliary system of chicken embryo trachea, although its ciliostatic potency is much less than that found for STDHF (0.5%), LPC (0.5%) and bile salts such as deoxycholate (0.2%), glycocholate (1.5%) and taurocholate (1.3%) (Merkus et al., 1993). Therefore, the long term clinical use of DM-β-CD as a potent absorption enhancer in nasal peptide formulations appears to be questionable and more studies are needed to verify its safety profiles.

Chitosan as potential nasal absorption enhancer of peptides

Chitosan is a polymer obtained from deacetylation of chitin, a naturally-occurring structural polymer abundant in crab and shrimp shells. Chitosan, or partially N-deacetylated chitin, is a cationic polysaccharide with linear chain consisting of two monosaccharides, i.e. N-acetyl-D-glucosamine and D-glucosamine, joining together by β-(1,4)-glycosidic linkage. The greater the extent of deacetylation, the smaller is the proportion of N-acetyl-D-glucosamine in the polymer chain. Figure 1 shows the chemical structures of chitin and chitosan. Since chitin and chitosan are obtained from crab and shrimp shells which are the waste products of

Thailand's marine food industry and can be manufactured and purified in large scale, any research attempt to increase the applicability of chitosan is always highly attractive.

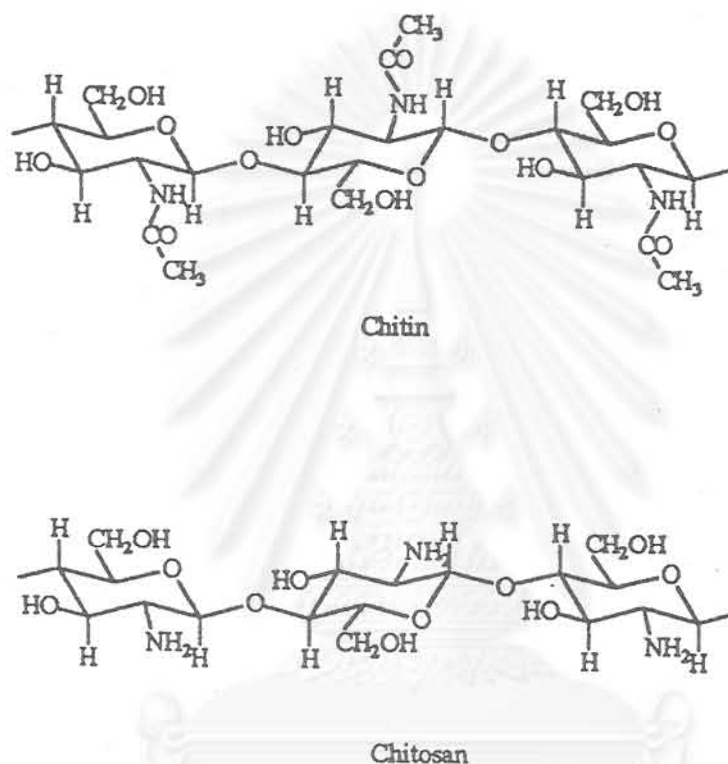

Figure 1 Chemical structures of chitin and chitosan

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There are several pharmaceutical applications of chitosan. For example, it has been used as a pharmaceutical excipient to increase water solubility of several oral drug formulations (Imai et al., 1991). It has also been used as polymer matrix in the sustained release drug preparations (Miyazaki et al., 1988). Being a substance of natural origin with biocompatibility, chitosan has found many applications in other areas such as food and cosmetic industries. Recently, Artursson et al. (1994) have studied the effects of chitosan on the transport of water-soluble molecules across the

cultured monolayer of intestinal epithelial cancer cells (Caco-2 cells) grown in vitro. They found that chitosan significantly increased the permeability of these cells. They also postulated that chitosan may react with the protein of the cellular tight junctions, leading to the opening of the tight junction and subsequent passage of hydrophilic macromolecules through the paracellular pathway. In the same year, Illum et al. (1994) studied the nasal administration of insulin in sheep and rats with and without chitosan. They found that inclusion of chitosan at concentrations from 0.1 to 1.0 % w/v in the nasal insulin solutions could significantly enhance the nasal absorption of this peptide over the control group (nasal insulin without chitosan). These preliminary results indicated the potential application of chitosan as nasal absorption enhancer of poorly absorbed drugs like peptides. However, more information is needed to characterize its safety and efficacy profiles. In our previous report (Tengamnuay et al., 1998), we have demonstrated that chitosan in both forms (free amine and soluble salt) is effective in enhancing the nasal absorption of L-Tyr-D-Arg, a model dipeptide, based on *in situ* rat nasal perfusion technique. Furthermore, the absorption enhancing activity appears to increase at the more acidic pH. This is particularly the case for the free amine chitosan, which requires protonation and hydration of the primary amine groups for its polymer chain to become uncoiled so as to obtain a better contact with the nasal membrane. However, the glutamate salt of chitosan does not appear to need that much acidity for its adjuvant activity. This is probably due to the high charge density of chitosan glutamate, which may be already present at a more neutral pH.

Since our previous results were based solely on the *in situ* experiments using a small dipeptide as a model drug, it is thus interesting to know if both forms of chitosan could similarly enhance the systemic nasal absorption of other larger peptides *in vivo*. In addition, the possible inhibitory effect of chitosan on the proteolytic enzymes of the nasal mucosa has not been investigated. Therefore, studies

on the enzyme inhibition may provide further information as to its mechanism of absorption enhancement.

Salmon calcitonin as a model drug for *in vivo* nasal administration

Salmon calcitonin (sCT) was chosen as a model polypeptide in this study for two reasons. First, this peptide is among the most widely studied peptide drugs for possible absorption through the alternative routes, particularly the nasal mucosa (Katagani et al., 1996; Morimoto et al., 1995; Pontiroli 1990). Approximate comparison between the results obtained in this study and other reported results would be readily possible. Secondly, improved methods of sCT delivery could significantly facilitate the treatment of Paget's disease, hypercalcemia, osteoporosis and postmenopausal disorders. sCT is a straight-chain polypeptide composed of thirty-two amino acids. It is produced synthetically. The hormone is joined at positions one and seven by a disulfide linkage and contains a proline amide at position thirty-two. The structure of sCT differs significantly from human and bovine calcitonin throughout the chain. This probably accounts for its greater potency and stability. All thirty-two amino acids are important for activity, as manipulation of the arrangement and studies with isolated portions of the chain resulted in reduced activity. The potency of sCT is expressed in International Units (IU), which are equal to Medical Research Council Units (MRC Units). One unit corresponds to 0.2 μg of the pure peptide.

Mechanism of action and uses of sCT

Hypercalcemia: sCT has been shown to effectively lower serum calcium concentrations in hypercalcemic patients with carcinoma, multiple myeloma, or, to a lesser degree, primary hyperparathyroidism. Two mechanisms have been proposed whereby calcitonin exerts its hypocalcemic effects. The primary action is through an

inhibition of bone resorption, a rapidly occurring process during the active stages of Paget's disease. It causes a downward shift in the number of osteoclasts formed (osteoclasts are elevated during bone resorption) in favor of an increase in osteoblast production (osteoblasts are elevated during bone formation) leading to a greater bone surface area with a reappearance of normal histological structure (Brodier et al., 1974). A secondary action occurs through an inhibition of the tubular reabsorption of both calcium and phosphorus resulting in intial hypercalciuria and hyperphosphouria.

Paget's disease of bone: sCT is effective in the treatment of Paget's disease. This disease is commonly found in axial skeleton, but may involve any bone. The earliest pathology lesion appears to be an increase in bone resorption. The resultant clinical symptoms include bone pain at the site of lesion, increased local skin temperature due to an increased vascularity of the affected bone and an increase in the incidence of fractures. Calcitonin reduces the rate of bone turnover, possibly by an initial blocking of bone resorption, resulting in decreases in serum alkaline phosphate (reflecting increased bone formation) and urinary hydroxyproline excretion (reflecting decreased bone resorption, i.e., breakdown of collagen).

Postmenopausal osteoporosis: sCT may be used in conjunction with adequate calcium and vitamin D intake in the management of postmenopausal osteoporosis to prevent progressive loss of bone mass. However, sCT alone appears to be ineffective in the management of osteoporosis.

Preparations and dosage

Salmon calcitonin is available for clinical use as Calcimar© or Miacalcic©, a synthetic preparation supplied as a metered-dose nasal spray containing 50 or 100 IU per ml. The recommended dosage (administered intranasally) is 200-400 IU daily in several divided doses for treatment of hypercalcemia and an initial dose of 200 IU twice daily is used for Paget's disease.

CHAPTER III
MATERIALS AND METHODS

The experiments were divided into two major parts:

1. Studies of chitosans as nasal absorption enhancer of sCT using *in vivo* nasal absorption technique in rats
   1.1 Studies to determine the optimum enhancing conditions for different types of chitosan (free amine chitosan and its glutamate salt)
   1.2 Comparison of the absorption enhancing efficacy of chitosans with that of cyclodextrin derivatives
2. Studies to evaluate the possible inhibitory effect of chitosans on the activity of two nasal proteolytic enzymes, leucine aminopeptidase and trypsin, using *in vitro* incubation technique

Materials:

Chitosan J (CSJ): Chitosan free amine (MW 300 –500 kDa), Kyowa Technos Co., Ltd., Japan.

Chitosan G (CSG): Chitosan glutamate (Seacure G 210+, MW 300-500 kDa), Lot. No. 206-409-03, Pronova Biopolymer, Drammen, Norway.

Salmon Calcitonin, Synthetic (sCT): Lot no. 84H4951 and Lot no. 84449512, Sigma Chemicals Co., St. Louis, MO, USA.

Salmon Calcitonin RIA kit: RIK-600, Lot no. 970311, Peninsula Lab. Inc., Belmont, CA, USA.

Calcium Kit: Lot no. 628756 and 958456, Clinag Co. Ltd., Bangplad, Bangkok, Thailand.

Hydroxypropyl-β-cyclodextrin (HPβCD): Lot no. 231026R, Aldrich Chemical Co., Inc., Milwaukee, USA.

2,6-Di-O-Methyl)-β-Cyclodextrin (DMβCD or Heptakis): Lot no. 56H0347, Sigma Chemicals Co., St. Louis, MO, USA.

Thiopental: Lot no. 1300495, Research Institute of Antibiotics and Biotransformation, Czech Republic.

Heparin: Lot no. 01976A, Leo Pharmaceutical Products, Ballerup, Denmark.

Sodium Chloride AR grade: Lot no. 4F027304F, Farmitalia Carlo Erba, Milan, Italy.

Sodium Hydroxide AR grade: Lot no. 191293, Eka, Sweden.

Ethanol Absolute GR grade: Lot no. 321K19712083, E. Merck, Darmstadt, Germany.

Disodium Hydrogenphosphate AR grade: Lot no. F997086, E. Merck, Darmstadt, Germany.

Sodium Phosphate Monobasic AR grade: Lot no. G37948D5560A, Farmitalia Carlo Erba, Milan, Italy.

Phosphoric acid 85% GR grade: Lot no. 931K42677273, E. Merck, Darmstadt, Germany.

Trypsin (from bovine pancreas): Lot no. 63H02802, Sigma Chemicals Co., St. Louis, MO, USA.

N-2-Benzoyl-DL-Arginine-p-Nitroanilide Hydrochloride (BAPA HCl): Lot no. 25H1133, Sigma Chemicals Co., St. Louis, MO, USA.

p-Nitroaniline: Lot no. 85K3406, Sigma Chemicals Co., St. Louis, MO, USA.

Trichloroacetic acid GR grade: Lot no. 637K2887707, E. Merck, Darmstadt, Germany.

Aprotinin (from bovine lung): Lot no. 75H7186, Sigma Chemicals Co., St. Louis, MO, USA.

Leucine Aminopeptidase (Type VI-S: from porcine kidney microsomes): Lot no. 61H7045, Sigma Chemicals Co., St. Louis, MO, USA.

L-Leucine-β-Napthylamide Hydrochloride: Lot no. 025H6220, Sigma Chemicals Co., St. Louis, MO, USA.

N-1-Naphthylethylenediamine DiHCl: Lot no. 105H6160, Sigma Chemicals Co., St. Louis, MO, USA.

Sodium nitrite: Lot no. 065H6131, Sigma Chemicals Co., St. Louis, MO, USA.

Ammonium sulfamate: Lot no. 105H6159 , Sigma Chemicals Co., St. Louis, MO, USA.

Alcoholic dye base: Lot no. 035H6211, Sigma Chemicals Co., St. Louis, MO, USA.

Bestatin: Lot no. 103H58601, Sigma Chemicals Co., St. Louis, MO, USA.

All other reagents were of analytical grade and were used as received.

Equipment:

UV Spectrophotometer, Model 7800, Jasco Corporation, Tokyo, Japan.

pH Meter, Model 420A, Orion Research Operation, Boston, MA, USA.

Analytical Balance, Sartorius 1615 MP, Gottingen, Germany.

Thermostatted Circulating Water Bat;h, Heto InterMed, Heto Birkerod, Denmark.

Vortex Mixer, Model G-560E, Scientific Industries Inc., New York, USA.

Ultrasonic Bath, Model 3210, Branson Ultrasonic Corporation, Danbury, CO, USA.

Osmometer, Osmomat 030-D, Gonotec, Berlin, Germany.

Refrigerated Centrifuge, Model Sigma-302K, Laboratory Centrifuges, Germany.

Gammatec II, Model Gamma 600B Gamma Counter, The Nucleus Inc., Oak Ridge, CA, USA.

Laminar Air Flow Hood, Model Airone 1000-GS, Safelab Systems Ltd., Nailsed, Great Britain.

Part I. *In Vivo* Studies of Chitosans as Nasal Absorption Enhancer in Rats

The purposes of this part were:

1. To confirm previous *in situ* results that chitosans can promote nasal absorption of peptide drugs using *in vivo* absorption technique in rats with salmon calcitonin (sCT) as a model peptide.
2. To determine optimum pH and concentration for the *in vivo* absorption enhancing activity for each type of chitosans, i.e. the free amine chitosan (CSJ) and the glutamate salt form (CSG).
3. To compare the *in vivo* absorption enhancing activity of the two chitosans with other novel enhancers, i.e., HPβCD and DMβCD.
4. To determine the systemic bioavailability of plasma sCT following nasal administration of sCT in the presence of an enhancer (chitosan or cyclodextrin) in relation to nasal sCT without enhancer and intravenous injection.

The *in vivo* nasal absorption technique was used to evaluate the effectiveness of different nasal formulations. This technique was first developed by Hirai et al. (1981) and has been successfully used by other researchers such as Huang et al. (1985). It may be useful in screening of a drug candidate for possible nasal absorption and to confirm that the drug is actually absorbed across the nasal mucosa into systemic circulation. The technique also allows for the quantitation of the extent of nasal absorption (absolute bioavailability) as compared to the control (intravenous administration). The *in vivo* nasal absorption of sCT was evaluated by its hypocalcemic effects and measurements of its plasma level after nasal administration to anesthetized rats. The following is a detailed description of the *in vivo* nasal absorption procedure, which is a slight modification of Hirai's method (Hirai et al., 1981).

Male Sprague-Dawley rats weighing 250-300 g were fasted for 16-18 hr prior to the experiments but allowed free access to water. They were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg, with additional doses given intraperitoneally as necessary. For surgical procedure, an incision was made at the neck of the rat to expose the trachea. A polyethylene tube was inserted about 1.5 cm deep into the trachea toward the lungs to maintain respiration during the experiment. The esophagus was also cannulated with another similar polyethylene tube, which was closed at the end with an adhesive agent (Elephant glue©) and inserted toward the posterior nasal cavity. Such manipulation was necessary to prevent the rat from swallowing the drug into the esophagus through nasopharynx. The nasopalatine was sealed with an adhesive agent to prevent drainage of the drug solution from the nasal cavity into the mouth. Diagrams of the surgical procedure for the *in vivo* nasal absorption experiment are illustrated in Figure 1.

Nasal Administration of sCT

Depending on the body weight of an individual rat, drug solution was administered through the right nostril into the nasal cavity at a volume of 80-100 µl via a microsyringe (Hamilton, Reno, Nevada) which was attached to a blunt needle. The microsyringe was inserted at a depth of 4-5 mm from the nare and the drug solution was slowly administered within 30 sec. In all cases, the nasal dose of sCT was 10 IU/kg body weight. Therefore, to achieve the dose and administration volume as required, the concentration of sCT in the final preparation was calculated to be 0.3 IU/ml (equivalent to 1.75 µM). Blood samples (0.2 ml each) were collected periodically from the jugular vein for 240 min (at 0, 10, 20, 30, 40, 60, 90, 120, 180 and 240 min) following nasal administration. At the end of each blood withdrawal, the same volume of sterile normal saline was put back into the circulation to maintain total blood volume and thus viability of the rats. Cannulation of the jugular vein was

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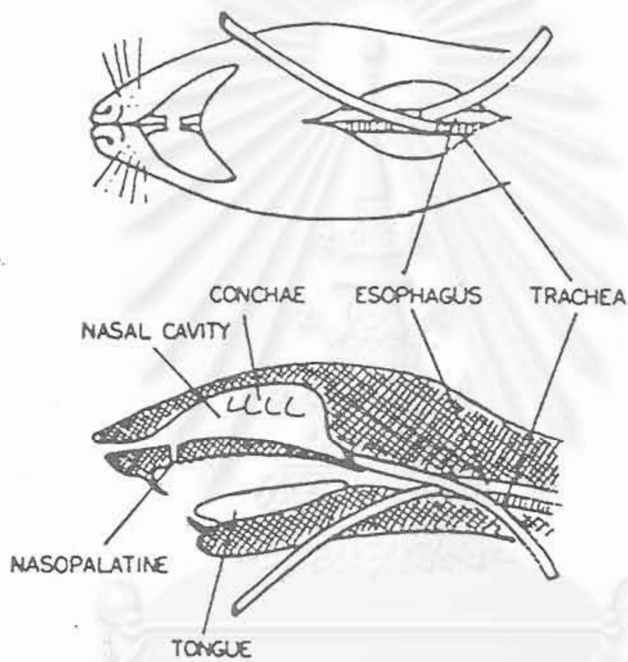

Figure 1/1. Diagrams of the surgical procedure of the *in vivo* nasal absorption experiment

adapted from the method of Harms and Ojeda (1974) using a catheter no. 20G (Jelco©, Italy), filled with heparinized physiological saline (20 IU/ml). This cannula served as a port for blood sampling, intravenous administration of sCT as well as for the fluid replacement.

Intravenous Administration of sCT

In order to determine the absolute bioavailability of nasally absorbed sCT, the intravenous administration of the drug was carried out. As an i.v. reference, sCT was injected intravenously to compare the areas under the plasma sCT concentration-time curves (AUC) with those after nasal administration. The i.v. pharmacokinetic of sCT in rats was determined by injecting a bolus dose of 0.15 IU/kg sCT in 0.15 M phosphate buffer (pH 4.0) into the jugular vein. To ensure complete dosing the injection cannula was flushed afterwards with 200 μl physiological saline. Blood samples (about 0.2 ml each) were withdrawn at 0, 5, 10, 15, 30, 60, 120 and 180 min after application and treated as described above.

Collection of Plasma Samples

In all cases (intravenous or intranasal), blood was collected into a heparinized microcentrifuge tube (Eppendorf® Safe-lock tube, Germany) and was centrifuged at 3,000 x g for 15 min using a refrigerated centrifuge. Plasma samples were separated and kept frozen at –20 °C for analysis of plasma calcium level. The absorption of sCT through the nasal mucosa was estimated indirectly from the reduction in plasma calcium (hypocalcemic effect). In some cases, the systemic absorption was directly determined by measuring the plasma levels of sCT using a specific RIA kit.

Preparations of Sample Solutions

The basic formulation of 3.5 μM sCT (6 IU/100 μl) was prepared by separately dissolving the peptide in 0.15 M isotonic phosphate buffer (IPB) solutions with four different pH values, i.e., pH 3.0, 4.0, 5.0, and 6.0. The stock solutions of the two chitosans (CSJ and CSG) were prepared at 2.0 % w/v concentration by dissolving each one in 1 % v/v acetic acid solution and allowing them to swell overnight. The pH was subsequently adjusted to the same as the sCT solution (pH 3.0, 4.0, 5.0, or 6.0) by dropwise addition of either 1 N hydrochloric acid or 1 N sodium hydroxide solution. The tonicity was then adjusted by stepwise addition of sodium chloride to obtain an isotonic solution, which was checked by osmometer (290-310 mosmole/kg). Each of the chitosan stock solutions was subsequently incorporated in the basic peptide formulation with corresponding pH at a 1:1 ratio prior to the absorption experiment. The resulting solutions contained 1.0 % w/v chitosan (CSJ or CSG) and 1.75 μM sCT in isotonic buffer of varying pH. The concentration of sCT (1.75 μM) was fixed throughout the entire experiments whereas the concentration of CSJ and CSG was also prepared at 0.25, 0.50, 0.75, and 1.25 % w/v. Similar method of preparation was applied by mixing chitosan stock solutions (0.5, 1.0, 1.5, and 2.5 % w/v) with 3.5 μM sCT solution at the same 1:1 ratio. Table 1 lists the sample solutions, which were prepared for investigation.

The effect of pH on the enhancing activity of both chitosans was first investigated by varying the pH of the sample solutions from 3.0 to 6.0 (concentration of chitosan fixed at 1.0 % w/v). Furthermore, the baseline groups were carried out by nasal administration of only the phosphate buffers at various pH (no sCT and no enhancers) in order to determine the baseline of calcium level at each pH. After determining the pH of optimum enhancing activity for each chitosan, the effect of chitosan concentration was subsequently studied in a range of 0.25 % to 1.25 % w/v. The sample solution also contained sCT at the final concentration of 1.75 μM, and

Table 1. Summary of sample preparations for the *in vivo* absorption experiments

Conditions	Dose of sCT (IU/Kg)	Enhancers	Conc. of enhancer (% w/v)	pH of sample solution in 0.15 M IPB				
				3.0	4.0	5.0	6.0	7.4
1. Baseline group	-	-	-	/	/	/	/	/
2. Control group	10	-	-	/	/	/	/	/
3. $sCT_{treated}$ group	10	CS J , CS G	1	/	/	/	/	-
(sCT with enhancers)		CS J , CS G	0.25 0.50 0.75 1.25	Optimum pH				
		DM-B-CD , HP-B-CD	5	-	-	-	-	/

IPB = Isotonic Phosphate Buffer

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was administered at the same dose (10 IU/kg). The same *in vivo* technique and analytical method were followed in order to determine the concentration which would give an optimum hypocalcemic effect. After the optimum chitosan concentrations had been established, the hypocalcemic effect of sCT, after coadministration with chitosans, were then compared with that after cyclodextrin derivatives (HPβCD and DMβCD). The reasons as to choosing the two compounds as reference enhancers are given in the next chapter. The reference enhancer solutions contained the same concentration of sCT (1.75 μM) and 5 % w/v of either HPβCD or DMβCD in isotonic phosphate buffer pH 7.4. The compositions of all the isotonic phosphate buffers employed in this study are given in Appendix I.

The absolute bioavailability of sCT relative to the intravenous injection was also determined by direct quantitation of plasma immunoreactive sCT following sCT nasal administration in the presence and absence of chitosan and DMβCD.

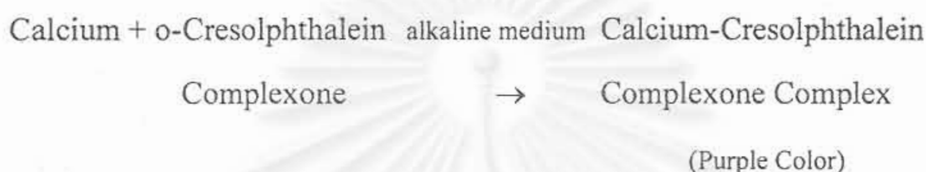
Analytical Method

Plasma Calcium Determination

The colorimetric method for determining plasma calcium levels with o-cresolphthalein complexone was introduced by Stern and Lewis (1957). The procedure was based on the interaction of the cationic calcium with a suitable chromogenic agent such as o-cresolphthalein complexone. This technique is very simple, direct and reliable.

Plasma calcium levels following nasal administration of sCT were determined colorimetrically with o-cresolphthalein complexone using a kit commercially available from Clinag (Bangkok, Thailand). The reagent contained an o-cresolphthalein complexone (phenolphthalein, 3', 3" bis-{[bis-(carboxymethyl) amino] methyl}-5', 5"-dimethyl) as a color reagent, which specifically complexes with calcium to form a purple colored complex in an alkaline environment that uses

ethanolamine as a buffer reagent. This purple colored complex, measured with a spectrophotometer at $\lambda = 570$ nm, is directly proportional to the calcium concentration in the sample. Standards representing equivalent calcium concentration of 2-12 mg/dl, are prepared in a similar manner. The concentration of the unknown is determined from a standard curve.



The assay procedures are as follows:

1. Set spectrophotometer wavelength at 570 nm and absorbance reading to zero with water as reference.
2. Set up a series of tubes for standard and sample.
3. Two ml of buffer agent and two ml of color agent (provided with the kit) was added to sample or standard tubes.
4. After mixing by vortex, the content of each tube was read within 30 min. The first absorbance (A1) indicated the extent of calcium, which may have contaminated the pipet or test tube.
5. Fifty µl of samples or standards was individually added to the tubes.
6. The tubes were mixed by gentle inversion and then incubated for 5 min at room temperature. The second absorbance (A2) was measured at 570 nm by using water as reference. A2 was expressed as the absorbance of total calcium (the combination of calcium which contaminated the container (pipet, test tube) plus calcium in plasma).

So the absorbance of calcium in plasma (A) = A2 – A1

Calculation of calcium concentration in the sample was as follows:

$$\text{Calcium concentration (mg/dl)} = \frac{\text{A sample}}{\text{A standard}} \times \text{Concentration of standard}$$

7. The observed changes in plasma calcium level were expressed as percent of the initial calcium value (0 min sample)

   or $\%$ plasma calcium remaining at any time $= \frac{\text{A sample at time t}}{\text{A sample at time 0}} \times 100\,\%$

   where A = Absorbance of calcium in plasma measured at 570 nm

Plasma sCT Determination

Plasma immunoreactive sCT was quantitated by radioimmunoassay using an RIA kit developed by Peninsula Laboratories (Belmont, CA.). This technique is essentially the double antibody assay and has a minimum detectable concentration ranging from 10 to 1,280 pg/ml depending on the plasma sample size. The principle is based upon the ability of a limited quantity of antibody to bind a fixed amount of radiolabeled antigen. It is assumed that both the labeled and unlabeled antigens have the same affinity for the antibody resulting in competitive binding between the two species. Therefore, the extent of the binding of radiolabeled antigen to antibody will depend on the amount of both labeled and unlabeled antigens. The greater the amount of unlabeled antigen present in the test sample, the smaller is the percentage of the bound radiolabeled antigen detected by a gamma counter. Separation of the bound and free radiolabeled antigens is necessary in order to determine the quantity of the unlabeled antigen. This can be achieved by precipitating the antigen-antibody complexes by adding a second antibody directed toward the immunoglobulin present

in the original antiserum. The quantity of unlabeled antigen in an unknown sample is then determined by comparing the radioactivity of the precipitate, after centrifugation, with the values established using known standards in the same assay system.

Briefly, the assay procedures are as follows :

A: Polystyrene disposable tubes (12 X 75 mm) were labeled with the following designations : Total Counts (TC), tubes 1 and 2 ; Non - specific Binding (NSB), tubes 3 and 4 ; Total Binding (TB), tubes 5 and 6 ; sCT Standards , tubes 7 through 22 ; tubes starting with number 23 and after were assigned for plasma samples and also prepared in duplicate.

B: Two hundred microliters of RIA buffer was added to TC tubes (tube 1& 2) and NSB tubes (tube 3 & 4), RIA buffer provided with the kit.

C: One hundred microliters of RIA buffer was added to TB tubes (tube 5 & 6).

D: One hundred microliters of each sCT standard (concentrations 10, 20, 40, 80, 160, 320, 640 and 1280 pg/ml) was added in duplicate to tubes 7 to 22. The standard solutions were also provided with the kit.

E: One hundred microliters of each plasma sample was added in duplicate to all tubes starting with number 23 and up.

F: One hundred microliters of primary antibody (rabbit anti-peptide serum) was added to all tubes except TC and NSB tubes.

G: All the tubes were gently vortexed, covered and incubated overnight for 16-24 hr at 4° C.

H: One hundred microliters of tracer (^{125}I-salmon calcitonin) solution was added to all tubes.

I: All the tubes were again vortexed, covered and incubated overnight for 16-24 hr at 4° C.

J: One hundred microliters of Goat Anti-Rabbit IgG serum (GARGG) was added to each tube in the RIA.

K: One hundred microliters of Normal Rabbit Serum (NRS) was added to each tube in the RIA.

L: Vortex the contents of each tube and allowed to stand at room temperature for 90 min.

M: Add five hundred microliters of RIA Buffer to each tube and vortex the contents.

N: They were centrifuged at 3,000 rpm (approx. 1,700 x g) for 20 min at 4° C using Sigma 320 K refrigerated centrifuge.

O: The supernatant of each tube (except TC) was carefully decanted into a radioactive waste container.

P: The radioactivity in all tubes were counted for one min using Gamatec II ™.

After the values in count- per- minute (CPM) were obtained for each tube , a correction for the nonspecific binding was made by subtracting the average NSB count from the average CPM of each tube to obtain average net CPM (Equation shown below). The percentage of bound radiolabeled antigen ($\%B/B_0$) was then determined from the following formula :

$$\text{Average net CPM} = \text{Average net } \overline{\text{CPM}} - \overline{\text{NSB}}$$

The percentage of bound radiolabeled antigen ($\%B/B_0$) for the duplicate standards and unknown samples use the following

calculation:

$$\%B/B_0 = [\text{net } \overline{CPM} \text{ std. or unknown / net } \overline{CPM}\ B_0] \times 100\ \%$$

$$B_0 = \overline{TB} - \overline{NSB}$$

Where $\overline{CPM}$ = average count per minute

$\overline{NSB}$ = average non-specific binding

$\overline{TB}$ = average total binding

% B/B_0 for each standard was then plotted as a function of standard concentration of unlabeled sCT on semilog graph paper. The concentration of sCT in the plasma samples could be read directly from the standard curve (n = 2 replications).

Data Analysis

Pharmacodynamic Data Analysis

The plasma calcium lowering effect in the individual sCT-treated rat was indicated by the total percent decrease in plasma calcium level during 0-240 min (% D) as compared to the appropriate baseline group (no sCT and enhancer) was calculated by a modification of Hirai et al. (1981) using the following equation:

$$\%D = \frac{[\overline{AUC}_{baseline} - AUC_{sCT\ treated}]}{\overline{AUC}_{baseline}} \times 100\ \%$$

Where $\overline{AUC}_{baseline}$ = average area under plasma calcium curve from 0-240 min after nasal administration of 0.15 M IPB at the corresponding pH to rats of the baseline group.

$$= \Sigma_{i=1 \rightarrow n} [(C_i + C_{i-1}) / 2]\ \Delta t$$

$AUC_{sCT\ treated}$ = area under plasma calcium curve from 0-240 min after nasal administration of sCT (with or without enhancer) to the individual rat.

% D = total percent decrease in plasma calcium

The areas under all the individual plasma calcium concentration versus time curves ($AUC_{0\text{-}240}$) were calculated by a linear trapezoidal rule from 0 to 240 min.

Determination of Absolute and Relative Nasal Bioavailabilities

All samples which showed sCT serum concentrations below the limit of quantitation (10 pg/ml) were set to zero concentration. The areas under the curves of plasma sCT concentration versus time from 0 to 180 min were calculated using the trapezoidal rule ($AUC_{0\text{-}180}$). The absolute and relative nasal bioavailabilities were calculated for each nasal preparation by comparing its $AUC_{0\text{-}180}$ to that following intravenous injection of 0.15 IU/kg sCT.

$$\% F_{abs} = \frac{\overline{AUC}_{(in)} \times Dose_{(iv)}}{\overline{AUC}_{(iv)} \times Dose_{(in)}} \times 100\ \%$$

$$\% F_{rel} = \frac{\overline{AUC}_{(in\ with\ enhancer)}}{\overline{AUC}_{(in\ no\ enhancer)}} \times 100\ \%$$

Where $\overline{AUC}$ = average area under the plasma sCT versus time curve

$\% F_{abs}$ = percent of absolute bioavailability

$\% F_{rel}$ = percent of relative bioavailability

$Dose_{(iv)}$, $Dose_{(in)}$ = intravenous and intranasal dose of sCT in IU/kg

Part II. *In Vitro* Evaluation of the Inhibitory Effect of Chitosans on the Activity of Two Nasal Proteolytic Enzymes, Leucine Aminopeptidase and Trypsin

Proteolytic activities of the enzymes in the absorptive membranes are perhaps the most important barriers and can significantly reduce the systemic bioavailability of most protein and peptide drugs. The mucosal membranes of the nasal cavity are known to have various types of peptidase and protease activities, including both exopeptidases and endopeptidases (Zhou, 1994). The nasal delivery of proteins and peptides has been hindered by the presence of these enzymatic barriers. The predominant enzymes appear to be aminopeptidases and their activity in the nasal mucosa was found to be similar to that of the ileal mucosa in its subcellular distribution (Sarkar, 1992). Since the major enzymes acting as important barriers to peptide absorption from the nasal mucosa are aminopeptidases, inhibition of their activity should improve the absorption of these susceptible molecules.

Morimoto and co-workers (1995) demonstrated that the proteolytic activities in homogenates of the nasal mucosa tissues of the rat were high and found to be in the following order: leucine aminopeptidase > dipeptidyl aminopeptidase > cathepsin > trypsin. Leucine aminopeptidase thus appears to be most abundant in the nasal mucosa. Interestingly, the same researchers also found that inhibition of trypsin activity could minimize sCT degradation (Morimoto et al., 1995). It is possible that trypsin may have specific activity for sCT despite its generally lower peptidase activity than other nasal enzymes. Therefore, the inhibitory effect of nasal absorption enhancers on leucine aminopeptidase and trypsin activities may provide additional mechanism for the enhancement of sCT absorption.

The aim of this part was to determine the potential of chitosans in inhibiting the proteolytic enzyme activity of the nasal mucosa. An *in vitro* enzyme inhibition technique was employed in this study using trypsin and leucine aminopeptidase as

model nasal proteases. Trypsin is an endopeptidase whereas leucine aminopeptidase is an exopeptidase. Both enzymes are important in peptide drug delivery since they are involved in the nasal degradation of many peptide and protein drugs (Zhou, 1994).

2.1 Degradation Studies with Trypsin (EC 3.4.21.4).

Principle

Determination of trypsin activity was carried out by the method of Erlanger (1961). Trypsin activity was determined with the chromogenic substrate N-α-benzoyl-DL-arginine-p-nitroanilide HCl (PABA) and the hydrolysis of this substrate, as catalyzed by trypsin, releases p-nitroaniline which is yellow in color and can be estimated colorimetrically at 410 nm.

Preparation of Solutions

Buffer Solution: The buffer system used in all the enzyme inhibition studies was 0.15 M phosphate buffer, pH 6.0.

Substrate Stock Solution (1mM): Degradation studies with trypsin were performed using N-α-benzoyl-DL-arginine-p-nitroanilide HCl (PABA) as the enzyme substrate. Its high activity and indicator-like properties (i.e., the release of color upon hydrolysis) make it ideal for the detection or quantitative determination of trypsin in biological systems (Erlanger et al., 1961). The substrate concentration in this experiment was 1.0 mM, which had been shown in the preliminary experiment to provide sufficient substrate for maximal hydrolytic activity measurements. This is necessary to provide a meaningful comparison of the enzyme activities. A concentration of 1.0 mM of the substrate was prepared by dissolving 43.5 mg of BAPA in 1.0 ml of dimethylsulfoxide, and the solution was brought to 100 ml with the phosphate buffer (pH 6.0). Care must be taken to dissolve all of the BAPA in

dimethylsulfoxide (Be sure no crystals remain). The temperature of the stock solution was always kept at 37 °C and freshly prepared.

Enzyme Stock Solution (0.2 mg/ml): The experiments were performed using the commercial trypsin enzyme, purified from bovine pancreas (Sigma). Each mg of solid enzyme was equivalent to 8,060 BAEE units, where each BAEE unit will produce a change in absorbance at 253 nm (ΔA_{253}) of 0.001 per min at pH 7.6 and 25 °C using BAEE as a substrate. BAEE is an abbreviated name for N-α-benzoyl-DL-arginine-ethylester. The enzyme solution at the concentration of 0.2 mg/ml was prepared by first dissolving 10 mg of trypsin in 10 ml of 0.001 M HCl and then diluting with the phosphate buffer (pH 6.0) to the volume of 50 ml. The stock solution was stored at 5 °C.

Test Solutions: The two chitosans (CSJ and CSG) were prepared at various concentrations (0.25, 0.5, 0.75, 1.0 and 1.25 % w/v) by dissolving each one in sufficient quantity of 1 % v/v acetic acid solution and allowing them to swell for several hours. The solutions were subsequently dispersed in phosphate buffer (pH 6.0) and diluted to the desired concentrations. The pH values were checked and adjusted to 6.0 with either 1 N NaOH or 1 N HCl.

In addition, a solution of aprotinin, a specific inhibitor of trypsin, was used for comparison with chitosans for its inhibitory effect on trypsin activity. Aprotinin, a bovine pancreatic kallikrein inhibitor, consists of a single-chain polypeptide containing 58 amino acid residues with a molecular weight of 6,500 Da. It has been used to inhibit plasmin, trypsin, chymotrypsin and various intracellular proteases (Zhou et al., 1991). The inhibitor was prepared from bovine lung (Sigma Chemicals) and its activity was 4.4 TIU/mg, i.e., each mg of solid substance is equivalent to 4.4 TIU, where TIU is a trypsin inhibitor unit. One TIU will decrease the activity of 2 trypsin unit by 50 % where one trypsin unit will hydrolyze 1.0 μmole of N-α-

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benzoyl-DL-arginine-p-nitroanilide HCl per min at pH 7.8 and at 25 °C. The inhibitor solution at concentration of 0.2 mg/ml (equivalent to 0.88 TIU/ml) was prepared by dissolving 1 mg of aprotinin in 5.0 ml of the phosphate buffer. The stock solution of the inhibitor was stored at 2 to 8 °C. For the control group, the phosphate buffer (pH 6.0) was used in place of the sample solutions at an equal volume.

Calibration Curve of p-nitroaniline

Ten mg of p-nitroaniline was accurately weighed and dissolved in 5 ml of distilled water. The solution was then adjusted to 100 ml with the phosphate buffer (pH 6.0) and used as a stock solution (0.1 mg/ml). The stock solution was pipetted at volumes of 1, 2, 3, 6, 9, 12 and 15 ml into a series of 50-ml volumetric flask and diluted to volume with the phosphate buffer, pH 6.0. The final concentrations of each solution were 2, 4, 6, 12, 18, 24 and 30 μg/ml, respectively.

The absorbance of each known standard concentration was determined using a UV-Visible spectrophotometer in a 1-cm cell at 410 nm. The spectrophotometer was set for zero absorbance with the phosphate buffer, pH 6.0. Each concentration was determined in triplicate.

Analytical Method

Inhibitor activity was assayed by monitoring the loss in enzyme activity with time following incubation of the enzyme and substrate with a varying amount of the inhibitor. The conversion of N-α-benzoyl-DL-arginine-p-nitroanilide HCl (BAPA) by trypsin to p-nitroaniline was measured as follows: At first, 40 ml of the substrate (1 mM) was incorporated with 10.0 ml of either chitosan or aprotinin preparations, respectively. The hydrolytic experiment was started by addition of 10.0 ml of trypsin solution. The test solution was then incubated in a thermostatically controlled bath at 37 °C. The final concentration of trypsin in the incubation mixture was 0.029 mg/ml.

The experiments were allowed to run for 240 min. A volume of 3.5 ml was withdrawn from the incubation mixture at predetermined time intervals (0, 10, 20, 30, 40, 60, 90, 120 and 240 min) and was added with 0.5 ml of 30 % v/v acetic acid to stop the trypsin activity. The absorbance of the withdrawn mixture was then measured at 410 nm for the content of p-nitroaniline formed. Buffer control tube (incubation of the substrate and enzyme without chitosan or inhibitor) was also run simultaneously. The control experiment was conducted by replacing the inhibitor solution with an equivalent volume of buffer and the same steps were followed.

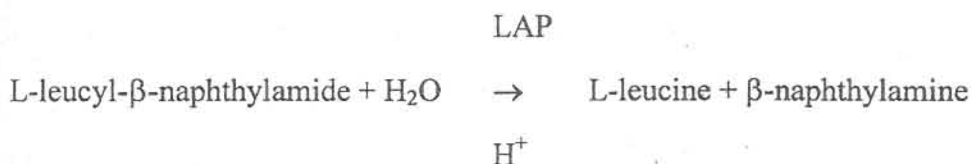
The enzyme inhibitory activity was determined by calculating the area under the metabolite (p-nitroaniline) concentration versus time curve up to 240 min using the trapezoidal rule. The values were subsequently compared with the control group.

2.2 Degradation Studies with Leucine Aminopeptidase (LAP, L-alkylpeptide hydrolase, EC 3.4.11.2)

Principle

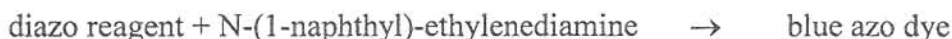
The method used was that developed by Goldberg and Rutenberg (1958) as modified by Takenaka and Takahashi (1962). L-leucyl-β-naphthylamide (L-Leu-β-NA) was used as the substrate. Its hydrolysis leads to the formation of β-naphthylamine, which is subsequently diazotized and coupled with sodium nitrite and N-(1-naphthyl)-ethylenediamine to produce a highly colored compound, which can be quantified by absorbance measurement at 580 nm. The spectrophotometric measurements were made in triplicate for each point during the incubation period.

LAP catalyzes the first of the following reactions:



$$\text{L-leucyl-}\beta\text{-naphthylamide} + H_2O \xrightarrow[H^+]{LAP} \text{L-leucine} + \beta\text{-naphthylamine}$$

β-naphthylamine + $NaNO_2$ → diazo reagent



diazo reagent + N-(1-naphthyl)-ethylenediamine → blue azo dye

The dye has an absorption maximum at 580 nm and its absorbance is proportional to the β-naphthylamine concentration, which, in turn, reflects the original LAP activity.

Preparation of Sample Solutions

Buffer solution: The buffer system used in all the enzyme inhibition studies was 0.15 M phosphate buffer, pH 6.0.

Substrate Stock Solution (0.2 mg/ml or 0.78 mM): L-leucyl-β-naphthylamide (L-Leu-β-NA) was used as a substrate for leucine aminopeptidase. Twenty mg of L-Leu-β-NA was dissolved in phosphate buffer and made up to 100 ml. The pH of the solution was adjusted to pH 6.0 with 1 N NaOH or 1 N HCl. The substrate solution was stored at a temperature below 0 °C and shaken well after thawing.

Enzyme Stock Solution (0.067 unit/ml): 2.1 mg (equivalent to 25 unit) of leucine aminopeptidase enzyme obtained from porcine kidney microsomes (Sigma Chemicals) was first dissolved in 5.0 ml phosphate buffer, pH 6.0 to obtain a solution. Each mg solid was equivalent to 12 units, where each unit was defined as being capable of hydrolyzing 1.0 μmol of L-leucyl-p-nitroanilide to L-leucine and p-nitroaniline per min at pH 7.2 and 37 °C. A 75-fold enzyme dilution was prepared by adding 0.1 ml of this solution to 7.4 ml of the phosphate buffer. The final concentration of the enzyme stock solution was 5.6 μg/ml (equivalent to 0.067 unit/ml). The stock solution was stored at 5 °C until use.

Test Solution: The inhibitory effect of chitosans on the LAP activity was studied at concentrations of 0.25, 0.5, 0.75, 1.0 and 1.25 % w/v. The enhancer

solutions were prepared by the same method as described for trypsin studies. A solution of bestatin, a specific inhibitor of leucine aminopeptidase, was used for comparison as a positive control. Bestatin, or [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-(S)-leucine, is an amino acid derivative isolated from the culture filtrate of *Streptomyces olivoreticuli* (MD 976-C7). It shows a strong inhibition of leucine aminopeptidase in a competitive manner (Suda et al., 1976) and thus was selected in this study as a reference inhibitor of LAP.

Bestatin solution at the concentration of 0.145 mM was prepared by dissolving 5 mg of bestatin, accurately weighed, in 10.0 ml of phosphate buffer, pH 6.0 and used as a stock solution. One ml of this solution was pipetted into 10-ml volumetric flask and diluted to volume with the same buffer. For the control group, 0.15 M phosphate buffer was used instead of the inhibitor/chitosan solutions at an equal volume.

Sodium Nitrite Solution (0.2 % w/v): The solution was prepared by dissolving 50 mg of sodium nitrite in 25.0 ml of water. The solution was prepared fresh daily.

Alcoholic Dye Base Solution: The solution was prepared by adding 110 ml of 95 % ethyl alcohol to 55 mg of N-1-naphthylethylenediamine and shake well. It can be stored at 2-8 °C for several months and should be discarded when the solution becomes colored, or if microbial growth becomes evident.

Calibration Curve of β-Naphthylamine

The β-naphthylamine standard stock solution contained 0.018 mg/ml of β-naphthylamine in phosphate buffer pH 6.0. The solution was individually pipetted into six tubes with various volumes as indicated in columns 2 and 3 of the diagram shown below. Appropriate volumes of 2 N HCl were added to each tube to make the total volume of 1.5 ml. The preparations were analyzed as described in steps 4 to 7 in the analytical method. The final nominal concentrations of β-naphthylamine in the

reaction mixture after adding sodium nitrite, ammonium sulfamate and alcoholic dye base solution were 0, 3.6, 7.2, 14.4, 21.6 and 28.8 mg/ml x 10^{-4}. The absorbance of the dye was determined in a 1-cm cell using a spectrophotometer at 580 nm.

Analytical Method

1. For test solution, 10.0 ml of L-leu-β-NA was mixed with 10.0 ml of either chitosan (0.25 to 1.25 % w/v) or bestatin (inhibitor) solution.
2. After adding 10.0 ml of leucine aminopeptidase enzyme, the reaction was allowed to run for 4 hr at 37 °C.
3. Samples of 2.0 ml each were withdrawn from the reaction mixture at predetermined intervals (0, 10, 20, 30, 40, 60, 90, 120 and 240 min) and quenched with 0.5 ml of 2 N HCl. The solution was mixed by gently shaking. The total volume of each withdrawn sample after quenching was 2.5 ml.
4. 0.5 ml sodium nitrite solution was added to each tube. The mixture was quickly agitated and kept for exactly 3 min at room temperature.
5. 1.0 ml ammonium sulfamate (provided with the assay kit) was added to each tube. The mixture was quickly agitated and kept for exactly 3 min at room temperature.
6. 2.0 ml alcoholic dye base solution (provided with the assay kit) was added to each tube. The mixture was agitated and kept for 45 $\pm$ 10 min at room temperature. The final volume in each sample was 6.0 ml.
7. The mixture was then transferred to a cuvette and the absorbance read at 580 nm using phosphate buffer as a blank. The buffer control tube was also similarly prepared. However, the chitosan or inhibitor solution was replaced with an equal volume of buffer pH 6.0.

The enzyme inhibitory activity was determined by calculating the area under the metabolite (β-naphthylamine) concentration versus time curve up to 240 min

using the trapezoidal rule. The values were subsequently compared with the control group.

Calibration solutions of β-naphthylamine

Tube #	β-naphthylamine Standard Solution (ml)	2N HCl (ml)	Sodium Nitrite (ml)	Ammonium Sulfamate (ml)	Alcoholic Dye Base Solution (ml)	Conc. of β-naphthylamine (mg/ml) x 10^{-4}
1	0.0	1.5	0.5	1.0	2.0	0
2	0.1	1.4	0.5	1.0	2.0	3.6
3	0.2	1.3	0.5	1.0	2.0	7.2
4	0.4	1.1	0.5	1.0	2.0	14.4
5	0.6	0.9	0.5	1.0	2.0	21.6
6	0.8	0.7	0.5	1.0	2.0	28.8

* Final volume of the reaction mixture for each standard conc. = 5.0 ml

Statistical Analysis

All data were expressed as mean ± SD. Statistical evaluations of the data were made by Student's t-test and analysis of variance (ANOVA), and by multiple comparison of the means using Duncan's test where appropriate. Differences between group means were considered significant at p-value < 0.05. The computation was performed using a statistical software package (SAS Inc.).

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CHAPTER IV
RESULTS AND DISCUSSION

I. Studies to Determine the Optimum Enhancing Conditions for Chitosans

The purpose of this part was to investigate the optimum nasal absorption enhancing conditions for two types of chitosans, i.e., the free amine chitosan (CSJ) and the glutamate salt form (CSG).

Determination of maximum absorption wavelength of o-cresolphthalein complexone (OCPC) color reagent containing 2.5 x 10^{-5} M calcium.

From the preliminary study, the absorption spectra of the o-cresolphthalein complexone (OCPC) solution in the presence of 2.5 x 10^{-5} M calcium at pH 10.0 is shown in Figure 2. The wavelength of maximum absorption was detected at 570 nm and the presence of other reagents in the reaction mixture did not interfere at this wavelength. Its absorptivity was found to be 458, which compared favorably with that of Mager et al.(1981), who reported the value of 525. As a result, the quantitative analysis of calcium was performed by colorimetric measurements at 570 nm.

Calibration curve of calcium assay.

Figure 3 illustrates a representative calibration curve of calcium, which was constructed by plotting the absorbance at 570 nm versus the total calcium concentrations at pH 10.0. The standard solutions contained 0 to 150 mg of calcium per dl, representing 0 to 12 mg calcium per dl of blood serum under test conditions. These concentrations were used to construct calibration curves in all the experiments. The curves were linear but did not pass through the origin. The equation for this representative curve is expressed as $y = 0.056x + 0.008$, where y is the absorbance and x is the concentration in mg/dl. The regression coefficient was 0.9999. All other

standard curves gave similarly good linearity with the r^2 values in the range of 0.99 – 0.999 in most cases. Consequently, the linear regression equation was always used to determine the calcium concentration of the unknown blood samples.

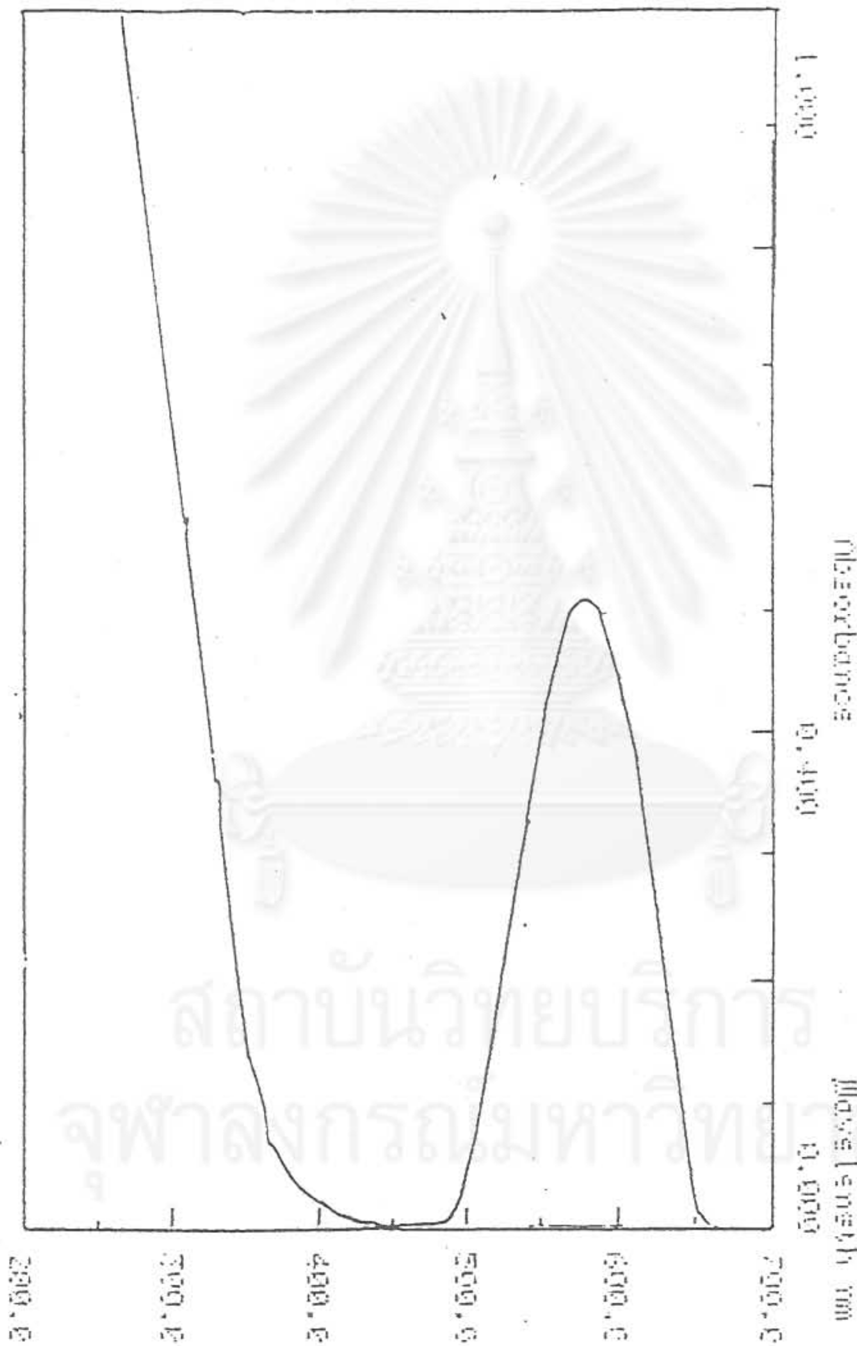

Figure 2. Absorption spectra of orthocresolphthalein complexone (OCPC) in the presence of calcium (2.5×10^{-5} M) at pH 10.0.

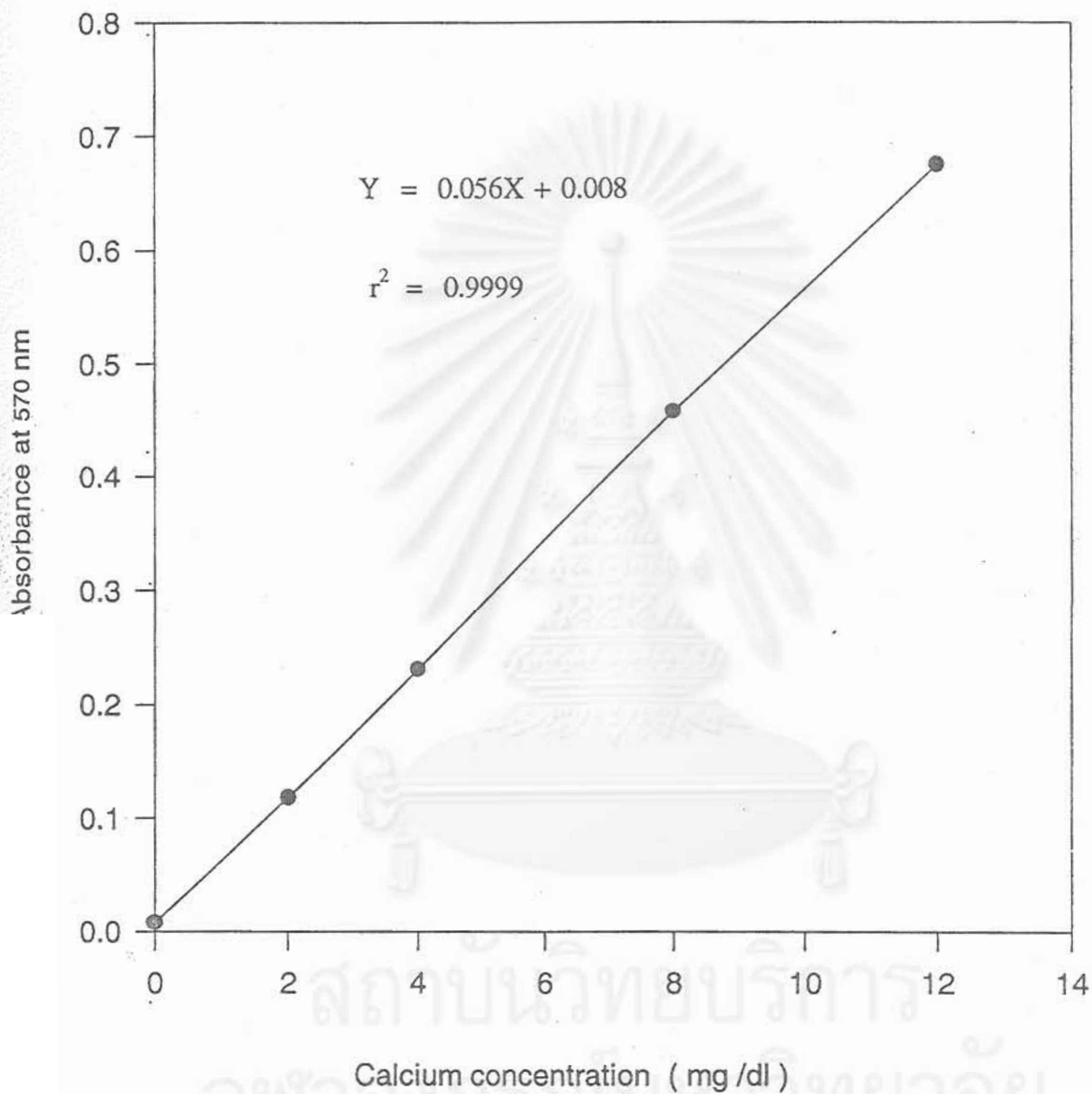

Figure 3. Representative calibration curve of orthocresolphthalein complexone (OCPC) color reagent containing calcium at different concentrations

1.1 *In Vivo* Nasal Absorption of Salmon Calcitonin (sCT): Effect of Varying pH on the Absorption Enhancing Activities of Chitosans in Rats

Four pH values (3.0, 4.0, 5.0, and 6.0) were selected in order to determine the pH of optimum enhancing activity for each type of chitosans. These values were chosen as a result of the ability of CSJ and CSG to dissolve in this pH range. Generally, chitosans can be dissolved in an acidic medium with a pH value below 6.5 (Sanford et al., 1991). The pH of the rat nasal mucosa is reported to be 7.39 (Hirai et al., 1981). The normal pH of the nasal secretions of human adults range approximately from 5.5 to 6.5, whereas in infants and young children, it ranges from 5.0 to 6.7 (Chien et al., 1989). Therefore, the pH values selected for the experiments (except for pH 3.0) were not too extreme for the nasal mucosa.

On the other hand, the experiments could not be set at the physiological pH (pH 7.4) due to precipitation of both CSJ and CSG when the pH of their solutions was raised above 6.5. The selected pH values of 4.0, 5.0, and 6.0 were considered to be relatively mild and close to the nasal secretions. More importantly, chitosans can be well dissolved in this pH region. In addition, the absorption experiments were also conducted at a more acidic pH of 3.0 since chitosans were most readily soluble at this pH and the maximum stability of sCT was reported to occur at about pH 3.3 (Lee et al., 1994). sCT encompasses several amino acids and functional groups that are susceptible to degradation. For instance, asparaginyl and/or aspartyl residue can undergo cyclization, deamidation and isomerization. The degradation reactions of sCT follow first-order kinetics and strongly depend on pH in the range of 3.0 and 6.0.

Moreover, Pujara et al. (1995) found that phosphate buffers with pH values between 3 and 10 caused minimal protein and enzyme release, while buffers with pH values above 10 and below 3 seemed to produce both membrane and intracellular damages. As a result, the pH range selected in this study (3.0 to 6.0) appeared to be

suitable for the study of the pH effect of chitosans on their nasal absorption enhancing activities.

On the other hand, the cyclodextrin derivatives (HPβCD and DMβCD) chosen as reference enhancers in this study can be dissolved at physiological pH. Therefore, the absorption of sCT with the two cyclodextrins was studied at pH 7.4 due to their good solubility, safety and enhancing activities at this pH (Gill et al., 1994).

The nasal preparations of the baseline groups (only buffers without sCT) and the sCT-treated groups at the dose of 10 IU/kg, with or without 1 % of the two chitosans, were nasally administered by the methods previously described. To prove that the nasally absorbed sCT was biologically active, measurements of the plasma calcium level were carried out and compared to that of the baseline buffer groups. The purpose of monitoring plasma calcium following nasal administration of only the pure buffer was to ensure that the decrease in plasma calcium level was due to the pharmacological effect of the absorbed sCT, not from the buffer effect, which may have caused changes in the endogenous calcitonin level. Also, the observation of stable plasma calcium levels in these baseline groups would confirm the absence of any surgical stresses and other experimental influences which may have affected the endogenous plasma calcium level which, in turn, may have interfered with the hypocalcemic effect to be observed with the sCT-treated groups.

The concentration of the plasma calcium after nasal administration was normalized as percent of the initial values. It is known that plasma calcium is subject to exquisite, fine-tuned regulation in a variety of circumstances. There is a three-fold control system for regulating plasma calcium using a combination of parathyroid hormone, calcitonin, and 1,25-dihydroxycholecalciferol. The three hormones regulate calcium by their actions on the intestine, bone and kidney functions (Robert et al., 1992). The level of plasma calcium also tends to vary in the same direction as the serum protein and inversely with the serum inorganic phosphorus. In addition, this

effect varies from rat to rat and may lead to a great variability in the initial calcium values. Therefore, the normalization of the plasma calcium levels with respect to the initial state appeared to be appropriate in this study.

Plasma calcium depression due to sCT absorption was evaluated in each rat based on three parameters, i.e, the total percent decrease in plasma calcium (%D), the minimum plasma calcium level (C_{min} or the maximum plasma calcium depression), and the time to reach minimum plasma calcium level (T_{min}). The value of %D reflects the extent of hypocalcemic effect produced by the nasally absorbed sCT. The value of C_{min}, on the other hand, reflects the maximum hypocalcemic effect observed in each rat whereas T_{min} indicates the rate at which it occurs.

Tables 2 - 5 show the average plasma calcium levels, expressed as percent of the initial values, after nasal administration of sample solutions at various pH's. The individual plasma calcium levels, along with the values of the above three parameters and $AUC_{0\text{-}240min}$, are provided in Appendix II. Summary of the average values of C_{min}, T_{min}, AUC, and %D for the baseline (buffer only), the control (sCT alone), and the chitosan-treated (sCT plus chitosan) groups is provided in Tables 6 and 7.

Figures 4-7 represent plots of percent plasma calcium versus time for each of the control and chitosan-treated (sCT plus chitosan) groups at pH 4.0, 5.0, 6.0, and 3.0, respectively, in comparison with their respective baseline groups. The first pH which was investigated was pH 4.0. From the data in Table 2, it can be seen that nasal administration of IPB pH 4.0 alone induced practically no changes in the percent plasma calcium over the 240 min period. This indicated that neither the surgical procedure nor the buffer itself had any effects on the plasma calcium during the experiment, resulting in a relatively stable calcium baseline. At 240 min, the value (mean ± SD) was about 98.17 ± 2.14 % of the initial level. Similarly stable plasma calcium baselines were also observed after nasal administration of IPB at other pH's, namely, 5.0, 6.0, and 7.4 (Table 2 except pH 3.0). It thus appeared that

of the initial value) of rats following na
buffer at various pH (Baseline groups)

0.15 M IPB	Time (min)										AUC$_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
pH 3.0	100.00 ± 0.00	101.79 ± 2.13	101.21 ± 2.24	100.18 ± 1.90	98.06 ± 2.29	94.64 ± 1.88	92.48 ± 1.28	93.31 ± 1.85	93.78 ± 1.49	94.76 ± 1.75	22,811.45 ± 194.71
pH 4.0	100.00 ± 0.00	102.18 ± 2.53	101.50 ± 1.70	101.58 ± 2.24	100.76 ± 1.26	100.55 ± 1.18	99.14 ± 0.99	97.94 ± 1.43	98.50 ± 1.29	98.17 ± 2.14	23,814.38 ± 269.47
pH 5.0	100.00 ± 0.00	102.12 + 2.60	102.73 ± 1.65	101.77 ± 1.65	103.09 ± 1.72	101.75 ± 1.38	100.52 ± 1.81	100.02 ± 2.18	98.36 ± 2.20	99.18 ± 1.24	24,049.35 ± 125.12
pH 6.0	100.00 ± 0.00	102.89 ± 0.92	102.02 ± 0.97	101.68 ± 0.97	101.42 ± 2.02	100.05 ± 1.73	100.55 ± 2.14	99.91 ± 1.91	98.82 ± 1.27	100.23 ± 1.04	24,037.27 ± 319.92
pH 7.4	100.00 ± 0.00	101.99 ± 2.90	101.43 ± 2.17	101.47 ± 2.13	100.29 ± 2.13	100.10 ± 2.01	100.97 ± 1.70	99.67 ± 1.84	100.64 ± 2.21	101.34 ± 1.75	24,148.43 ± 256.81

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Table 3. Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of sCT alone (10 IU/kg) at various pH (Control groups)

sCT alone	Time (min)										$AUC_{(0\text{-}240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
pH 3.0	100.00 ± 0.00	98.98 ± 2.52	94.41 ± 2.25	88.24 ± 2.80	84.85 ± 2.11	88.02 ± 2.53	89.19 ± 2.64	89.51 ± 0.87	89.86 ± 1.08	89.86 ± 1.15	21,564.16 ± 208.32
pH 4.0	100.00 ± 0.00	101.28 ± 1.76	99.20 ± 1.86	99.64 ± 1.86	95.28 ± 1.83	89.00 ± 2.01	91.21 ± 2.64	92.78 ± 2.59	93.66 ± 2.24	93.05 ± 1.28	22,477.78 ± 272.04
pH 5.0	100.00 ± 0.00	101.78 + 2.54	101.60 ± 1.46	99.32 ± 2.30	96.09 ± 1.90	92.24 ± 2.24	94.30 ± 1.39	92.78 ± 1.97	94.03 ± 1.70	93.78 ± 2.54	22,733.23 ± 305.96
pH 6.0	100.00 ± 0.00	101.39 ± 2.78	100.54 ± 1.48	98.44 ± 1.44	95.85 ± 1.12	93.21 ± 1.51	94.78 ± 1.76	94.78 ± 1.76	94.67 ± 2.29	93.82 ± 1.44	22,797.40 ± 213.15
pH 7.4	100.00 ± 0.00	101.22 ± 2.30	99.95 ± 2.29	97.80 ± 1.50	95.62 ± 1.38	93.80 ± 1.86	94.02 ± 0.93	94.44 ± 1.55	94.57 ± 1.70	94.68 ± 1.46	22,854.02 ± 230.27

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Table 4. Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of sCT (10 IU/kg) with 1 % CSJ at various pH

1% CS J	Time (min)										$AUC_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
pH 3.0	100.00 ± 0.00	94.01 ± 3.14	85.52 ± 4.02	73.47 ± 4.22	76.64 ± 1.97	81.74 ± 2.46	84.05 ± 2.41	85.21 ± 2.25	87.34 ± 2.69	86.05 ± 0.97	20,400.98 ± 384.39
pH 4.0	100.00 ± 0.00	101.49 ± 2.21	90.70 ± 2.42	83.03 ± 3.26	74.45 ± 2.59	81.19 ± 4.21	87.30 ± 4.12	93.14 ± 1.64	91.31 ± 1.58	92.71 ± 1.47	21,469.01 ± 282.93
pH 5.0	100.00 ± 0.00	101.04 + 1.94	96.37 ± 2.36	90.22 ± 2.16	84.09 ± 2.74	88.49 ± 2.97	91.80 ± 2.81	93.29 ± 0.87	93.31 ± 0.99	93.39 ± 1.30	22,201.42 ± 246.33
pH 6.0	100.00 ± 0.00	100.94 ± 1.95	97.88 ± 1.75	95.31 ± 1.66	91.14 ± 2.99	86.40 ± 1.95	91.98 ± 1.95	93.01 ± 1.84	93.59 ± 1.42	93.07 ± 1.41	22,307.88 ± 82.37

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Table 5. Plasma calcium levels (expressed as % of the initial value) of rats following nasal administration of sCT (10 IU/kg) with 1 % CSG at various pH

1% CS G	Time (min)										AUC$_{(0\text{-}240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
pH 3.0	100.00 ± 0.00	95.08 ± 1.90	87.75 ± 3.74	83.26 ± 1.46	85.74 ± 3.74	86.00 ± 2.80	86.45 ± 1.49	87.74 ± 1.43	89.18 ± 0.78	88.81 ± 0.67	21,1560.52 ± 37.43
pH 4.0	100.00 ± 0.00	99.95 ± 1.69	95.02 ± 1.99	90.08 ± 3.20	85.47 ± 2.28	88.01 ± 3.01	91.83 ± 1.75	91.97 ± 2.01	93.40 ± 1.35	92.59 ± 2.02	22,107.33 ± 130.35
pH 5.0	100.00 ± 0.00	99.79 + 1.50	97.20 ± 1.89	90.54 ± 4.18	82.57 ± 1.85	86.16 ± 3.00	92.01 ± 3.00	94.81 ± 1.20	94.56 ± 0.80	94.51 ± 1.67	22,305.08 ± 88.20
pH 6.0	100.00 ± 0.00	100.46 ± 3.22	95.58 ± 4.23	88.86 ± 4.65	79.98 ± 2.70	84.04 ± 2.11	89.92 ± 3.19	92.39 ± 1.93	94.76 ± 1.74	93.96 ± 1.76	22,008.99 ± 162.21

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Table 6. Comparison of the total percent decrease in plasma calcium level following intranasal administration of different baseline and control groups at various pH

Route of Administration	Dose (IU/Kg)	Adjuvants	pH	C_{min} (% of initial value)	T_{min} (min)	$AUC_{0\text{-}240min}$ [% . min]	%D
i.n.	-	none (baseline group)	pH 3.0	-	-	22,811.45 ± 194.71	-
			pH 4.0	-	-	23,814.38 ± 269.47	-
			pH 5.0	-	-	24,049.35 ± 125.12	-
			pH 6.0	-	-	24,037.27 ± 319.92	-
			pH 7.4	-	-	24,148.43 ± 256.81	-
i.n.	10	sCTalone (control group)	pH 3.0	84.85 ± 2.11	38.00 ± 4.00	21,564.16 ± 208.32	5.45 ± 0.91
			pH 4.0	89.00 ± 2.01	66.00 ± 12.00	22,477.78 ± 272.04	5.61 ± 1.14
			pH 5.0	92.04 ± 2.24	132.00 ± 69.97	22,733.23 ± 305.96	5.47 ± 1.27
			pH 6.0	93.21 ± 1.51	126.00 ± 48.00	22,797.40 ± 213.15	5.16 ± 0.89
			pH 7.4	93.80 ± 1.86	98.00 ± 72.77	22,854.02 ± 230.27	5.36 ± 0.95

Each Values = mean ± SD. (n = 5 rats/group)

Table 7. Comparison of the total percent decrease in plasma calcium level following intranasal administration of sCT with 1 % chitosans at various pH

Route of Administration	Dose (IU/Kg)	Adjuvants	pH	C_{min} (% of initial value)	T_{min} (min)	$AUC_{0\text{-}240min}$ [% . min]	%D
i.n.	10	1% CS J	pH 3.0	72.66 ± 2.86	32.4 ± 4.00	20,400.98 ± 384.39	10.57 ± 1.69
			pH 4.0	73.47 ± 1.47	44.00 ± 8.00	21,469.01 ± 282.93	9.85 ± 1.19
			pH 5.0	83.37 ± 2.27	44.00 ± 8.00	22,201.42 ± 246.33	7.68 ± 1.02
			pH 6.0	86.27 ± 1.77	56.00 ± 8.00	22,307.88 ± 82.37	7.19 ± 0.34
i.n	10	1% CS G	pH 3.0	82.04 ± 1.63	38.00 ± 11.66	21,156.52 ± 37.43	7.25 ± 0.17
			pH 4.0	84.79 ± 1.39	44.00 ± 8.00	22,107.33 + 130.35	7.17 ± 0.55
			pH 5.0	81.55 ± 0.94	44.00 ± 8.00	22,305.08 ± 88.20	7.25 ± 0.37
			pH 6.0	79.38 ± 1.82	44.00 ± 8.00	22,008.99 ± 162.21	8.44 ± 0.68

Each Values = mean ± SD. (n = 5 rats/group)

Figure 4. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 4.0. Each point represents mean ± SD (n = 5 rats/group).

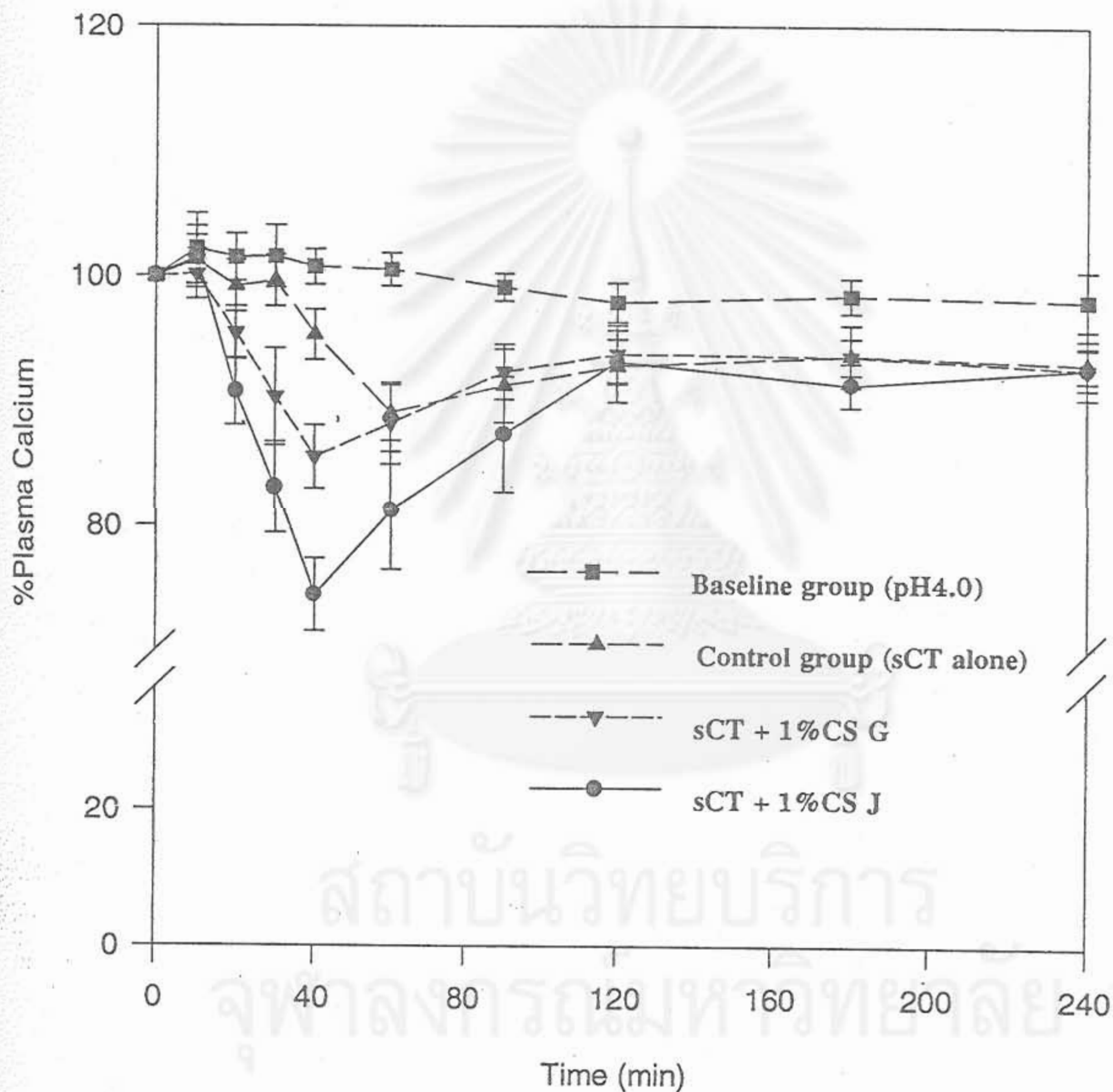


Figure 5. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 5.0. Each point represents mean ± SD (n = 5 rats/group).

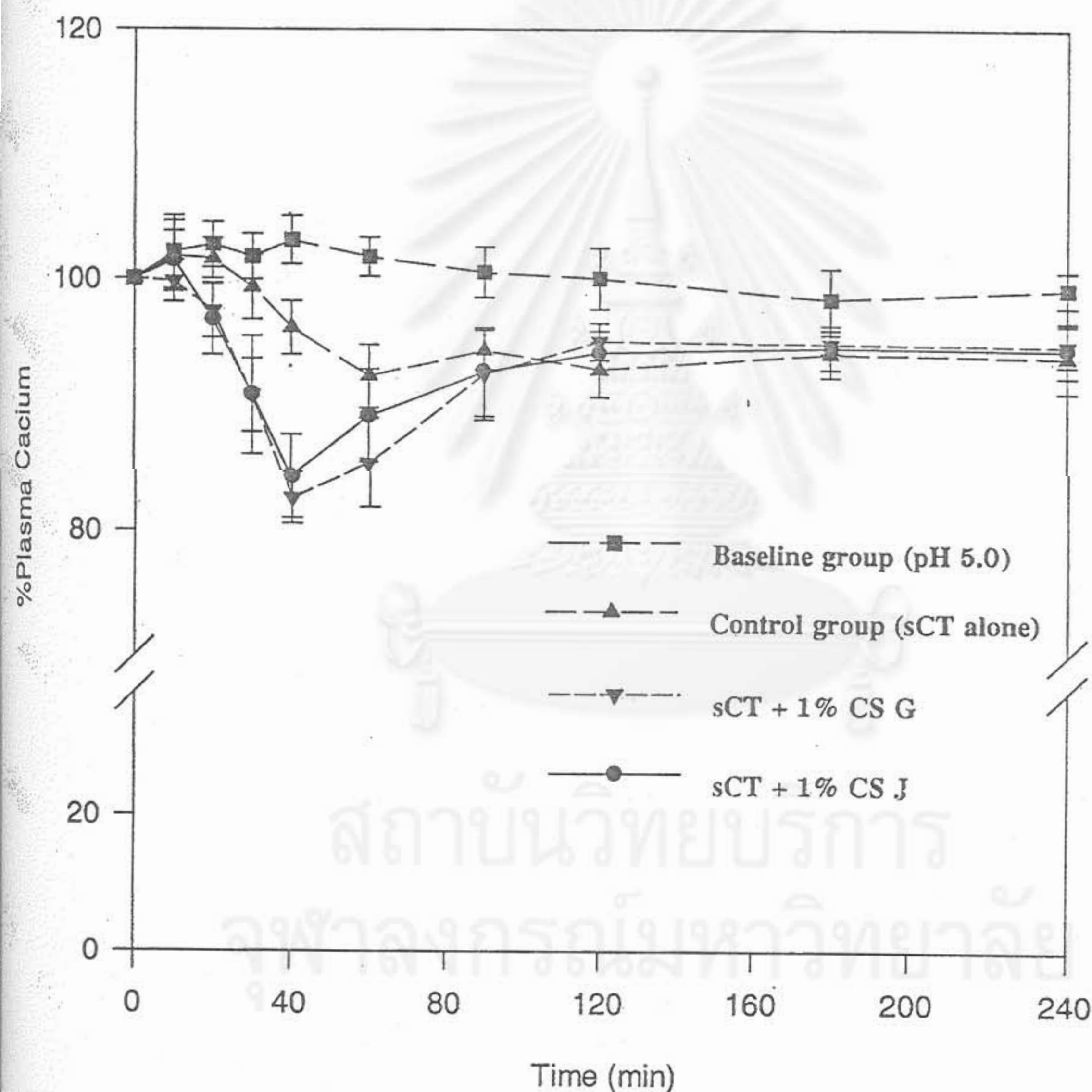


Figure 6. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 6.0. Each point represents mean ± SD (n = 5 rats/group).

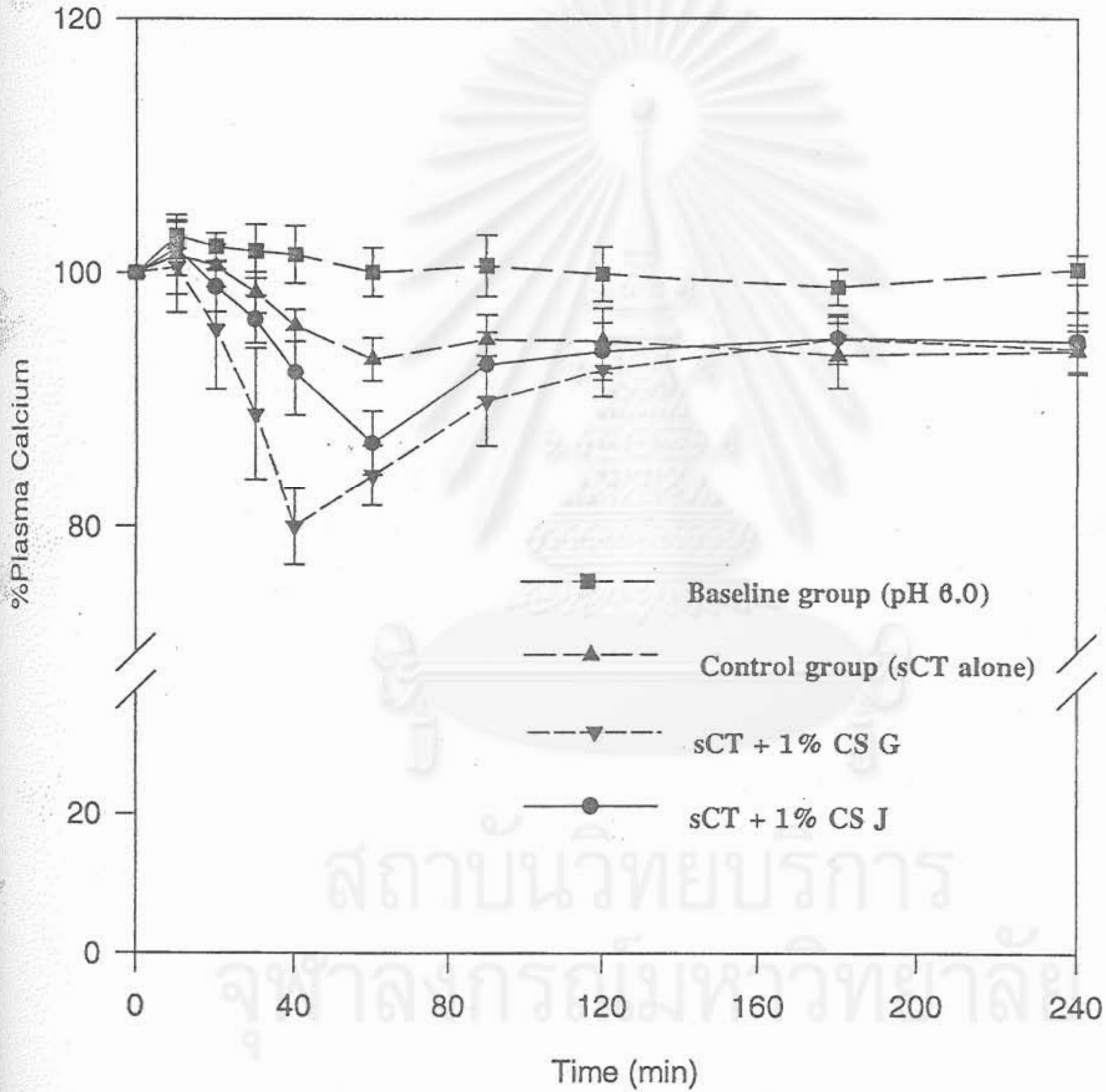
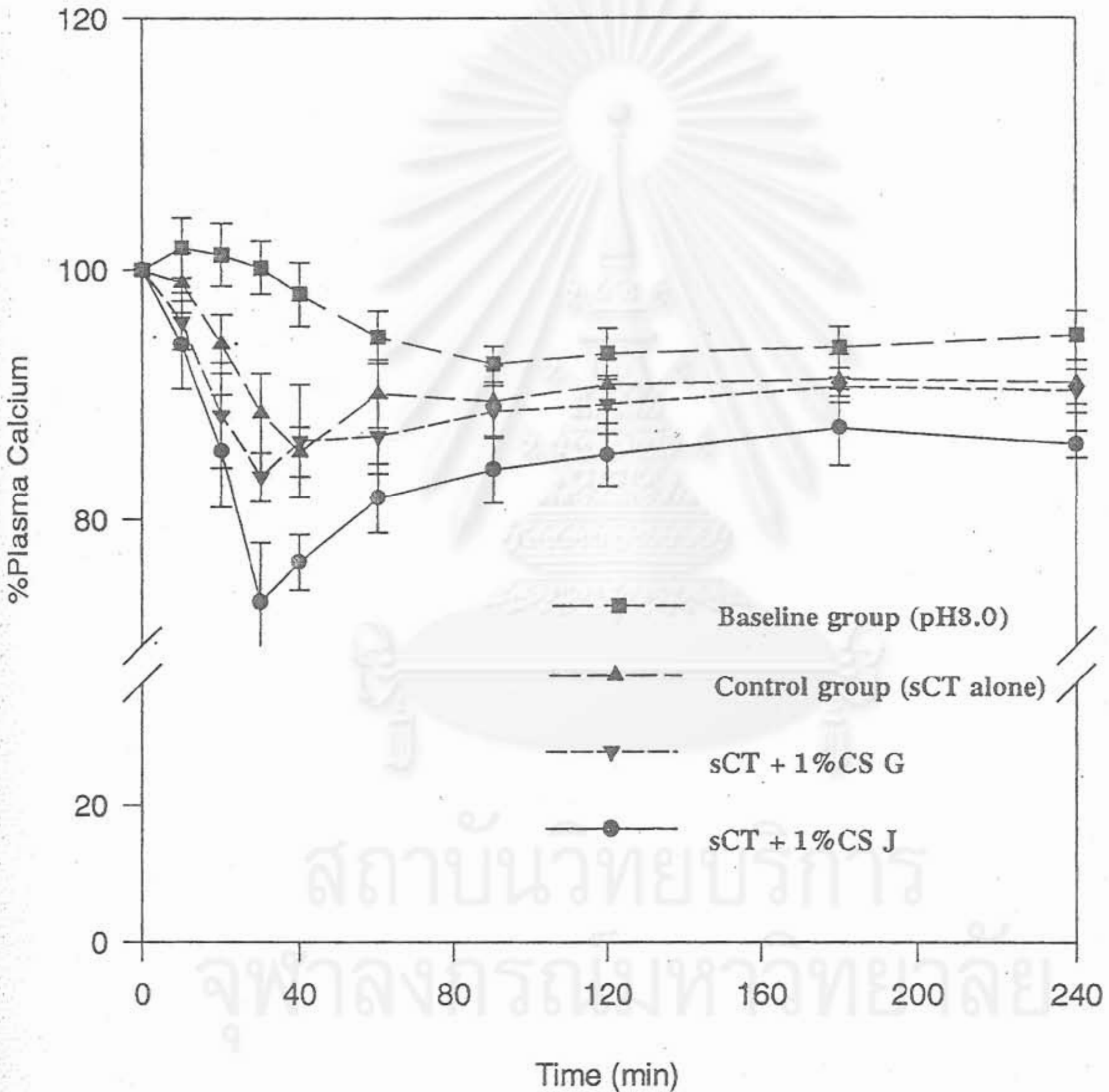


Figure 7. Percent of plasma calcium versus time after nasal administration of sCT with or without 1 % w/v chitosans and comparison with the baseline group at pH 3.0. Each point represents mean ± SD (n = 5 rats/group).



there was no buffer effect on the rat plasma calcium in this pH range (4.0-7.4) and that the experimental procedures employed in this study, including the surgery, did not cause any noticeable changes in the plasma calcium level. As a result, a drop in plasma calcium following nasal administration of sCT (with and without enhancers) in these buffers should be only due to the hypocalcemic activity of the absorbed peptide.

Results in Table 3 and Figure 4 reveal that nasal instillation of 10 IU/kg of sCT alone in IPB pH 4.0 (control group) caused a small decrease in plasma calcium, with a minimum calcium level observed at about 60 min. The average C_{min} and T_{min} were found to be 89.00 ± 2.01 % and 66.00 ± 12.00 min, respectively, whereas the total percent decrease in calcium over 4 hr period (%D) at this pH was calculated to be only 5.61 ± 1.14 % (Table 6). However, when 1 % w/v CSJ was included in the nasal sCT formulation at this pH, a further drop in plasma calcium was observed when compared to the control group (sCT alone, Figure 4). The percent decrease in plasma calcium (%D) was found to be 9.85 ± 1.19 %. The maximum hypocalcemic effect was more potent and occurred at an earlier time, with the average C_{min} of 73.47 ± 1.47 % observed at 44.00 ± 8.00 min (Table 7). This was about 20 minutes faster than the control group. Addition of 1 % w/v CSG at pH 4.0 also caused the same enhancement, with the average %D of 7.17 ± 0.55 %. The mean C_{min} and T_{min} were found to be 84.79 ± 1.39 % and 44.00 ± 8.00 min, respectively. The maximum calcium lowering effect of CSG, as judged from the C_{min} value, was somewhat less than CSJ (84.79 vs 73.47 %). However, the similar T_{min} values (44 min) indicated that both CSJ and CSG enhanced the nasal absorption of sCT at about the same rate.

To confirm whether the enhancing effect of the two chitosans was significant, analysis of variance (ANOVA) was performed on the values of %D among the three groups, namely, the control (sCT alone), sCT plus CSJ, and sCT plus CSG, at 5 % significance level. The total percent decrease in plasma calcium (%D) was selected as

the main parameter for statistical comparison since its represents the changes in the plasma calcium over the entire period of study (4 hr). In addition, the calculation also took into account the AUC of the baseline group to ensure that any possible fluctuation of the baseline calcium level would be corrected for the entire period. Therefore, this parameter appeared to be most appropriate for evaluation of the hypocalcemic effect produced by nasally administered sCT.

ANOVA results revealed that there were significant differences in %D among the three groups at pH 4.0 ($p < 0.05$). Duncan's New Multiple Range test was further applied to these data at the same significance level in order to rank this difference. The ranking result, in an increasing order, was

	Control (pH 4.0)	< CSG	< CSJ
%D	5.61	7.17	9.85 %

From the Duncan's test results, it can be seen that both chitosans significantly enhanced the nasal absorption of sCT over the control groups ($p < 0.05$). In addition, CSJ was more effective than CSG in enhancing the nasal absorption of sCT at this pH ($p < 0.05$).

The second pH to be investigated was pH 5.0. The data were found to be similar to that at pH 4.0. As can be seen from Table 2 and Figure 5, the basal calcium level remained relatively unchanged over 4 hr after nasal administration of only IPB pH 5.0. At 240 min, the value of plasma calcium was 99.18 ± 1.24 %, indicating that there was no buffer effect, nor fluctuation of the plasma calcium due to experimental stresses.

The nasal administration of sCT alone in this buffer (control group) resulted in the average C_{min} of only 92.04 ± 2.24 %, which occurred very slowly at 132.00 ± 69.97 min (Table 6). The total percent decrease in plasma calcium was only 5.47 ±

1.27 %. However, when 1 % w/v of CSJ or CSG was included in the sCT solutions, the absorption was markedly enhanced, as can be seen from a sharp drop in the plasma calcium level (Figure 5). CSJ rapidly caused maximum hypocalcemic effect at 44.00 ± 8.00 min, with the average C_{min} value of 83.37 ± 2.27 %. The total percent decrease in plasma calcium (%D) was found to be 7.68 ± 1.02 %. CSG also enhanced nasal absorption of sCT with similar rate and extent to that produced by CSJ, with the values of T_{min}, C_{min}, and %D of 44.00 ± 8.00 min, 81.55 ± 0.94 %, and 7.25 ± 0.37 %, respectively (Table 7). When ANOVA was applied to analyze %D at 5 % significance level, it is obvious that there was a significant difference in this parameter among the three groups. Duncan's New Multiple Range test was further applied at the same significance level. The ranking of %D in an increasing order was

	Control (pH 5.0)	<	<u>CSG</u>	<u><</u>	<u>CSJ</u>
%D	5.47		7.25		7.68 %

The line underneath CSG and CSJ signifies that there were no significant differences between the two chitosans with respect to their absorption enhancing activity at this pH ($p > 0.05$). However, both chitosans significantly enhanced sCT nasal absorption over the control group ($p < 0.05$).

The next pH investigated was pH 6.0. This pH is relatively close to the nasal and physiological pH (about 5.6 and 7.4). Both CSJ and CSG were still dissolved at this pH. As can be seen from the data in Table 2 and Figure 6, the plasma calcium of the baseline group receiving only IPB pH 6.0 remained constant and close to the initial value throughout the entire study period, thus indicating the stability of the basal calcium level. However, when sCT was nasally administered in this buffer, some absorption of the peptide was observed, as demonstrated by a slow decrease in the plasma calcium (Figure 6). The hypocalcemic effect occurred very slowly, with

average C_{min} of only 93.21 ± 1.51 % observed at 126.00 ± 48.00 min (Table 6). The total percent decrease in plasma calcium (%D) was found to be only 5.16 ± 0.89 %. On the other hand, when chitosans were included in the nasal formulations of sCT, a pronounced hypocalcemic effect was noticed for both CSJ and CSG at this pH. CSJ (1% w/v) effectively increased the nasal absorption of sCT, as demonstrated by a sharp decrease in plasma calcium, reaching a minimum of 86.27 ± 1.77 % at 56.00 ± 8.00 min, with the average %D of 7.19 ± 0.34 % (Table 7). CSG appeared to be even more effective at this pH. The data in Table 5 reveal that 1 % w/v CSG induced rapid nasal absorption of sCT, which resulted in a sharp drop of plasma calcium (Figure 6), with the average values of C_{min}, T_{min}, and %D of 79.38 ± 1.82 %, 44.00 ± 8.00 min, and 8.44 ± 0.68 %, respectively (Table 7). When the values of %D were analyzed by ANOVA at 5 % level, significant differences were found among the three groups ($p < 0.05$). Duncans' test was again applied to rank the enhancing effect with respect to %D. The ranking result, in an increasing order, was

	Control (pH 6.0)	< CSJ	< CSG
%D	5.16	7.19	8.44 %

The above ranking indicated that both chitosans were effective in enhancing the nasal absorption of sCT over the control group ($p < 0.05$). Moreover, CSG was found to be more effective than CSJ at this pH ($p < 0.05$).

Because of the good stability of sCT solution at acidic pH, its nasal absorption in the presence of chitosans was also studied at pH 3.0 for comparison with other pH's. The results of these experiments are illustrated in Tables 2-5. It is interesting to note that the nasal administration of IPB pH 3.0 alone did not result in a stable calcium baseline as opposed to other baseline groups (Table 2 and Figure 8). In fact, the plasma calcium appeared to decrease, although somewhat very slowly, reaching

the minimum value of about 92.48 ± 1.28 % at 90 min and slightly increasing to 94.76 ± 1.75 % at 240 min (Table 2). AUC_{0-240} was used as a parameter to indicate the extent of plasma calcium fluctuation among the five baseline groups. ANOVA was applied at 5 % significance level and the result revealed that there was a significant difference in this parameter among the five buffers studied ($p < 0.05$). Duncan's test was further applied to rank this difference. The ranking result, in an increasing order, was

Baseline groups:	pH 3.0	< pH 4.0	< pH 6.0	< pH 5.0	< pH 7.4
AUC_{0-240} (%.min)	22,811	23,814	24,037	24,049	24,148

The line underneath the letters indicates that the four baseline groups consisting of IPB pH 4.0, 5.0, 6.0, and 7.4 did not differ significantly in the AUC_{0-240} values ($p > 0.05$), whereas the value at pH 3.0 was significantly lower than other pH values ($p < 0.05$). It can be assumed at this point that nasal administration of IPB at pH 3.0 alone was able to cause some fluctuation in the baseline calcium level. Therefore, the buffer effect was significant at pH 3.0. The reasons for the observed decrease in plasma calcium after nasal administration of IPB at this pH are not presently known. It is possible that the high acidity of this buffer may have caused some stresses to the rats and resulted in slight changes in the levels of endogenous calcitonin or parathyroid hormone, which play a key role in the regulation of calcium metabolism. Moreover, the mucosa-irritating pH 3.0 may have induced substantial absorption of phosphate ions from the buffer into systemic circulation. The increase in plasma phosphate ions might have influenced the calcium-phosphate mineral balance and resulted in changes in plasma calcium. These effects may have interfered with the measurements of hypocalcemic responses observed with sCT. However,

more evidence is needed to substantiate this postulation about increased phosphate absorption and its interference with acid-base balance and calcium metabolism. Therefore, the data at pH 3.0 were excluded from further analysis in order to minimize the confounding effect during the selection of optimum pH condition.

Although the nasal administration of only IPB pH 3.0 did not result in a stable calcium baseline, determination of the hypocalcemic extent exerted by nasal sCT (%D) was still possible because any possible buffer effects, if existed, would be accounted for in the calculation of this parameter. For example, Figure 9 is a histogram comparing the AUC_{0-240} of plasma calcium between the baseline and the control groups at their respective pH. Nasal administration of sCT resulted in a decrease in the AUC value over its corresponding baseline group regardless of the pH studied. Since the AUC values of various baseline groups may vary from each other, as was the case with pH 3.0, direct comparison of the AUC values among different sCT-treated groups could be confounded by the different baselines. Therefore, comparison based on %D appeared to be more appropriate since the individual AUC value of the sCT-treated rat must be subtracted from the average AUC value of the corresponding baseline group in order to obtain the actual decrease in plasma calcium induced by sCT. As can be seen from the plasma calcium profiles of the various control groups in Figure 10, the nasal absorption of sCT at pH 3.0 seems to be much more pronounced than at other pH's. However, when ANOVA was applied to analyzed %D, it is obvious that there was no significant difference in %D ($p > 0.05$) among the five groups.

As seen from Table 6, nasal administration of sCT in IPB pH 3.0 induced some absorption of peptide, with the mean C_{min},T_{min}, and %D of 84.85 ± 2.11 %, 38.00 ± 4.00 min, and 5.45 ± 0.91%, respectively. As expected, inclusion of either 1% w/v of CSJ or CSG in sCT nasal formulations further increased its absorption

(Figure 7 and Table 7). At pH 3.0 CSJ induced the highest rate and extent of hypocalcemic effect, with the respective C_{min}, T_{min}, and %D of 72.66 ± 2.86 %, 32.40 ± 4.00 min, and 10.57 ± 1.69 % (Table 7). CSG also rapidly enhanced nasal sCT absorption at this pH, with the relatively similar T_{min} of 38.00 ± 11.66 min. However, the extent of hypocalcemic effect was somewhat smaller than CSJ, with the average C_{min} and %D of 82.04 ± 1.63 % and 7.25 ± 0.17 %, respectively (Table 7).

When ANOVA was applied at 5 % level, significant difference was observed in the values of %D among the three groups ($p < 0.05$). Duncan's test was further applied and the ranking result, in an increasing order, was

	Control (pH 3.0)	<	CSG	<	CSJ
%D	5.45		7.25		10.57 %

The ranking results were exactly the same as that of pH 4.0 in which the free base CSJ was the most effective absorption enhancer of sCT ($p < 0.05$). CSG, although exerted the enhancing effect which was significantly smaller than CSJ, its effect was still significant when compared to the control group ($p < 0.05$). Similar ranking results in %D at pH 3.0 and 4.0 seem to suggest that the adjuvant activity of CSJ may be higher in a more acidic pH. On the other hand, the ranking results at pH 6.0 indicate that CSG tended to be more effective at higher pH value. The average %D of the three sCT-treated groups (control, CSJ, and CSG) at various pH's are summarized graphically for overall comparison in Figure 11.

Data in Table 6 also indicate that the hypocalcemic extent, as judged by %D, appeared to be similar among various control groups receiving only sCT at different pH. The average %D ranged from 5.16 to 5.61 % with no significant differences ($p > 0.05$). However, the T_{min} values tended to decrease as the pH was lowered, from 98 min at pH 7.4 to 38 min at pH 3.0. The C_{min} values also decreased from 93.8 % at pH

Figure 8. Comparison of percent plasma calcium following nasal administration of isotonic phosphate buffers (baseline groups) at different pH's. Each point represents mean ± SD (n = 5 rats/group).

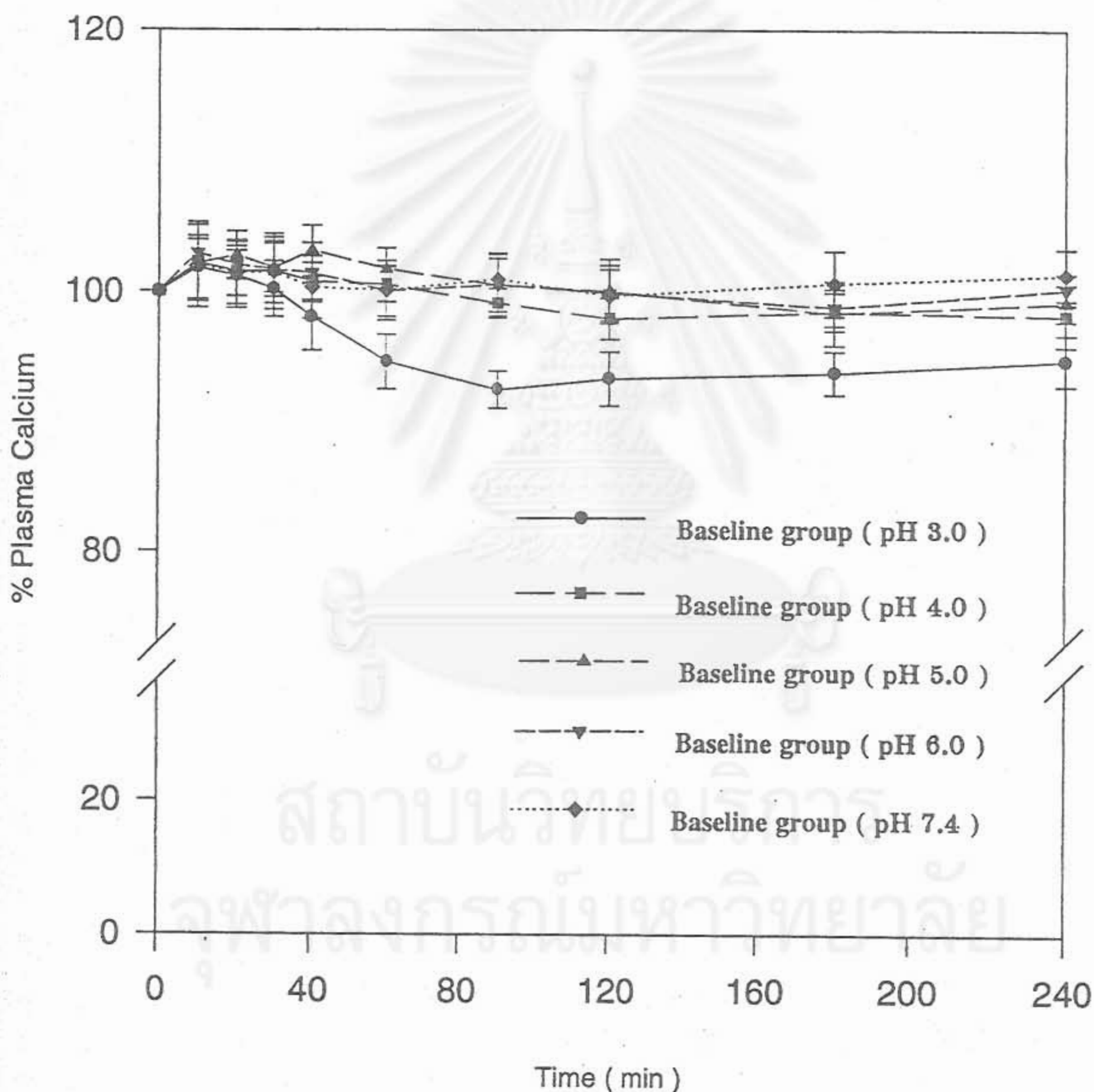


Figure 9. Comparison of area under plasma calcium vs time curve (AUC_{0-240}) after nasal administration to baseline and control groups at various pH's (4.0-7.4). Data = mean ± SD (n = 5 rats/group).

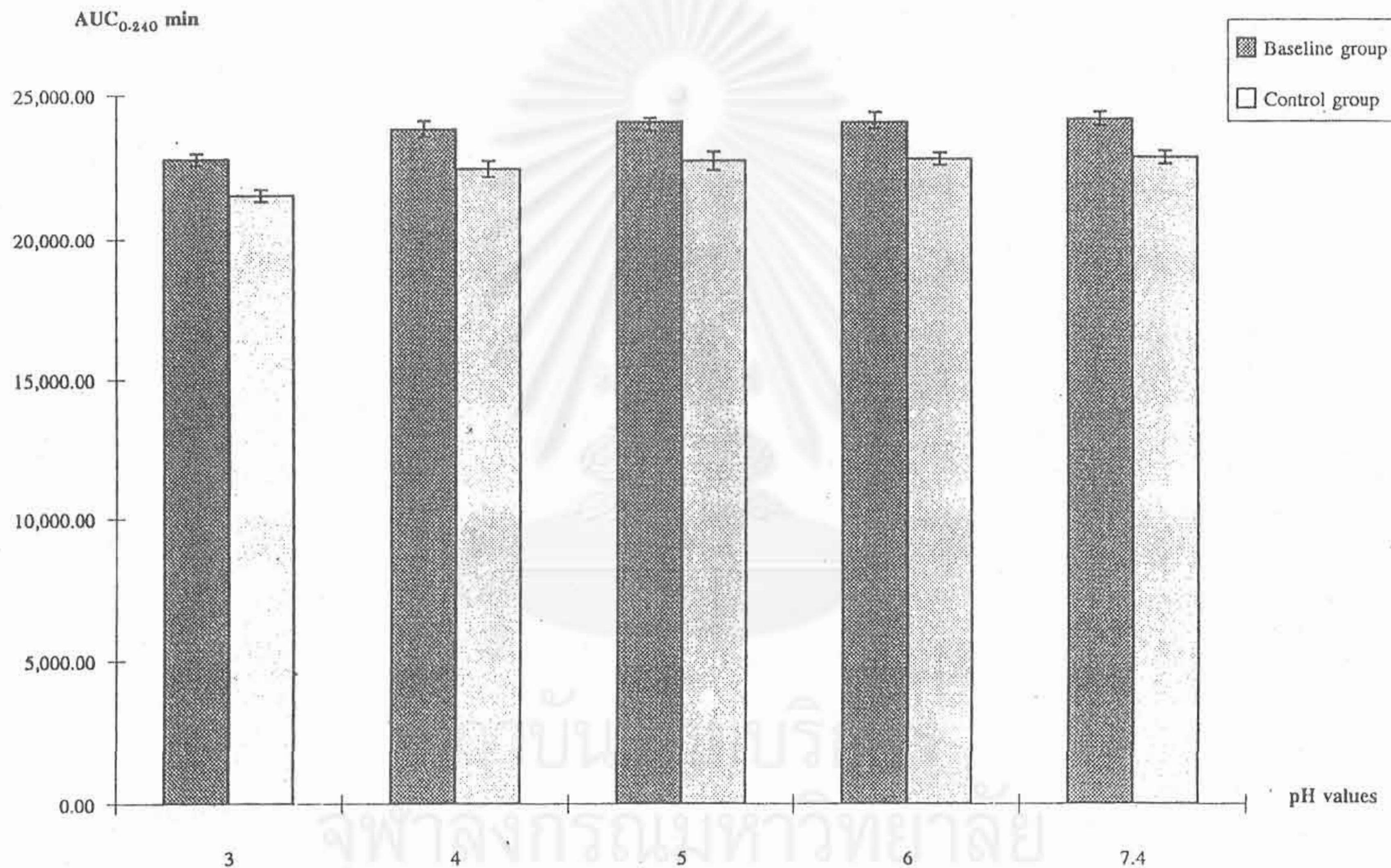
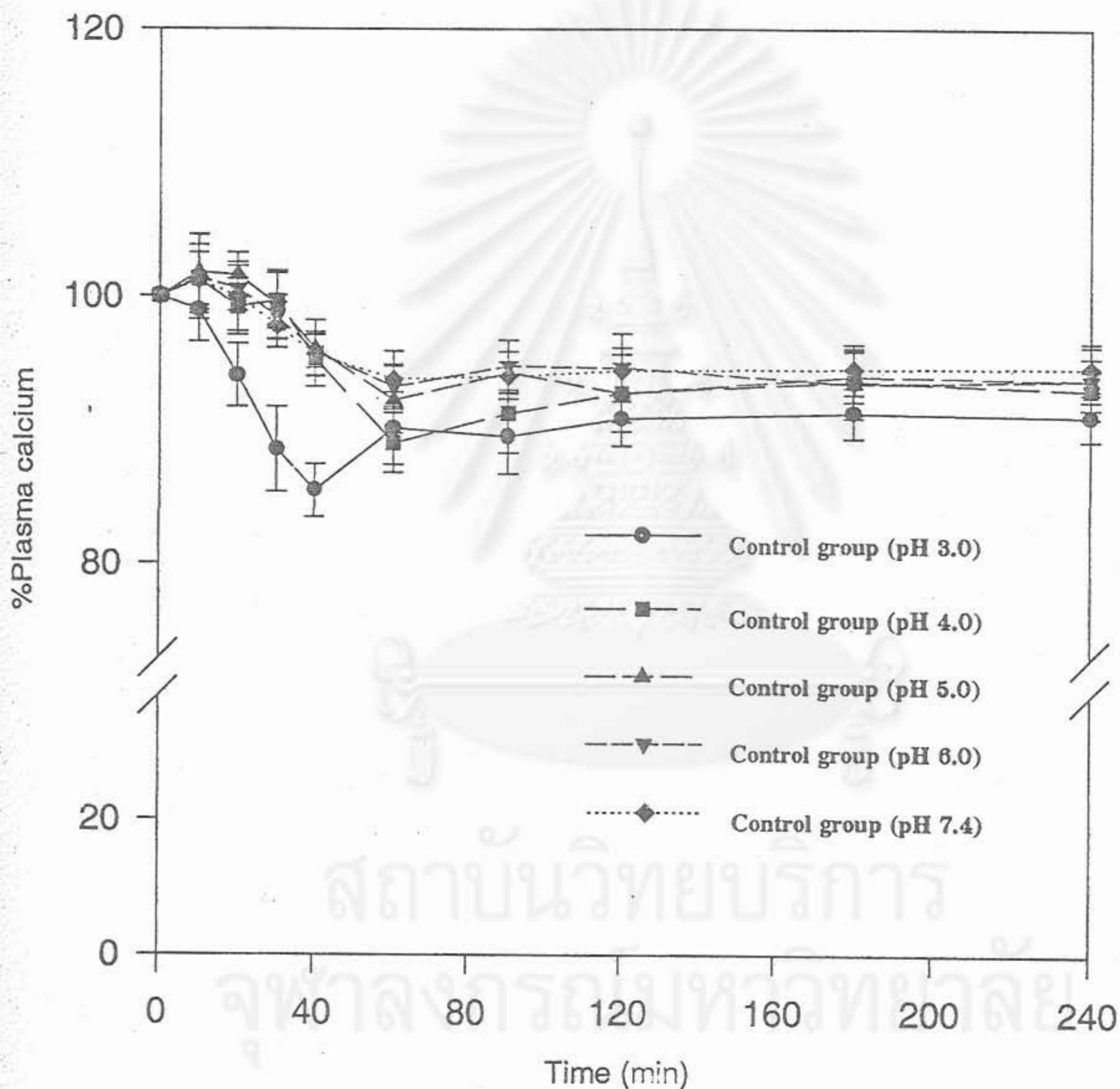


Figure 10. Percent of plasma calcium vs time after nasal administration of sCT without enhancers (control groups) at various pH (3.0-7.4). Each point represents mean ± SD (n = 5 rats/group).



7.4 to 84.8 % at pH 3.0. This indicates that, even in the absence of an enhancer, the rate of sCT nasal absorption may be increased in a more acidic solution. It is well known that sCT was most stable at relatively acidic pH and maximum stability was achieved by adjusting the pH to 3.3 (Lee et al., 1992). Furthermore, it is possible that the highly acidic pH like pH 3.0 may have caused some injuries to the nasal mucosa, leading to the increased membrane permeability, which might allow easier passage of peptide drugs like sCT. The results are in agreement with previous data (Tengamnuay et al., 1998). Using the *in situ* perfusion of the rat nasal cavity, we found that perfusion of L-Tyr-D-Arg, a model dipeptide, in IPB pH 3.0 without any adjuvant resulted in its significant absorption whereas perfusion of this dipeptide alone at higher pH values did not cause any nasal absorption. The absorption at pH 3.0 was attributed to be due to the high acidity of the buffer, which may have caused some damages to the nasal mucosa. By quantitating the extent of membrane protein and phosphorus release from the rat nasal mucosa after perfusion with IPB of different pH values, we found that IPB pH 3.0 induced the most pronounced leakage of these membrane components. On the other hand, IPB pH 4.0, 5.0, 6.0, and 7.4 produced only minimal release, indicating rather mild effects on the mucosa.

Similar to our previous results, the more rapid nasal absorption of sCT observed with CSJ and CSG at the most acidic pH in this study (pH 3.0) may not be due solely to the effect of the enhancers. It may as well be due to the deleterious effects of this buffer. Fluctuating plasma calcium baseline already reflected the extent of stress and membrane irritation, to which this buffer may have imposed on the rat during the experiment. For these reasons, the absorption at pH 3.0 was not further studied. The conditions appeared to be too acidic and may have caused injuries to the rat nasal mucosa, thereby making it unsuitable in clinical practice to use this pH value for nasal administration.

All the results up to the present pointed out that the two chitosans, at 1 % w/v, were always effective over the control groups regardless of the pH conditions in enhancing the nasal absorption of sCT, as judged from its plasma calcium lowering effect. Subsequently, the data were analyzed again by one-way ANOVA to determine the pH of optimum enhancing activity for each chitosan based on the values of %D. Figure 12 shows the plots of percent plasma calcium versus time after nasal administration of sCT with 1 % CSJ in IPB of various pH's. From this figure and the data in Table 7, it appears that the enhancing effect of CSJ was better in the more acidic conditions (pH 3.0 and 4.0) than at pH 5.0 and 6.0. The average %D was calculated to be 10.57 ± 1.69, 9.85 ± 1.19, 7.68 ± 1.02, and 7.19 ± 0.34 %, respectively. The average C_{min} and T_{min} also tended to decrease as the pH was lowered, indicating the higher adjuvant activity of CSJ at the more acidic pH. As previously discussed, however, the high acidity of the solution containing sCT and CSJ at pH 3.0 may have interfered with the plasma calcium levels. Therefore, the data at pH 3.0 were excluded from statistical analyses in order to minimize the confounding effect from this buffer. Only data obtained at pH 4.0, 5.0, and 6.0 were used for statistical comparison. ANOVA results at 5 % significance level revealed that there was a strong difference in the values of %D among the three pH's studied ($p < 0.05$). The ranking results after Duncan's test at the same significance level were as follows

	<u>pH 6.0 <</u>	<u>pH 5.0</u> <	pH 4.0
%D	7.19	7.68	9.85 %

Duncan's test thus indicated that, at the same concentration, CSJ was significantly more effective at pH 4.0 than at pH 5.0 and 6.0 ($p < 0.05$). On the other hand, the effect of CSJ at pH 5.0 and 6.0 was comparable ($p > 0.05$). Thus, CSJ

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appeared to be most effective at pH 4.0. The general tendency is that the free amine CSJ shows increasing adjuvant activity when the pH of the solution is lowered and vice versa. This phenomenon is most likely due to the pH-dependent change in the molecular configuration in conjunction with the degree of ionization of the chitosan molecule (Artursson et al., 1994). Chitosan has an apparent pKa of about 5.6. At higher pH values the chitosan molecule exists in a more coiled configuration. But as the pH decreases and the molecule becomes more ionized, the molecule uncoils and assumes a more elongated shape with greater extent of hydration (Filar et al., 1977). Hence, at lower pH values, chitosan has a higher charge density and will have a better possibility for intimate contact with the epithelial membrane (Artursson et al., 1994). The results obtained here also agree well with our previous study (Tengamnuay et al., 1998), who reported that CSJ, at 0.5 % w/v in IPB pH 4.0, was more effective in enhancing the nasal absorption of L-Tyr-D-Arg than at pH 5.0 or 6.0 ($p < 0.05$).

ANOVA was also applied to the values of %D comparing the enhancing effect of CSG at pH 4.0, 5.0, and 6.0. (The data at pH 3.0 was also excluded by the same reasons). Figure 13 shows the plots of percent plasma calcium versus time after nasal administration of sCT in the presence of 1 % w/v CSG at various pH's. It can be seen from this figure that the glutamate salt CSG tended to be more effective as the pH of the solution was increased in contrast to CSJ. ANOVA results revealed a significant difference in %D among the three pH's studied ($p < 0.05$). Further analysis using Duncan's test at the same significance level led to the following ranking result

	<u>pH 4.0 < pH 5.0</u>		< pH 6.0
%D	7.17	7.25	8.44 %

Thus, CSG appeared to be most effective at pH 6.0 ($p < 0.05$) whereas its enhancing effect at pH 4.0 and 5.0 was comparable as demonstrated by the adjoining

Figure 11. Comparison of the total percent decrease in plasma calcium (%D) after nasal administration of sCT with or without 1 % w/v chitosans (CSJ and CSG) at various pH's. Data = mean ± SD (n = 5 rats/group).

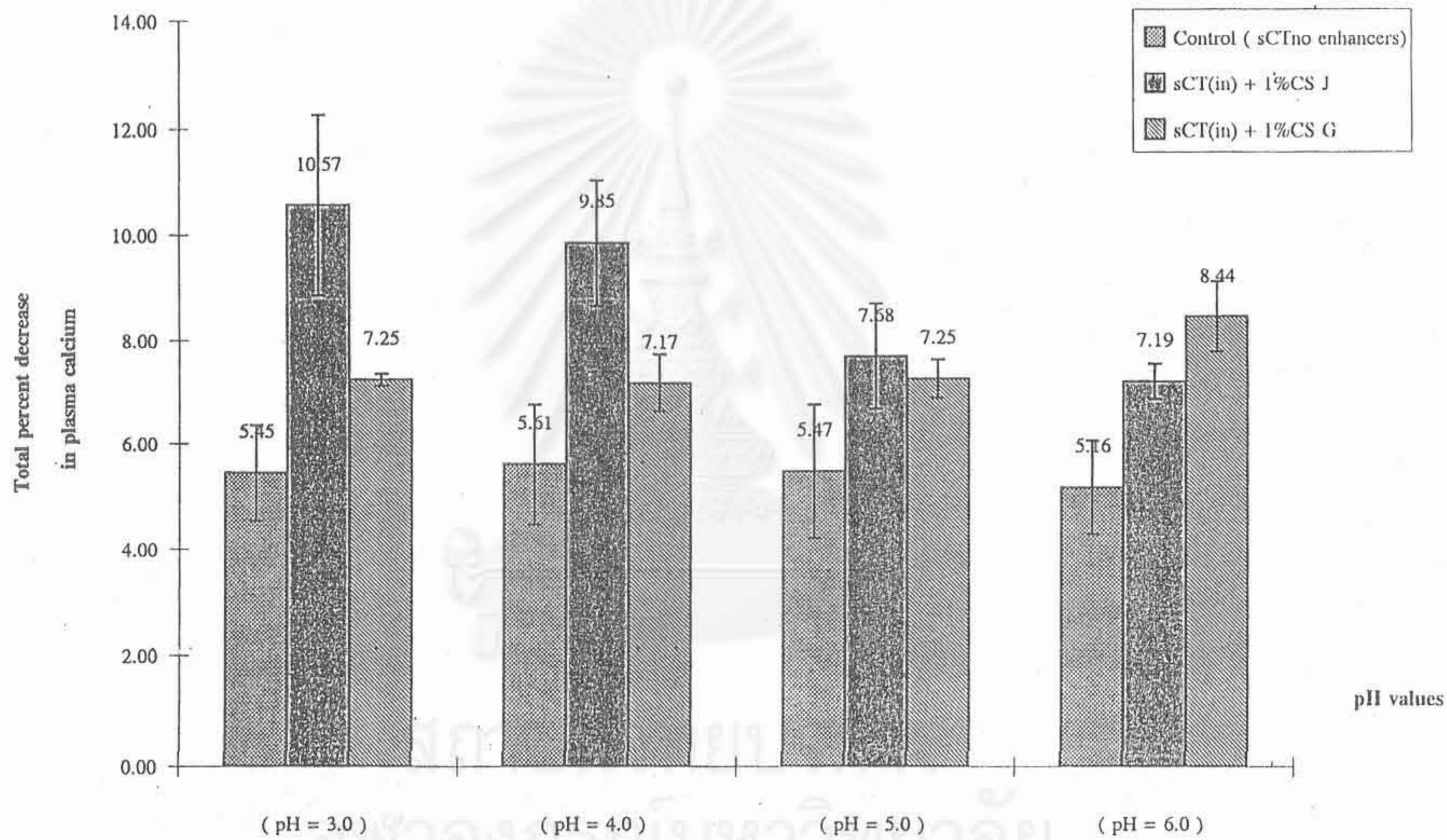


Figure 12. Percent plasma calcium after nasal administration of sCT with 1 % w/v CSJ at various pH's (3.0-6.0). Each point represents mean ± SD (n = 5 rats/group).

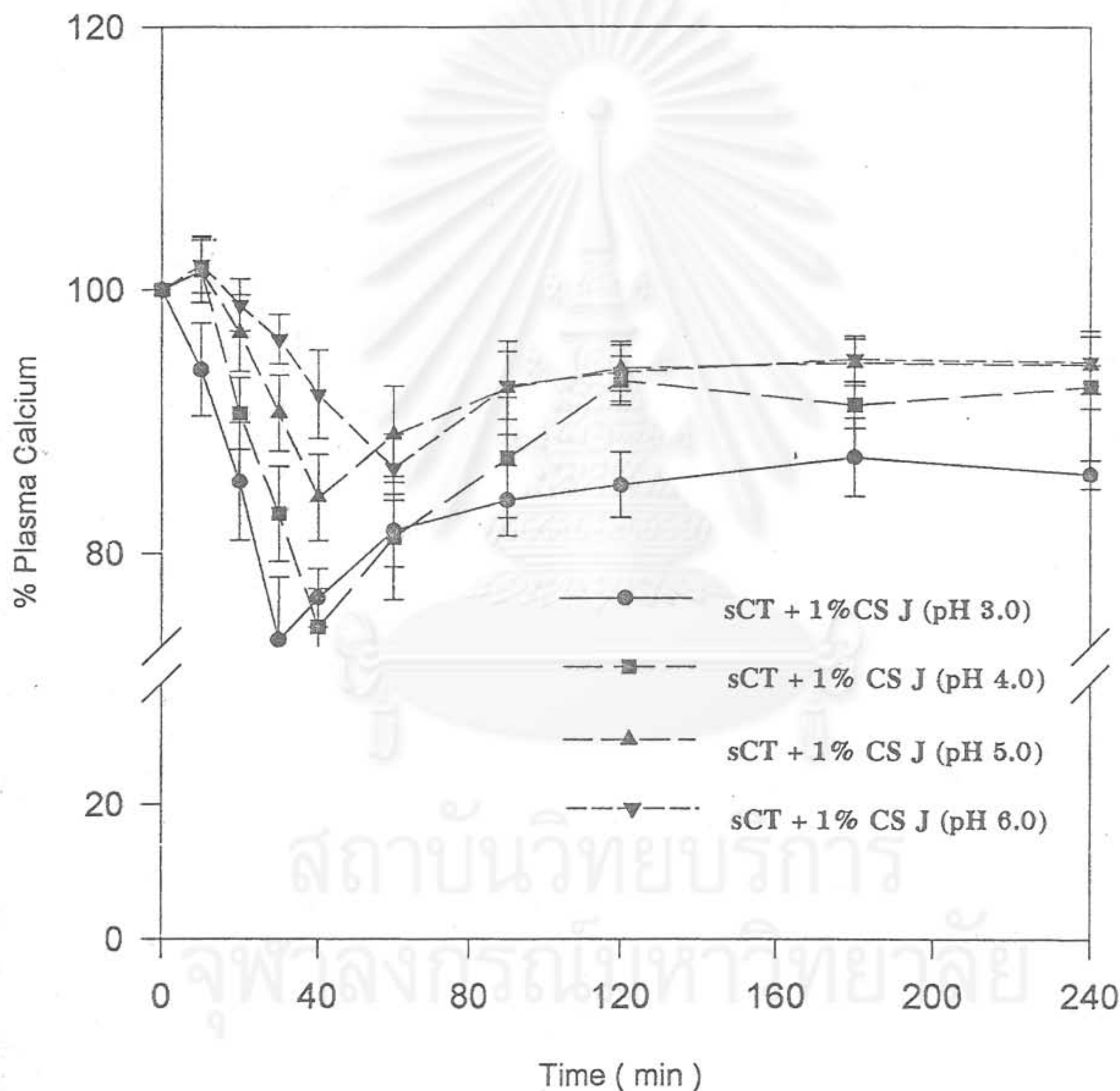
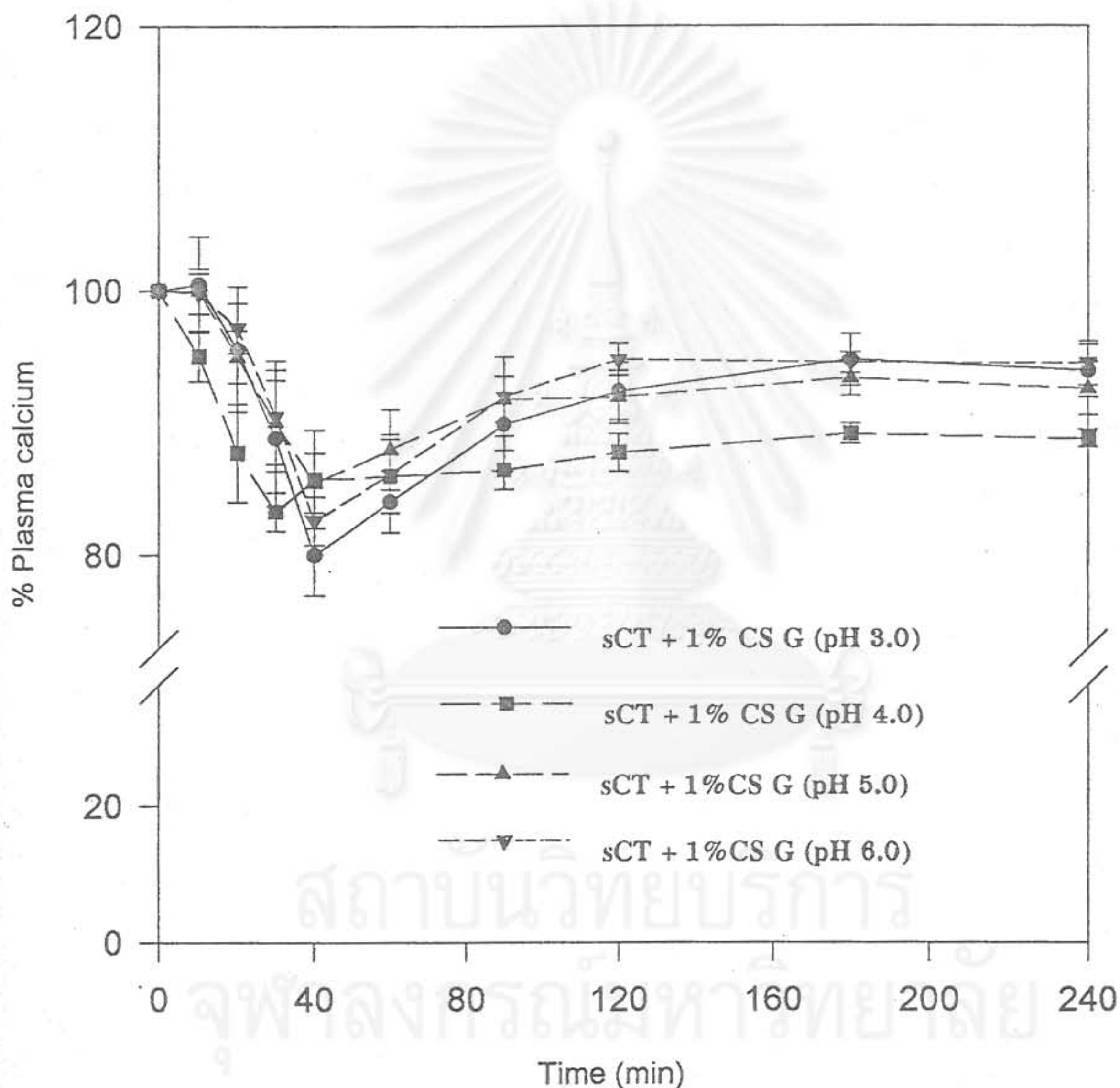


Figure 13. Percent plasma calcium after nasal administration of sCT with 1 % w/v CSG at various pH's (3.0-6.0). Each point represents mean ± SD (n = 5 rats/group).



underline ($p > 0.05$). Although our previous study (Tengamnuay et al., 1998) found that the effect of CSG in enhancing the nasal absorption L-Tyr-D-Arg was independent of pH in the range of 4.0 to 6.0 ($p > 0.05$), the extent of dipeptide absorption was also highest at pH 6.0 and tended to decrease as the pH was lowered, thereby yielding a similar ranking result.

It is interesting to note that the ranking order of CSG with respect to the pH effect was opposite to CSJ. The reason for the discrepancy could be partly explained by the difference in the chemical form of the two chitosans. CSJ exists in a free amine form, which normally requires an acidic condition for ionization, hydration and dissolution to occur in order to be able to interact with the nasal mucosa. On the other hand, CSG is already in a soluble salt form. It may not need that much acidity to hydrate or dissolve. Lehr et al. (1992) found that the glutamate salt of chitosan was the most readily soluble of all chitosans studied. It could be dissolved in water whereas the other chitosans must be dissolved in acid to obtain a solution. It thus appears that CSG may be able to retain its absorption enhancing activity at a pH which is less than acidic. It is possible that CSG is still able to assume the highly ionized, elongated shape, which helps maintain their adjuvant activities at higher pH values. In fact, its activity was even better at pH 6.0 than at pH 3.0, 4.0, or 5.0 (Table 7). The true explanations as to the reverse order observed with CSG are not clearly known at this point. The two chitosans were used as received in this study. A great deal of information was not available regarding their specifications and physicochemical properties such as percent deacetylation, molecular weight, and viscosity, etc.

In addition, the solubility of CSG used in this study was different from the previously reported results. This lot of CSG was more difficult to swell or dissolve in water than the one used by Lehr et al. (1992) despite coming from the same manufacturer. It is possible that lot-to-lot variation in the production of CSG may

have contributed to the observed discrepancies. Apparently, more studies with the new lot of CSG are needed to clarify this observation. Figures 9 and 10 show comparison of percent total decrease in plasma calcium after nasal administration of sCT, with or without 1 % w/v chitosans. The result shows the different tendency of each chitosan to enhance the nasal absorption of sCT. CSJ is effective in acidic pH (about 3.0-4.0) whereas CSG is effective in a more alkaline pH (about 6.0).

Based on the currently available data, the pH values which apparently give the most enhancing activities of CSJ and CSG are pH 4.0 and 6.0, respectively. For this reason, CSJ at pH 4.0 and CSG at pH 6.0 were selected for the next studies.

1.2 Determination of Optimal Enhancing Concentration for Chitosan

The next part of experiments was to evaluate the effect of varying chitosan concentration on its absorption enhancing activity. Since CSJ was shown to be most effective at pH 4.0, this pH was selected as the optimal pH for subsequent studies with CSJ. On the other hand, CSG gave the highest absorption enhancement at pH 6.0. This value was thus considered to be its optimal pH condition and was used in all subsequent experiments. The concentration of CSJ and CSG was varied from 0.25 % to 1.25 % w/v at their respective optimal pH so as to determine the concentration that produced optimal enhancing results. The data were then compared to two cyclodextrin type enhancers, i.e., HPβCD and DMβCD at 5 % w/v, which were reported to have minimal and intermediate deleterious effects on the rat nasal mucosa, respectively (Shao et al., 1992).

The potential use of natural cyclodextrins and their synthetic derivatives has been extensively studied to improve certain properties of the drugs, such as solubility, stability, and/or bioavailability. The enhancement of drug activity and selective transfer or the reduction of side effects can be achieved by inclusion complex

formation. Complexation with cyclodextrins enables the development of drug formulations that are difficult to formulate using the conventional pharmaceutical excipients (Irie et al., 1992). Recently, cyclodextrins have been studied by several researchers as potential novel nasal absorption enhancers of poorly absorbed drugs like peptides and proteins (Shao et al., 1992; Merkus et al., 1991). Of all the cyclodextrins studied, DMβCD was found to be the most active by dramatically enhance the permeability of large molecular weight peptides and proteins whereas HPβCD had a rather small or no enhancing effect on the permeability of these compounds (Merkus et al., 1993). However, DMβCD at 5 % also caused extensive leakage of 5'-nucleotidase, a membrane-bound enzyme, as well as the time-dependent linear release of the intracellular enzyme lactate dehydrogenase into the nasal perfusates (Shao et al., 1992). On the other hand, the less potent HPβCD at 5 % caused only minimal release of these enzymes. Our previous results also agree with them in that 5 % HPβCD induced only a minimal release of total protein and phosphorus from the rat nasal mucosa, with the effect equivalent to that caused by the phosphate buffer, pH 7.4 (Tengamnuay et al., 1998). For this reason, DMβCD was chosen as a representative of the more potent cyclodextrin derivative whereas HPβCD represented the enhancer with a more safe and weaker adjuvant activity.

The experiments were set similarly to the previous section (1.1). However, the concentration of both CSJ and CSG were varied from 0.25 to 1.25 % w/v. The sCT solution was administered at the same dose of 10 IU/kg. The experiments of CSJ were conducted at pH 4.0 and those of CSG at pH 6.0, which are their respective optimal pH conditions. Tables 8 and 9 show the changes in percent plasma calcium level with time following nasal administration of sCT with varying concentration of CSJ and CSG, respectively. The data are graphically represented in Figures 14 and 15 and comparison of the extent of hypocalcemic effect (%D) is given in Table 10 for both chitosans. The individual plasma calcium data are provided in Appendix III.

Table 8. Plasma calcium level (percent of initial value) in rats following nasal administration of sCT (10 IU/kg) with various concentrations of CSJ (pH 4.0)

CS J (pH 4.0)	Time (min)										$AUC_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
0.25 % w/v	100.00 ± 0.00	100.82 ± 1.43	98.45 ± 1.58	95.43 ± 0.96	86.01 ± 1.98	81.49 ± 1.73	88.56 ± 2.50	92.59 ± 2.75	93.72 ± 1.39	91.79 ± 1.61	21,974.50 ± 239.05
0.50 % w/v	100.00 ± 0.00	101.38 ± 2.32	98.52 ± 2.33	89.97 ± 4.59	81.03 ± 3.29	83.53 ± 1.93	85.09 ± 2.17	93.03 ± 1.78	92.89 ± 1.80	92.76 ± 1.60	21,818.56 ± 175.25
0.75 % w/v	100.00 ± 0.00	101.84 + 3.00	98.42 ± 2.78	93.82 ± 4.98	80.50 ± 4.22	80.97 ± 5.14	84.64 ± 1.72	89.86 ± 2.06	93.96 ± 1.74	93.44 ± 1.43	21,731.73 ± 180.50
1.00 % w/v	100.00 ± 0.00	101.49 ± 2.21	90.70 ± 2.42	83.03 ± 3.26	74.45 ± 2.59	81.19 ± 4.21	87.30 ± 4.12	93.14 ± 1.64	91.31 ± 1.58	92.71 ± 1.47	21,469.01 ± 282.93
1.25 % w/v	100.00 ± 0.00	99.33 ± 2.46	96.94 ± 2.24	89.91 ± 3.51	75.64 ± 0.89	83.08 ± 1.58	82.94 ± 2.80	89.29 ± 5.30	91.04 ± 1.39	91.94 ± 2.06	21,400.55 ± 401.90

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Table 9. Plasma calcium level (percent of initial value) in rats following nasal administration of sCT (10 IU/kg) with various concentrations of CSG (pH 6.0)

CS G (pH 6.0)	Time (min)										$AUC_{(0\text{-}240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
0.25 % w/v	100.00 ± 0.00	100.20 ± 2.43	98.70 ± 2.56	95.82 ± 2.82	89.59 ± 2.91	86.51 ± 2.08	91.14 ± 2.10	94.64 ± 2.85	95.27 ± 0.41	95.46 ± 0.96	22,528.87 ± 123.12
0.50 % w/v	100.00 ± 0.00	100.70 ± 2.52	97.45 ± 1.79	91.98 ± 1.89	83.60 ± 1.75	88.67 ± 2.46	93.83 ± 1.30	94.93 ± 1.61	93.59 ± 1.61	93.81 ± 1.14	22,388.39 ± 140.24
0.75 % w/v	100.00 ± 0.00	100.38 + 2.25	97.44 ± 2.13	92.89 ± 3.01	82.35 ± 1.78	86.19 ± 3.20	90.91 ± 2.01	93.42 ± 1.23	94.50 ± 1.45	94.41 ± 2.22	22,255.45 ± 112.61
1.00 % w/v	100.00 ± 0.00	100.46 ± 3.22	95.58 ± 4.23	88.86 ± 4.65	79.98 ± 2.70	84.04 ± 2.11	89.92 ± 3.19	92.39 ± 1.93	94.76 ± 1.74	93.96 ± 1.76	22,008.99 ± 162.21
1.25 % w/v	100.00 ± 0.00	100.30 ± 2.33	96.48 ± 2.80	90.65 ± 3.59	81.31 ± 1.94	86.45 ± 2.76	91.14 ± 1.81	92.24 ± 1.57	94.03 ± 0.68	92.87 ± 0.39	22,063.39 ± 90.32

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Table 10. Comparison of plasma calcium pharmacokinetic parameters and the percent total decrease in plasma calcium (%D or hypocalcemic extent) following nasal administration of sCT (10 IU/kg) with various concentrations of CSJ and CSG at their respective optimal pH.

Route of Administration	Dose (IU/Kg)	Adjuvants	Conc.	C_{min} (% of initial value)	T_{min} (mins)	$AUC_{0\text{-}240min}$ [% . min]	%D
i.n.	10	sCTalone (pH 4.0)	-	89.00 ± 2.01	66.00 ± 12.00	22,477.78 ± 272.04	5.61 ± 1.14
		CS J (pH 4.0)	0.25%	81.49 ± 1.73	56.00 ± 8.00	21,974.5 ± 239.05	7.72 ± 1.00
			0.50%	82.55 ± 1.11	68.00 ± 19.39	21,818.56 ± 175.25	8.47 ± 0.68
			0.75%	76.73 ± 3.24	48.00 ± 9.80	21,731.73 ± 180.50	8.75 ± 0.76
			1.00%	73.47 ± 1.47	44.00 ± 8.00	21,469.01 ± 282.93	9.85 ± 1.19
			1.25%	75.64 ± 0.89	48.00 ± 9.80	21,400.55 ± 401.90	10.14 ± 1.69
		sCTalone (pH 6.0)	-	93.21 ± 1.51	126.00 ± 48.00	22,797.40 ± 213.15	5.16 ± 0.89
		CS G (pH 6.0)	0.25%	84.84 ± 1.91	62.00 ± 16.00	22,528.87 ± 123.12	6.28 ± 0.52
			0.50%	83.14 ± 1.13	44.00 ± 8.00	22,388.39 ± 140.24	6.86 ± 0.58
			0.75%	81.89 ± 1.33	44.00 ± 8.00	22,255.45 ± 112.61	7.41 ± 0.47
			1.00%	79.38 ± 1.82	44.00 ± 8.00	22,008.99 ± 162.21	8.44 ± 0.68
			1.25%	80.74 ± 1.19	44.00 ± 8.00	22,063.39 ± 90.32	8.21 ± 0.38

Each Values = mean ± SD. (n = 5 rats/group)

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Figure 14. Percent of plasma calcium versus time following nasal administration of sCT (10 IU/kg) with various concentrations of CSJ (pH 4.0) to rats. Each point represents mean ± s.d. (n = 5 rats/group).

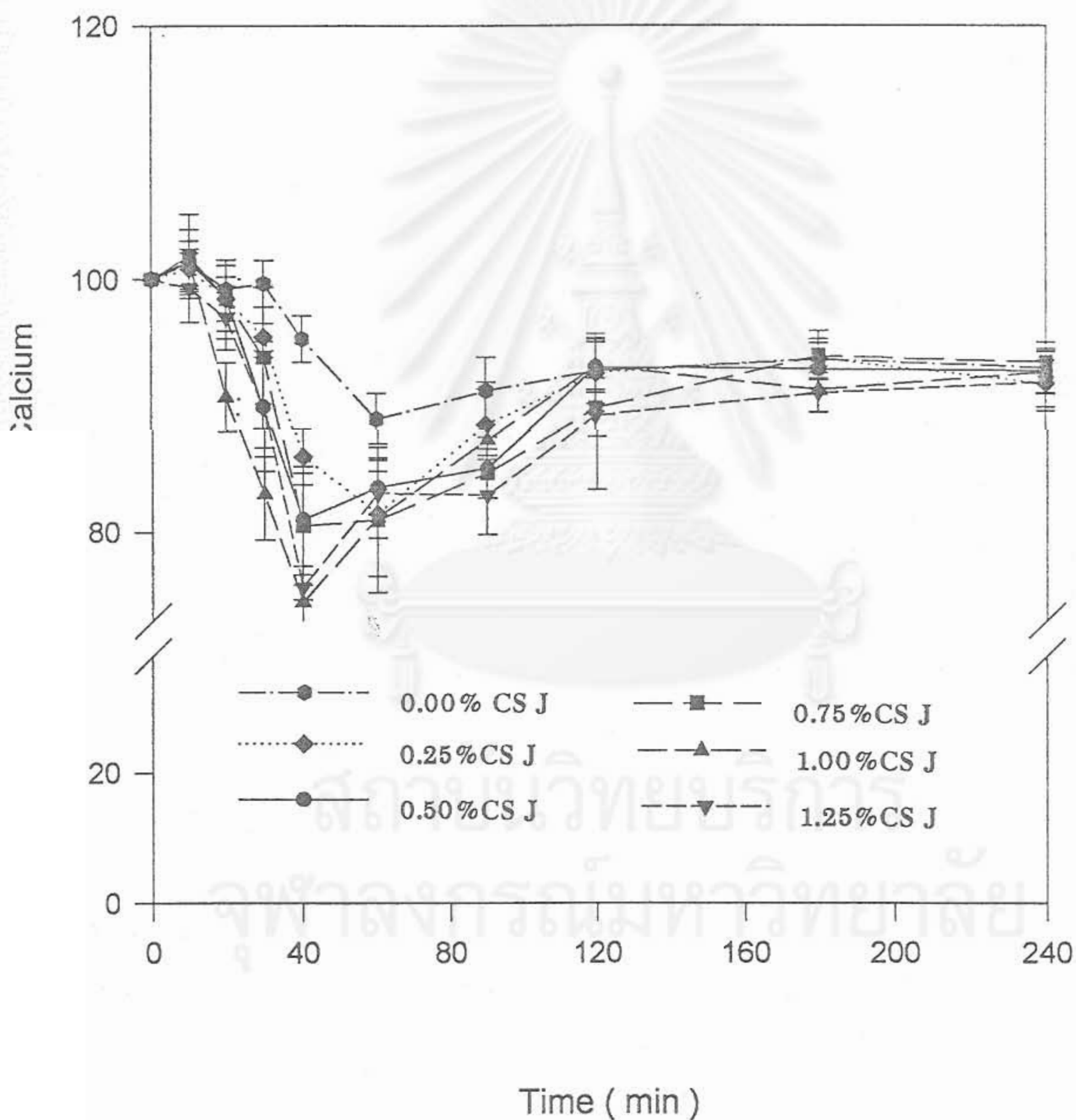
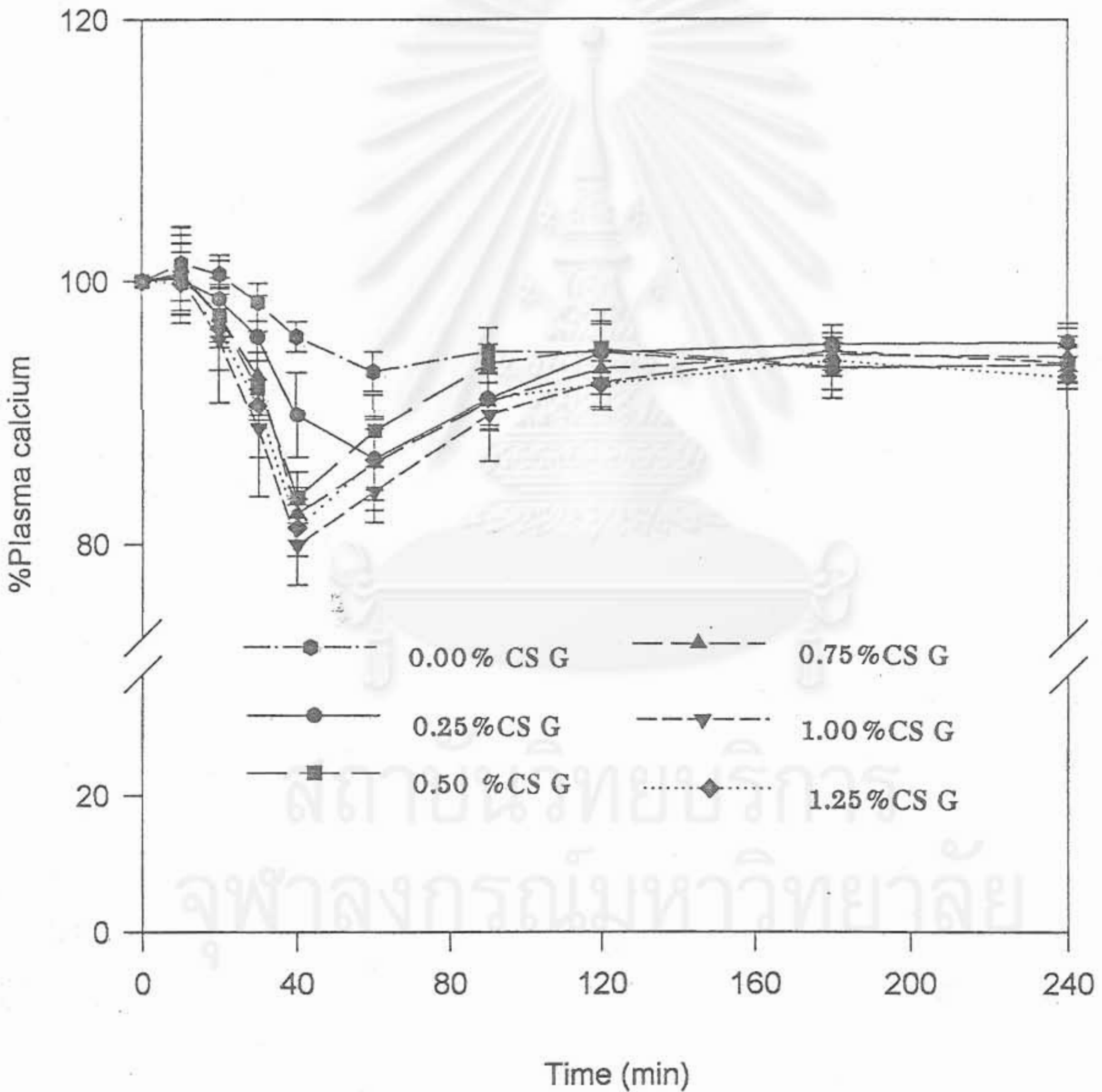


Figure 15. Percent of plasma calcium versus time following nasal administration of sCT (10 IU/kg) with various concentrations of CSG (pH 6.0) to rats. Each point represents mean ± s.d. (n = 5 rats/group).



From Figures 14 and 15, it can be seen that the drop in plasma calcium tends to be more pronounced as the concentration of both chitosans is increased from 0 to 1.25 % w/v. For CSJ, the average %D at CSJ concentrations of 0.0, 0.25, 0.5, 0.75, 1.0 and 1.25 % w/v are 5.61 ± 1.14, 7.72 ± 1.00, 8.47 ± 0.68, 8.75 ± 0.76, 9.85 ± 1.19 and 10.14 ± 1.69 %, respectively (Table 10). When ANOVA was applied to these six groups at $\alpha = 5$ %, significant difference was found ($p < 0.05$). Further comparison using Duncan's test revealed that all concentrations of CSJ under study were able to enhance sCT nasal absorption over the control group (no CSJ) at the same significant level ($p < 0.05$). Moreover, the hypocalcemic effect of 1.0 and 1.25 % CSJ was found to be the most effective, with the average %D significantly greater than 0.25 and 0.5 % ($p < 0.05$). On the other hand, the effect of 0.75 % CSJ was intermediate between 0.5 % and 1.0 % concentration. Since the values of %D for 1.0 and 1.25 % CSJ did not differ statistically (9.85 vs 10.14 %, $p > 0.05$ using Duncan's test), the enhancing effect of CSJ appears to saturate at the concentration above 1.0 %.

Similar saturable increase in the hypocalcemic response were also observed with CSG, with the average %D at CSG concentrations of 0.0, 0.25, 0.5, 0.75, 1.0 and 1.25 % w/v calculated to be 5.16 ± 0.89, 6.28 ± 0.52, 6.86 ± 0.58, 7.41 ± 0.47, 8.44 ± 0.68, 8.21 ± 0.38 %, respectively (Table 10). ANOVA and subsequent Duncan's test on %D also revealed that all the enhancer concentrations were capable of further increasing the nasal absorption of sCT over the control group (no CSG) at 5 % significance level ($p < 0.05$). The enhancing effect of 1.0 and 1.25 % CSG was also more pronounced than 0.25 and 0.5 % ($p < 0.05$), whereas the effect of 0.75 % was in between. It is also interesting to note that the effect of 1.0 % CSG (%D = 8.44 %) was even slightly greater than 1.25 % concentration (%D = 8.21 %). However, this was not statistically significant ($p > 0.05$). This observation suggests that the absorption promoting effect of CSG may be saturable at concentration higher than 1.0 % w/v similar to that previously observed for CSJ.

Thus, the absorption enhancing effect of both chitosans appear to depend on their concentration, with the increasing enhancer concentration resulting in increased absorption of sCT, as seen from the increase in %D values. However, the effect appears to be saturable at concentration higher than 1.0 % w/v for both types of chitosans. This is in agreement with the results of Artursson et al. (1994), who reported that CSG can promote the *in vitro* transport of hydrophilic molecules across the CaCO-2 cell monolayers in a concentration-dependent and saturable manner, as opposed to other types of absorption enhancers like bile sats.

Since 1.25 % concentration did not produce any superior absorption enhancement to that produced by 1.0 %, the latter concentration was considered to be the optimal enhancing concentration for both CSJ and CSG. Their absorption enhancing activity at this concentration was subsequently compared with that of 5 % HPβCD and 5 % DMβCD, the novel cyclodextrin-type enhancers used as references. Since the two compounds are neutral molecules and their absorption enhancing mechanisms do not appear to depend on pH (Shao et al., 1992), the studies with the two cyclodextrins were thus conducted at physiological pH 7.4, which was considered to be their optimal pH condition. Furthermore, previous studies with the control groups (nasal administration of only sCT in buffers with pH ranging from 3.0 to 7.4) have shown that the absorption of sCT, as judged from the values of % D, was similar regardless of the pH studied (see part 1.1). Therefore, comparison of the effect of these enhancers on % D can be made directly under their respective optimal pH conditions.

Figure 16 is a plot showing changes in the percent plasma calcium with time following nasal administration of sCT (10 IU/kg) in the presence of 5 % DMβCD and 5 % HPβCD. The calcium levels of the control and the baseline groups are also shown for comparison. The average plasma calcium data are given in Tables 2, 3 and 11, whereas the individual plasma calcium values for the two cyclodextrins are

Table 11. Changes in plasma calcium level (percent of initial value) following nasal administration of sCT (10 IU/kg) in rats with 5 % DMβCD and 5 % HPβCD as absorption enhancers.

CD (pH 7.4)	Time (min)										$AUC_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
DM-*B*-CD	100.00 ± 0.00	101.30 ± 2.65	97.32 ± 2.43	89.02 ± 4.13	77.85 ± 2.06	83.46 ± 2.91	89.11 ± 3.38	91.88 ± 2.30	92.99 ± 1.73	92.83 ± 1.66	21,810.41 ± 74.37
HP-*B*-CD	100.00 ± 0.00	101.31 ± 1.86	97.40 ± 2.24	92.91 ± 2.72	83.21 ± 1.68	86.51 ± 1.68	90.89 ± 1.45	92.04 ± 1.11	94.99 ± 1.23	93.62 ± 0.72	22,203.70 ± 110.93

The data show mean ± SD (n = 5 rats/group).

IPB = Isotonic Phosphate Buffer

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Figure 16. Percent of plasma calcium versus time following nasal administration of sCT with 5 % DMβCD and 5 % HPβCD as absorption enhancers in comparison with the control and baseline groups. Each point represents mean ± s.d. (n = 5 rats/group).

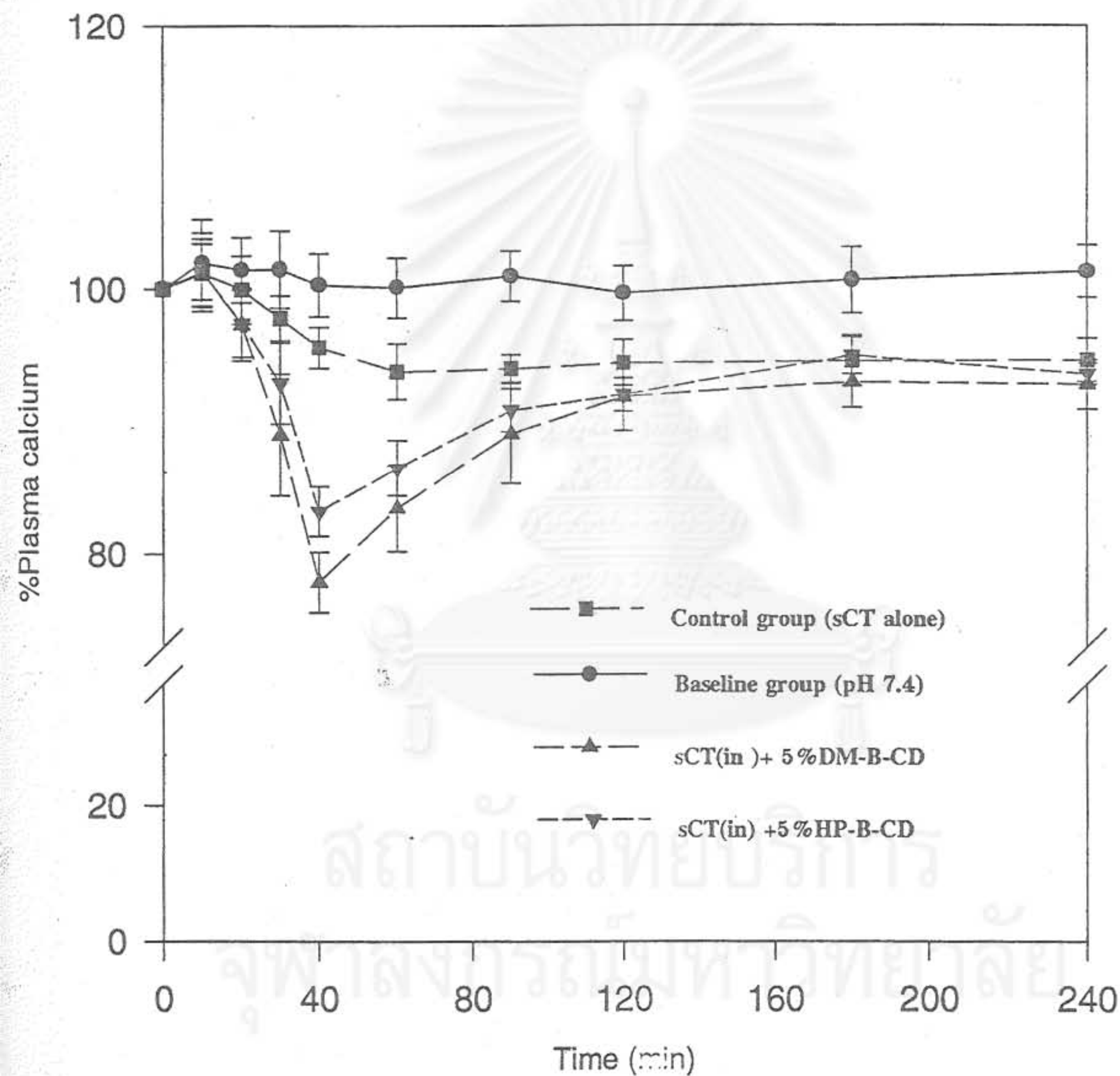
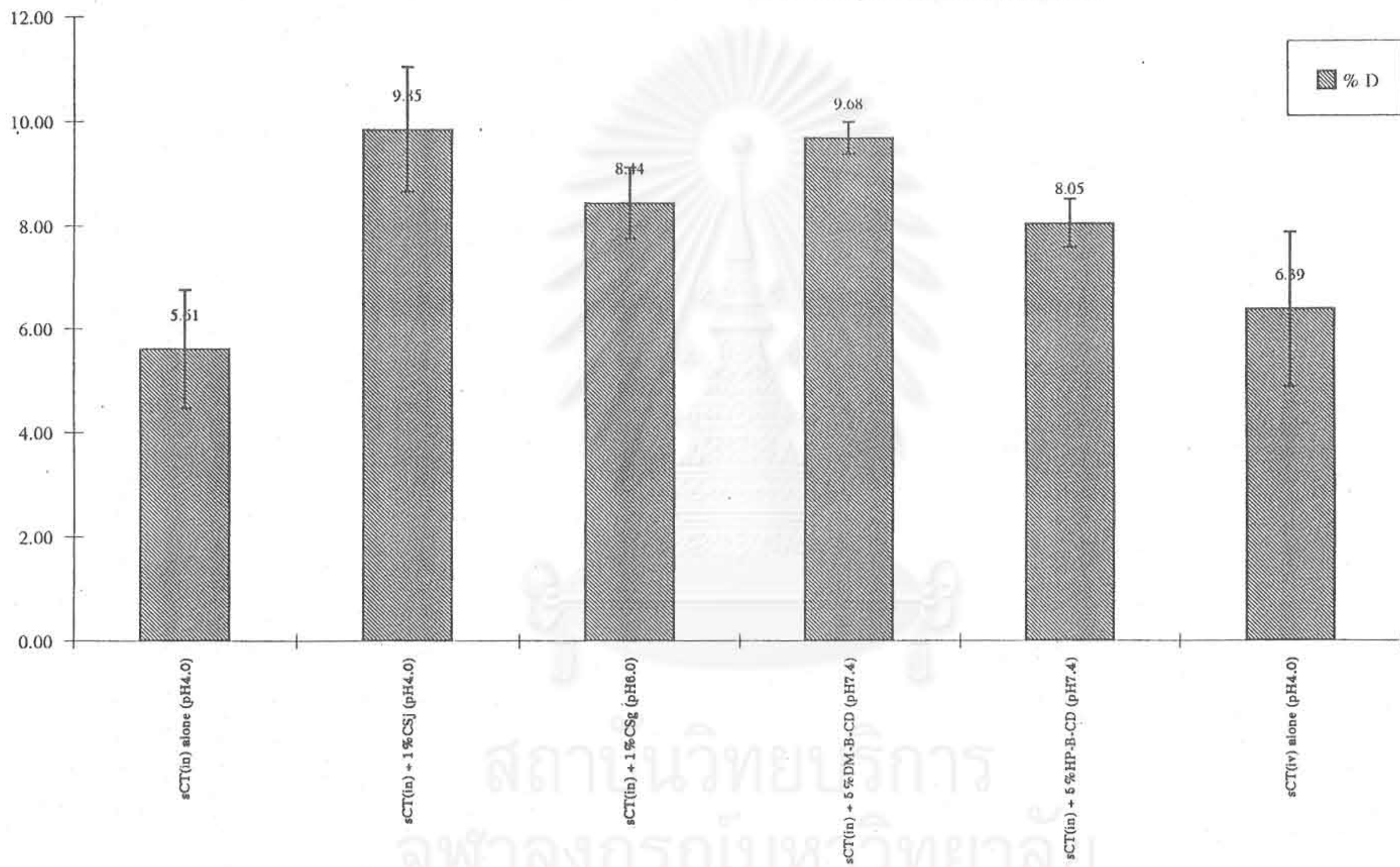


Figure 17. Histogram comparing the average total percent decrease in plasma calcium (% D) following nasal administration of sCT with different enhancers under their corresponding optimal pH and concentration.



provided in Appendix IV. It can be seen from this figure that nasal administration of the pure phosphate buffer pH 7.4 or only sCT in this buffer did not result in any marked decrease in plasma calcium. On the other hand, inclusion of either DMβCD or HPβCD at 5 % w/v caused a sharp drop in the calcium levels. Furthermore, the hypocalcemic effect of DMβCD appears to be more pronounced than HPβCD, with the average % D of 9.68 ± 0.31 and 8.05 ± 0.46 %, respectively.

ANOVA was then applied on the values of % D to compare the effect of 1 % CSJ (pH 4.0) and 1 % CSG (pH 6.0) with that of the two cyclodextrins (5 %, pH 7.4). Significant difference was found among the four groups ($p < 0.05$) and Duncan's test was further applied to rank their absorption enhancing activity. The results, in an increasing order, are as follows:

	HPβCD pH 7.4 $<$ CSG pH 6.0		$<$ DMβCD pH 7.4 $<$ CSJ pH 4.0	
% D	8.05	8.44	9.68	9.85 %

The lines signify that there was no significant difference among the two groups on the same line ($p > 0.05$). Therefore, Duncan's test results indicated that the four enhancers could be divided into two groups of different enhancing activities. The first group, which consists of 1.0 % CSJ and 5.0 % DMβCD, is more effective than the second group, which consists of 1.0 % CSG and 5.0 % HPβCD. Therefore, according to the available data on plasma calcium, the absorption enhancing effect of CSJ, under its optimal pH and concentration (pH 4.0, 1.0 % w/v), appears to be highest and equivalent to that of 5 % DMβCD (pH 7.4). On the other hand, the adjuvant activity of its glutamate salt (CSG pH 6.0, 1.0 % w/v) is lower and similar to that of 5 % HPβCD. Comparison of the average % D values among the four groups is also graphically represented in Figure 17. Since our previous studies have shown that both CSJ and CSG was less membrane-irritating than DMβCD based on the nasal

protein and LDH release studies (Tengamnuay et al., 1998), CSJ thus appears to have a promising potential as a safe and effective nasal absorption enhancer of sCT.

1.3 Plasma sCT Determination

To prove that sCT is absorbed intranasally into the systemic circulation, measurements of the plasma sCT levels following nasal administration were made and compared to the intravenous administration. CSJ at 1.0 % (pH 4.0) and DMβCD at 5.0 % (pH 7.4) were selected for study in this part due to their similar absorption enhancing activity based on Duncan's test results from previous experiments. Plasma sCT was quantitated by a specific radioimmunoassay technique using a standard kit available from Peninsular Laboratory. Figure 18 is a representative standard curve for sCT determined by radioimmunoassay. It is a semilogarithmic plot between percent bound radiolabeled sCT (%B/Bo) on a linear scale versus standard sCT concentration (pg/ml) on a logarithmic scale. The curve is sigmoidal indicating the saturable nature of the binding process. The standard curve was prepared for every experiment and all the curves obtained were very similar suggesting a low variation between assays.

Figure 19 shows the plasma sCT concentration-time profiles following nasal administration of sCT (10 IU/kg) with and without adjuvants, in comparison with the profile obtained from 0.15 IU/kg intravenous administration. The values (means $\pm$ s.d.) are also tabulated in Table 12 whereas the individual plasma sCT data are given in Appendix V, together with the AUC, C_{max} and T_{max} values. From data in Table 12, it is apparent that elimination of plasma sCT following intravenous injection was much more prolonged than that of the intranasal administration, with plasma sCT concentration being practically unchanged during 60 to 180 min period. This result indicated that elimination profiles of sCT may be different among various routes of administration. The nasal administration of sCT (10 IU/kg) dissolved in 0.15 M

isotonic phosphate buffer pH 4.0 without enhancers resulted in very low plasma sCT concentrations. The obtained pharmacokinetic and bioavailability parameters are listed in Table 13. The maximum plasma sCT level (C_{max}) remained very low about 37.53 ± 4.50 pg/ml at 40 min. The absolute bioavailability ($\%F_{abs}$), calculated based on the ratio of the dose-corrected AUC value relative to that of 0.15 IU/kg intravenous sCT was only 1.22 %. This observation is in accordance with the results of Lee et al. (1994) who found that sCT administered intranasally without enhancers at pH 4.0 was only slightly absorbed, with the absolute nasal bioavailability of only 1.16 %. The low extent of sCT nasal absorption reported here is also in agreement with the small hypocalcemic effect previously observed (%D = 5.61 ± 1.14 %). Therefore, the inclusion of suitable absorption enhancers appears necessary in the nasal formulations of sCT.

When sCT was intranasally administered at the same dose in the presence of 1% CSJ (pH 4.0), the absorption was considerably enhanced. As seen from Figure 19 and Table 12, sCT was rapidly absorbed into the systemic circulation, reaching a maximum plasma level of 90.59 ± 4.71 pg/ml at 30 min. This value is about 2.4 fold over the control group (intranasal without the enhancer). Intranasal administration of sCT with 5% DMβCD also resulted in the elevated peak plasma sCT concentration of 75.16 ± 4.47 pg/ml at 30 min. After 40 min post-nasal administration with either of the two enhancers, the plasma sCT levels gradually declined in parallel and remained relatively high even at 180 min (about 30 pg/ml). ANOVA and subsequent Duncan's test on the C_{max} values among the three nasal administration modes further confirmed that the two enhancers were effective in increasing the C_{max} over the control group (sCT alone without enhancers) ($p < 0.05$). In addition, Duncan's test result also showed that the average peak plasma level of sCT was significantly greater with 1 % CSJ (90.59 pg/ml) than with 5 % DMβCD (75.16 pg/ml) ($p < 0.05$).

Figure 18. Representative standard binding curve for the determination of sCT in plasma

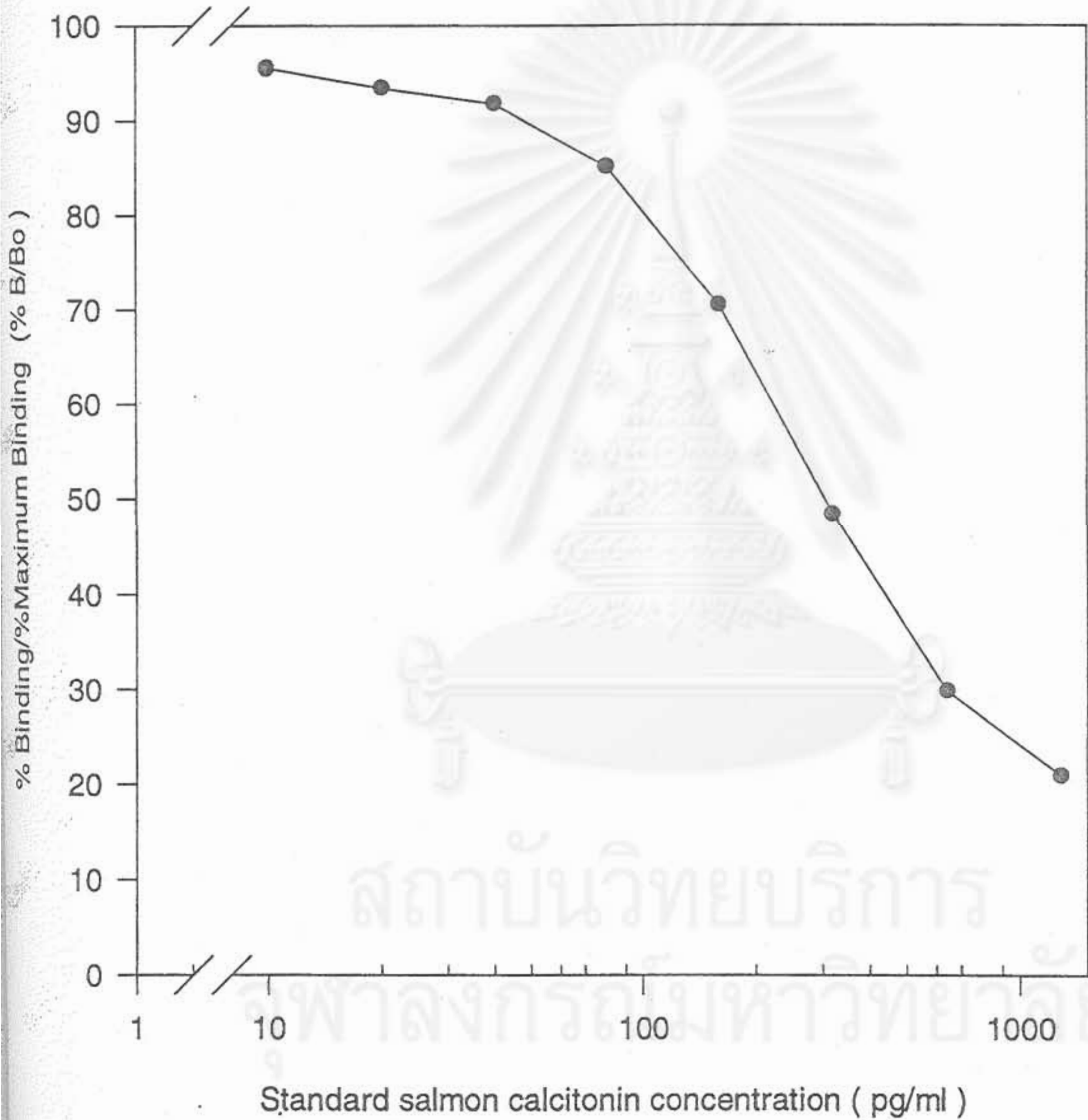


Table 12. Plasma sCT concentrations in rats following intravenous and intranasal administration of sCT with or without enhancers

Condition	Time (min)									AUC$_{(0-180)min}$
	0	5	10	15	30	40	60	120	180	[pg/ml].min
sCT(iv) alone : pH 4.0	76.47 ± 7.85	53.97 ± 5.67	42.16 ± 5.15	30.72 ± 4.28	23.36 ± 2.84	23.36 ± 2.84	22.75 ± 2.40	21.30 ± 2.75	20.96 ± 3.33	4,488.33 ± 180.45
sCT(in) alone : pH 4.0	0.00 ± 0.00	0.00 ± 0.00	15.24 ± 2.74	19.25 ± 3.91	26.65 ± 6.06	37.53 ± 4.50	22.88 ± 3.12	17.02 ± 2.85	18.08 ± 2.01	3,643.88 ± 423.05
sCT(in) + 1% CS J : pH 4.0	0.00 ± 0.00	20.23 ± 3.31	28.63 ± 6.86	45.79 ± 5.95	90.59 ± 4.71	80.39 ± 6.07	48.54 ± 8.55	27.75 ± 5.75	22.79 ± 5.75	7,330.85 ± 1,054.52
sCT(in) + 5% DM-B-CD : pH 7.4	0.00 ± 0.00	21.95 ± 6.72	29.24 ± 7.08	34.68 ± 7.43	75.16 ± 4.47	53.39 ± 5.09	36.71 ± 6.28	22.57 ± 5.93	17.89 ± 1.97	5,702.11 ± 890.49

The data show mean ± SD (n = 3 rats/group).

IPB = Isotonic Phosphate Buffer

i.v. = intravenous

i.n. = intranasal

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Figure 19. Plasma sCT concentration-time profiles following intranasal administration of sCT (10 IU/kg) with or without enhancers in comparison with the intravenous injection (0.15 IU/kg)

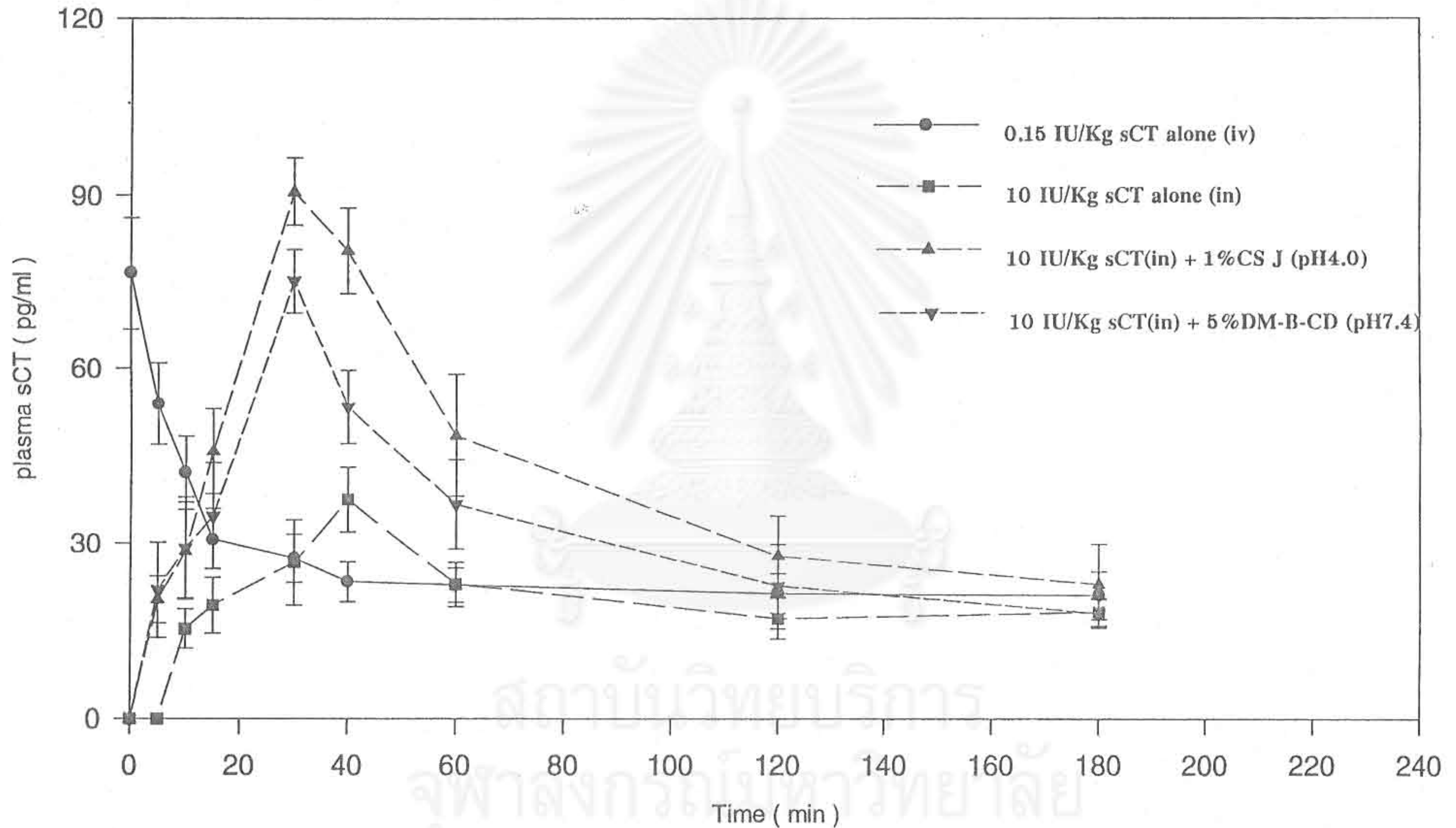

Table 13. Pharmacokinetic and bioavailability parameters of sCT following intranasal (with and without enhancers) and intravenous administration in rats

Route of Administration	Dose (IU/Kg)	Adjuvants	C_{max} (pg/ml)	$AUC_{0\text{-}180min}$ [pg/ml].min	$\%F_{abs}$ absolute bioavailability	$\%F_{rel}$ relative bioavailability
i.v	0.15	none	76.47 ± 7.85	4,488.33 ± 180.45	100	-
i.n.	10	none	37.53 ± 4.50	3,643.88 ± 423.05	1.22	100.00
i.n	10	1% CS J pH 4.0	90.59 ± 4.71	7,330.85 ± 1,054.52	2.45	201.18
i.n.	10	5% DM-*B*-CD pH 7.4	75.16 ± 4.47	5,702.11 ± 890.49	1.91	156.48

Each Values = mean ± SD. (n = 3 rats /group)

$AUC_{0\text{-}180min}$ = area under the plasma calcium concentration vs time curve from 0 to 180 minutes

$\%F_{abs}$ = Absolute bioavailability of plasma sCT during 0 - 180 minutes

$$= \frac{AUC_{in}}{AUC_{in}} \times \frac{Dose_{iv}}{Dose_{in}} \times 100\ \%$$

$\%F_{rel}$ = Relative bioavailability of plasma sCT during 0 - 180 minutes

$$= \frac{AUC_{in(with\ enhancer)}}{AUC_{in(no\ enhancer)}} \times \frac{Dose_{in(no\ enhancer)}}{Dose_{in(with\ enhancer)}} \times 100\ \%$$

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The area under the plasma sCT curve during the 180 min period (AUC_{0-180}) were also compared among the three nasal administration groups using ANOVA and Duncan's test at the same significance level. The average values are 7,330.8 ± 1,054.5, 5,702.1 ± 890.5, and 3,643.9 ± 423.1 [pg/ml].min for CSJ, DMβCD and the control group, respectively. In agreement with the C_{max} results, both 1 % CSJ and 5 % DMβCD were able to increase the AUC_{0-180} over the control group ($p < 0.05$). However, the Duncan's test result did not detect any significant difference in the AUC_{0-180} values between 1 % CSJ and 5 % DMβCD ($p > 0.05$), indicating the same extent of sCT nasal absorption. The similarity in the AUC values may, in turn, be responsible for the same hypocalcemic effect (% D) previously observed for the two enhancers (see part 1.2).

Despite the similarity in % D and AUC_{0-180}, the significantly higher C_{max} value for CSJ suggests the possibility that CSJ, under its optimal pH condition employed here (1 %, pH 4.0), may exhibit a slightly greater enhancing activity on sCT absorption than 5 % DMβCD (pH 7.4). This observation was also supported by the plasma calcium data which show that the maximum drop in plasma calcium, as judged from the value of C_{min}, was more pronounced for CSJ than for DMβCD, CSG and HPβCD ($p < 0.05$, Duncan's test on the four enhancer-treated groups). The ranking of C_{min} is as follows:

	1 % CSJ <	5 % DMβCD <	1 % CSG <	5 % HPβCD
C_{min} (%)	73.47	77.51	79.38	82.83 %

(5 % DMβCD < 1 % CSG underlined)

The line joining DMβCD and CSG indicates that the values of C_{min} are not significantly different between the two enhancers ($p > 0.05$). However, 1 % CSJ induced a significantly greater drop in C_{min} than 5 % DMβCD and other enhancers.

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The slightly better enhancing activity of CSJ over DMβCD could be partly explained by the difference in pH conditions between the two enhancers (pH 4.0 for CSJ and pH 7.4 for DMβCD). Figure 10 and data in Appendix IIb indicate that the more acidic pH (3.0 and 4.0) tends to facilitate the nasal absorption of sCT as demonstrated by the significantly lower C_{min} values, even in the absence of any enhancer ($p < 0.05$, Duncan's test on the control groups with different pH). The ranking after Duncan's test is as follows:

Control (sCT only)	pH 3.0 <	pH 4.0 <	pH 5.0 <	pH 6.0 <	pH 7.4
C_{min} (%)	84.04	88.16	90.26	91.67	92.76 %

It can be seen from the above ranking that % C_{min} values at pH 3.0 and 4.0 are significantly lower than at pH 7.4 ($p < 0.05$) since there is no line joining pH 3.0, 4.0 and 7.4 together. On the other hand, the values at the less acidic pH 5.0 and 6.0 are not significantly different from pH 4.0 and 7.4 ($p > 0.05$). Thus, there appears to be a slight influence from the medium acidity, which may partly contribute to the somewhat greater absorption enhancing effect observed with CSJ at pH 4.0 than with DMβCD at pH 7.4.

As previously discussed, the more acidic pH of the nasal solution such as pH 4.0 may have a direct effect on the nasal membrane permeability, or may have helped stabilize sCT in the formulation, as well as provided some protection against enzymatic degradation in the nasal cavity, thereby leading to shorter T_{min} and lower C_{min} when compared to pH 7.4 (Figure 10 and Appendix IIb). Nevertheless, whether such mechanisms did exist, the effect was very small and it needs more studies to confirm the above explanations. Studies with HPβCD and DMβCD were conducted at physiological pH of 7.4 because their enhancing effect was reported to be optimal at this pH (Hsieh, 1994). Previous perfusion studies by Tengamnuay et al. (1998) of

the effect of cyclodextrins on the nasal absorption of L-Tyr-D-Arg were also carried out at pH 7.4. Therefore, the results obtained in this study with the two cyclodextrins could be directly compared with our previously report that the promoting effects of 1% CSJ (pH 4.0) and 1% CSG (pH 6.0) were significantly better than 5% HPβCD at pH 7.4 ($p < 0.05$). In addition, perfusion of the rat nasal mucosa with only the buffers, either at pH 4.0 or 7.4, resulted in similar minimal release of mucosal protein and phospholipid ($p > 0.05$, Tengamnuay et al., 1998). These data, in conjunction with the histological evaluation of the rat nasal mucosa, revealed that the two buffers with different pH values produced no deleterious effect to the rat nasal mucosa and appeared to be suitable for use in the nasal formulations.

The absolute bioavailability (F_{abs}) of intranasal sCT was calculated for each group by comparing the AUC_{0-180} values to that after an intravenous injection. As shown in Table 13, the absolute nasal bioavailabilities in the presence of CSJ and DMβCD were found to be 2.45 % and 1.91 %, respectively. On the other hand, the absolute bioavailability for the nasal control group was only 1.22 %. Although the absolute nasal bioavailabilities of the enhancer-treated groups seem to be low when compared to the intravenous administration, the inclusion of 1 % CSJ resulted in a two-fold increase in the AUC_{0-180} value over the control group. This was equivalent to 201.18 % relative bioavailability (F_{rel}) when compared to the control group at the same nasal dose (10 IU/kg). Addition of 5% DMβCD also led to the relative nasal bioavailability of 156.48 % or a 1.56 fold increase in absorption over the control group. Therefore, inclusion of 5 % DMβCD or 1 % CSJ in the nasal formulation can increase the nasal absorption efficiency of extremely expensive sCT by at least 56 to 100 %.

All the results from this experiments indicate that chitosans could remarkably increase the absorption of polypeptides like sCT across the rat nasal mucosa. Both types of chitosans under study gave this enhancing effect when compared with their

corresponding control groups. The enhancing effect of chitosans occurred according to the proposed combinatorial mechanisms of mucoadhesion and their effect on the gating properties of the tight junctions (Illum et al., 1994). Chitosan is a cationic polysaccharide shown to have a mucoadhesive property, which is probably mediated by ionic interactions between the positively charged amino groups of the chitosan molecule and the negatively charged sialic acid residues of the mucus (Lehr et al., 1992). This effect decreases the rate of clearance of the drug from the nasal cavity and thereby allows a longer contact time with the absorptive epithelium. The other mechanism, i.e., the effect on the gating properties of the tight junctions, is described by the interaction of chitosan with the cytoskeletal filamentous actin (F-actin) that induces a simultaneous increase in paracellular permeability. F-actin is directly or indirectly associated with the proteins in the tight junctions. The parallel changes in F-actin distribution induce structural separation of the tight junctions (Anderberg et al., 1993). Therefore, chitosan can directly affect the permeability of the tight junctions, which results in an increase in the paracellular absorption of the drug across the epithelium.

Furthermore, Artursson et al. (1994) proposed that chitosan might be able to displace cations from electronegatives sites such as tight junctions on a membrane, which requires coordination with cations (such as calcium) for dimensional stability. Removal of these "pivot" ions could result in a loosening or opening of the tight junction. However, this hypothesis of absorption enhancement needs further proof of evidence.

In addition, other unknown mechanisms of absorption enhancement may exist for chitosans such as inhibition of the proteolytic enzymes in the nasal cavity. Thus, more studies need to be carried out regarding the possible mechanisms of absorption enhancement caused by chitosans, particularly their possible inhibitory effect on the nasal proteolytic enzyme activities such as trypsin and leucine aminopeptidase.

Part II. Possible Inhibitory Effects of Chitosans on Nasal Proteolytic Enzymes Activities

2.1 Degradation Studies with Trypsin (EC 3.4.21.4)

Preliminary results have established the optimal conditions for substrate hydrolysis such as the pH of preparation, substrate concentration and wavelength of maximum absorption.

The pH for the maximum trypsin activity on BAPA hydrolysis was originally determined to be 8.0. However, chitosans could not be dissolved at this pH. Therefore, pH 6.0 was chosen instead as the optimal pH at which the trypsin activity was sufficiently high and could be accurately quantitated. The optimal substrate (BAPA) concentration was determined to be 1 mM since the rate of hydrolysis was saturated at concentrations above this value. Measurement of p-nitroaniline, the product of BAPA hydrolysis, was made at its optimal wavelength of 410 nm.

Effect of Chitosans on Trypsin Activity

Determination of trypsin activity was carried out by measuring the extent and rate of metabolite formation (p-nitroaniline) during the incubation (0-240 min), as reflected by the values of AUC and the initial rate (k). AUC was defined as area under the metabolite curve versus time, whereas k was defined as the initial rate of product formation (mg $.ml^{-1}$ min^{-1} x 10^{-3}). The degradation study was carried out using trypsin (0.2 mg/ml) in the presence of substrate (1 mM BAPA) and the reaction mixtures were incubated at 37° c for 240 min during which the samples were periodically taken and analyzed as described in the analytical method (experiment 2.1 in Chapter III). A representative calibration curve of p-nitroaniline is shown in Figure 20. This curve was plotted between the p-nitroaniline concentration and the

absorbance at 410 nm. The inhibitory effect of chitosans on the tryptic activity was studied at various concentrations of the polymers. Figures 21 and 22 are the plots of p-nitroaniline concentration versus time showing the formation of p-nitroaniline following incubation of BAPA and trypsin with and without CSJ and CSG, respectively. The plot of aprotinin, a specific trypsin inhibitor, was also provided in each figure for comparison. The average values are tabulated in Tables 14 and 15. Analysis of the data from these tables indicated that both CSJ and CSG , at all concentrations studied, did not induce any significant reduction in the extent of p-nitroaniline formation in comparison with the control (no enhancer) ($p > 0.05$, ANOVA on AUC_{0-240} values with chitosan concentration varying from 0 to 1.25 %). The results revealed the non-linear p-nitroaniline concentration versus time profiles similar to the control experiments.

At the concentrations between 0.25 and 1.25 % of CSJ, the initial rate of hydrolysis as judged from the k values also did not significantly differ from the control group of 0.47 ± 0.03 mg/ml/min x 10^{-3} ($p > 0.05$, ANOVA on k with CSJ concentration varying from 0 to 1.25 %). However, ANOVA on the k values of CSG showed significant differences among various CSG concentrations ($p < 0.05$). Duncan's test was further applied and the result revealed that the CSG concentrations of 0.25, 0.5, 0.75 and 1.0 % gave the k values similar to that of the control (0.47 ± 0.03 mg/ml/min x 10^{-3}) ($p > 0.05$). On the other hand, the highest CSG concentration of 1.25 % yielded the average k of 0.34 ± 0.05 mg/ml/min x 10^{-3}, which was significantly smaller than the control and other concentration groups ($p < 0.05$, Table 15). Although not significant, the k values of 1.25 % CSJ was also observed to be smaller than at other concentrations, with the average value of only 0.35 ± 0.04 mg/ml/min x 10^{-3} (Table 14).

The slight decrease in the hydrolysis rate observed with 1.25% CSJ and 1.25% CSG may be due to the formation of the gel matrix at this relatively high polymer

Figure 20. Representative calibration curve of p-nitroaniline in 0.15 M phosphate buffer (pH 6.0)

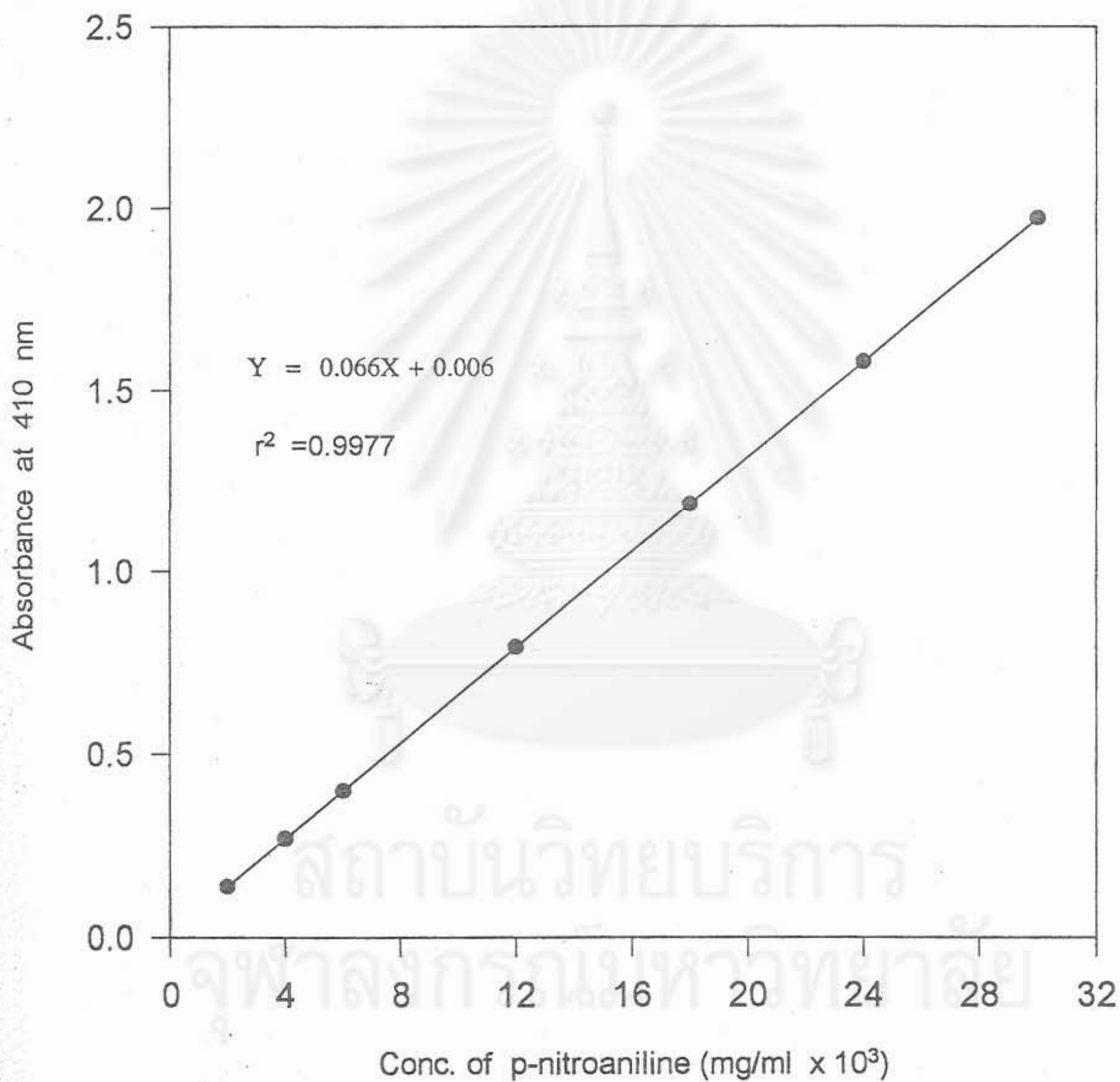

Table 14. Effects of various concentrations of CSJ on trypsin activity as determined from the formation p-nitroaniline

[p-nitroaniline] (mg/ml x 10^3)	Time (min)									$AUC_{0-240min}$ [mg/ml].min x 10^3	k mg/ml/min x 10^3
	0	10	20	30	40	60	90	120	240		
Control	0.44 ± 0.02	10.91 ± 0.97	15.05 ± 1.46	18.011 ± 1.5	20.239 ± 1.1	22.36 ± 2.08	23.96 ± 2.14	24.68 ± 1.65	24.83 ± 1.81	5,364.29 ± 178.05	0.47 ± 0.03
Aprotinin	0.43 ± 0.02	0.433 ± 0.03	0.43 ± 0.04	0.413 ± 0.02	0.43 ± 0.02	0.445 ± 0.01	0.43 ± 0.08	0.445 ± 0.03	0.43 ± 0.02	104.56 ± 5.60	0.00 ± 0.00
0.25% CSJ	0.44 ± 0.04	12.82 ± 1.59	15.74 ± 0.85	20.72 ± 1.00	22.01 ± 1.74	22.73 ± 1.82	24.91 ± 0.85	25.77 ± 1.24	26.69 ± 1.92	5,514.28 ± 260.73	0.51 ± 0.04
0.50% CSJ	0.44 ± 0.04	10.45 ± 1.63	14.82 ± 1.14	18.43 ± 1.33	20.82 ± 1.69	21.93 ± 1.03	23.82 ± 2.07	25.01 ± 2.24	25.37 ± 2.05	5,412.80 ± 286.61	0.49 ± 0.05
0.75% CSJ	0.45 ± 0.05	10.85 ± 1.07	13.73 ± 1.37	16.72 ± 1.07	18.82 ± 3.07	21.76 ± 1.91	22.73 ± 1.90	24.12 ± 1.01	24.62 ± 1.91	5,205.24 ± 111.67	0.43 ± 0.05
1.00% CSJ	0.42 ± 0.08	11.17 ± 1.20	14.04 ± 1.08	18.01 ± 1.90	21.63 ± 2.05	23.62 ± 2.20	24.77 ± 1.89	25.02 ± 0.92	25.24 ± 2.31	5,483.10 ± 64.941	0.49 ± 0.07
1.25% CSJ	0.41 ± 0.05	11.52 ± 2.12	12.51 ± 0.74	14.47 ± 1.52	16.21 ± 0.69	18.69 ± 1.75	21.82 ± 2.36	24.22 ± 0.58	25.64 ± 0.71	5,107.76 ± 130.85	0.35 ± 0.04

Data show mean + S.D. (n = 3)

The value show the concentration of p-nitroaniline (x 10^3 mg/ml)

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Table 15. Effects of various concentrations of CSG on trypsin activity as determined from the formation p-nitroaniline

[p-nitroaniline] (mg/ml x 10^3)	Time (min)									$AUC_{0\text{-}240min}$	k
	0	10	20	30	40	60	90	120	240	[mg/ml].min x 10^3	mg/ml/min x 10^3
Control	0.44 ± 0.02	10.91 ± 0.97	15.05 ± 1.46	18.011 ± 1.5	20.239 ± 1.1	22.36 ± 2.08	23.96 ± 2.14	24.68 ± 1.65	24.83 ± 1.81	5,364.29 ± 178.05	0.47 ± 0.03
Aprotinin	0.43 ± 0.02	0.433 ± 0.03	0.43 ± 0.04	0.413 ± 0.02	0.43 ± 0.02	0.445 ± 0.01	0.43 ± 0.08	0.445 ± 0.03	0.43 ± 0.02	104.56 ± 5.60	0.00 ± 0.00
0.25% CSG	0.44 ± 0.01	11.53 ± 0.93	14.93 ± 0.85	18.79 ± 0.83	20.89 ± 1.52	21.98 ± 1.70	23.86 ± 1.64	25.61 ± 0.78	25.51 ± 1.18	5,485.03 ± 244.78	0.48 ± 0.03
0.50% CSG	0.43 ± 0.01	11.63 ± 0.81	13.72 ± 0.69	18.84 ± 0.73	21.80 ± 1.43	22.75 ± 1.46	24.25 ± 1.34	24.87 ± 1.64	24.96 ± 1.63	5,430.18 ± 217.70	0.50 ± 0.03
0.75% CSG	0.48 ± 0.04	10.66 ± 1.24	14.92 ± 0.87	17.73 ± 1.14	20.02 ± 1.42	22.76 ± 1.03	23.92 ± 1.53	24.72 ± 1.87	24.35 ± 2.28	5,336.86 ± 306.58	0.46 ± 0.05
1.00% CSG	0.428 ± 0.00	11.043 ± 0.7	15.35 ± 1.80	19.25 ± 1.63	21.68 ± 1.28	22.81 ± 2.24	24.76 ± 1.74	24.52 ± 1.19	25.17 ± 2.01	5,445.82 ± 124.98	0.51 ± 0.03
1.25% CSG	0.46 ± 0.01	12.03 ± 0.95	12.81 ± 0.85	14.64 ± 1.43	16.31 ± 1.53	18.75 ± 0.92	20.88 ± 1.65	22.92 ± 1.60	26.53 ± 1.68	5,047.69 ± 128.39	0.34 ± 0.05

Data show mean + S.D. (n = 3)

The value show the concentration of p-nitroaniline (x 10^3 mg/ml)

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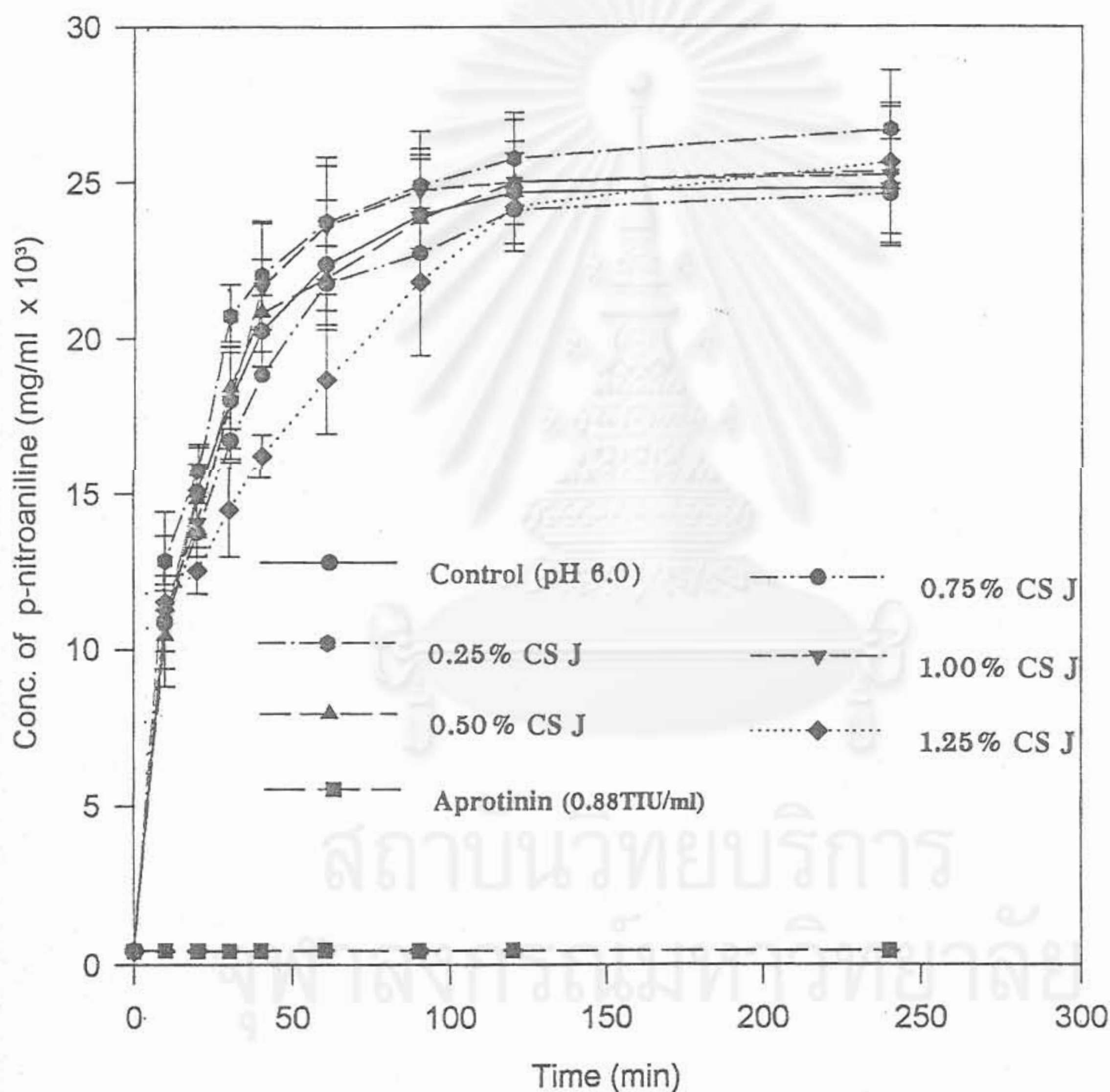
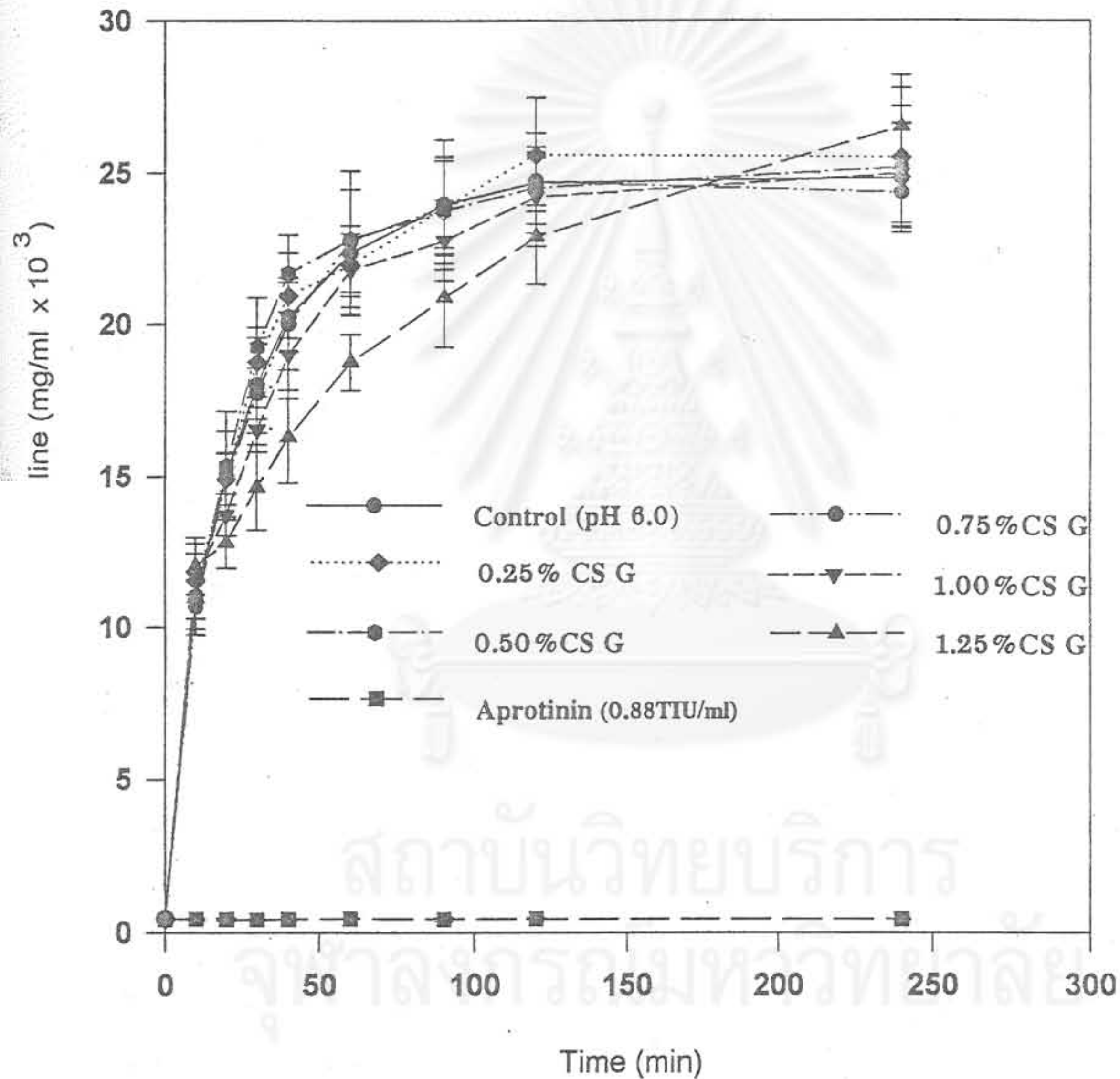
Figure 21. Formation of p-nitroaniline following incubation of BAPA with trypsin in the presence of varying concentrations of CSJ. The reactions were carried out in 0.15 M phosphate buffer pH 6.0



Each value = mean + S.D. (n = 3 determination)

Figure 22. Formation of p-nitroaniline following incubation of BAPA with trypsin in the presence of varying concentrations of CSG. The reactions were carried out in 0.15 M phosphate buffer pH 6.0



Each value = mean + S.D. (n = 3 determinations)

concentration. The highly viscous microenvironment created by chitosans may have markedly reduced the diffusion of enzyme and BAPA, thus slowing down the rate of substrate hydrolysis. On the other hand, incubation of substrate-enzyme mixture in the presence of aprotinin (0.88 TIU/ml) resulted in a highly significant sharp decrease in trypsin activity from the control buffer group, as judged from the AUC and k values ($p < 0.05$, Student's t-test). By comparison with aprotinin, it is evident that chitosans in the concentration range of 0.25-1.25 % hardly possess any inhibitory effect on the *in vitro* activity of trypsin. The slight decrease in the trypsin activity at 1.25 % was rather an indirect effect due to an increase in viscosity of the microenvironment than a direct inhibitory effect on the enzyme. However, more evidence is needed to prove this hypothesis.

2.2 Degradation Studies with Leucine Aminopeptidase

Preliminary study to determine the wavelength of maximum absorption of β-naphthylamine, after diazotization and coupling reaction, was performed by spectrophotometric scanning in the range of 400-600 nm. β-Naphthylamine was diazotized and coupled with sodium nitrite and naphthylethylene diamine. The amount of β-naphthylamine liberated, as analyzed from the above reactions, was thus a measure of the LAP activity.

The wavelength for the maximum absorption of β-napthylamine was determined to be 580 nm. Moreover, other reagents present in the reaction mixture did not absorb nor interfere at this wavelength. Thus, the quantitative analysis of LAP activity by colorimetric measurements was performed at this wavelength and the color of the final azo dye solution was blue.

Similar to trypsin, the pH for the maximum LAP activity on L-Leu-β-naphthylamide hydrolysis has previously been found to be around pH 8.0. Since

chitosans could not be dissolved at this alkaline pH, the lower pH value of 6.0 was chosen as the optimal pH, at which the LAP activity was still sufficiently high and could be accurately quantitated. The optimal substrate (L-Leu-β-naphthylamide) concentration was determined to be 0.78 mM in phosphate buffer at pH 6.0 since the rate of hydrolysis was saturated above this concentration.

Effect of Chitosans on Leucine Aminopeptidase (LAP) Activity

The degradation experiment was started by adding LAP to the mixture of substrate in the presence and absence of CSJ or CSG. The concentration of β-naphthylamine formed was then measured periodically during the 240-min incubation period at 37 ^{0}C. After quenching the reaction with 0.5 ml of 2 N HCl, the samples were analyzed as described in steps 4 to 7 of the analytical method.

Figure 23 shows a representative calibration curve of β-naphthylamine by making a plot of absorbance at 580 nm versus the concentration of β-naphthylamine. The curve is expressed by a linear equation Y = 0.077X, with the regression coefficient of 0.9997, where Y = absorbance at 580 nm and X is the concentration of β-naphthylamine in mg/ml x 10^{-4}.

Figure 24 and 25 show the formation of β-naphthylamine at various times following the incubation of L-Leu-β-naphthylamide and LAP enzyme in the presence of various concentrations (0 to 1.25 %) of CSJ and CSG, respectively. The plot of bestatin, a specific LAP inhibitor, was also provided in each figure for comparison. The average values of AUC and k, which indicate the extent and the initial rate of β-naphthylamine formation, are shown in Table 16 and 17. As seen from these figures, the LAP activity was not inhibited by both CSJ and CSG. ANOVA results on the AUC and k values at 5 % significance level indicated that both chitosans at all concentrations studied did not differ significantly from the control group ($p > 0.05$). The data thus suggest that the two chitosans did not possess any inhibitory effect on

Figure 23. Representative calibration curve of β-naphthylamine in 0.15 M phosphate buffer (pH 6.0)

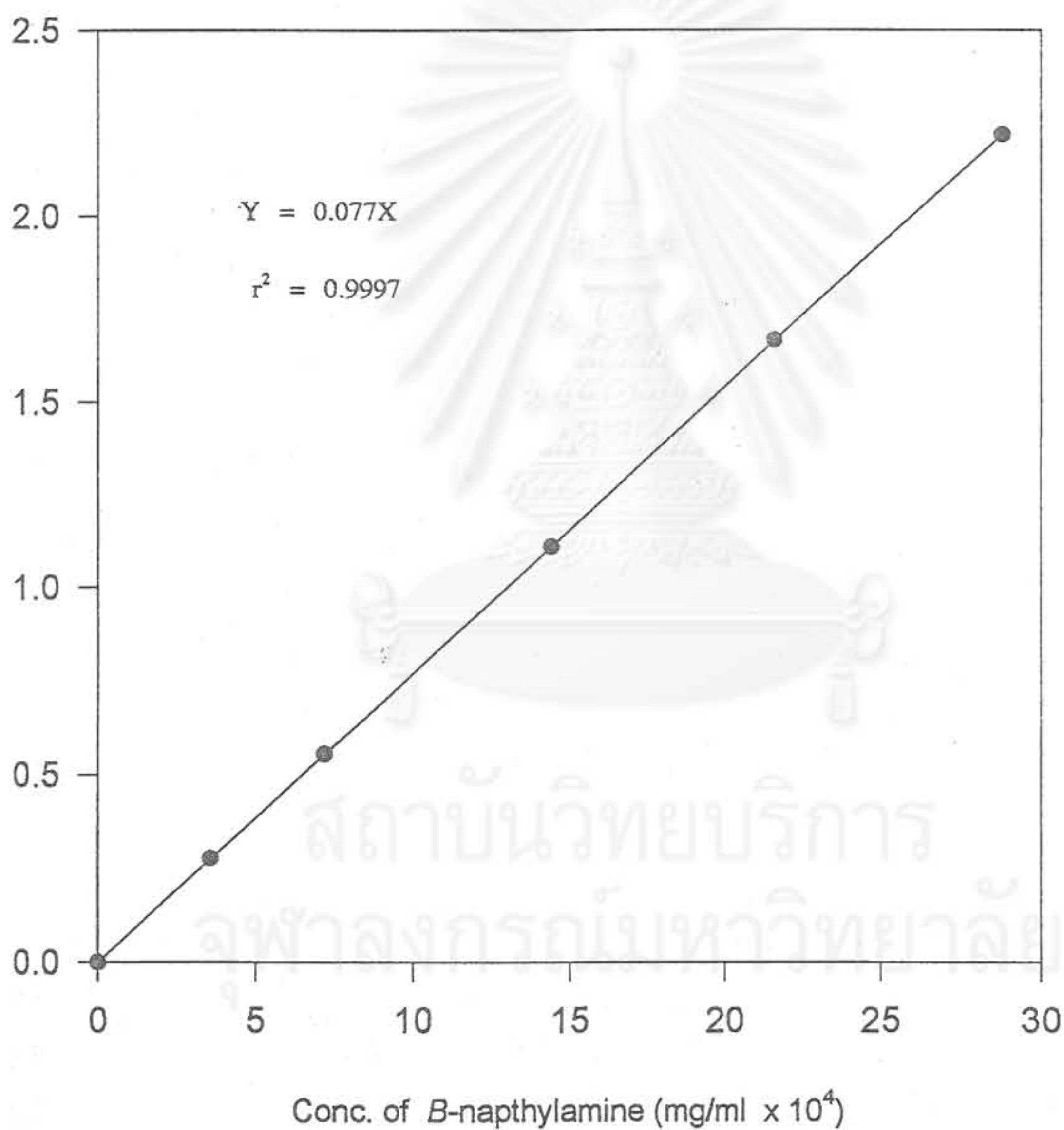


Table 16. Effects of various concentrations of CSJ on LAP activity as determined from the formation of β-naphthylamine

B-naphthylamine (mg/ml x 10^4)	Time (min)									$AUC_{0-240min}$ [mg/ml].min x 10^4	k mg/ml/min x 10^4
	0	10	20	30	40	60	90	120	240		
Control	0.12 ± 0.02	3.07 ± 0.08	5.16 ± 0.43	6.85 ± 0.78	10.56 ± 0.81	14.73 ± 1.34	17.36 ± 0.93	20.01 ± 1.67	20.86 ± 1.26	3,951.23 ± 256.89	0.247 ± 0.01
Bestatin	0.78 ± 0.08	2.34 ± 0.23	4.11 ± 0.42	5.20 ± 0.73	5.53 ± 1.01	5.83 ± 0.98	5.66 ± 0.96	5.877 ± 0.76	5.82 ± 1.04	1,309.15 ± 47.65	0.124 ± 0.02
0.25% CSJ	0.065 ± 0.01	3.057 ± 0.37	4.58 ± 0.80	6.68 ± 0.71	10.45 ± 0.49	14.17 ± 0.67	17.66 ± 0.65	18.47 ± 1.17	21.08 ± 1.57	3,834.74 ± 260.73	0.24 ± 0.01
0.50% CSJ	0.12 ± 0.02	2.47 ± 0.33	4.50 ± 0.74	6.48 ± 0.49	10.04 ± 0.7	13.28 ± 1.03	15.90 ± 1.14	18.54 ± 1.27	21.13 ± 1.09	3,756.56 ± 223.48	0.24 ± 0.02
0.75% CSJ	0.09 ± 0.02	3.02 ± 0.24	4.96 ± 0.60	7.14 ± 0.56	10.19 ± 0.92	14.28 ± 0.93	17.77 ± 1.33	19.32 ± 1.06	21.55 ± 1.09	3,936.99 ± 123.29	0.243 ± 0.02
1.00% CSJ	0.08 ± 0.02	2.79 ± 0.40	4.38 ± 0.75	6.66 ± 0.54	11.30 ± 1.03	15.88 ± 1.03	18.97 ± 1.19	21.57 ± 1.49	21.77 ± 1.56	4,197.81 ± 153.85	0.26 ± 0.02
1.25% CSJ	0.12 ± 0.01	3.76 ± 0.21	5.66 ± 0.59	8.06 ± 0.84	11.85 ± 1.21	15.73 ± 1.08	19.30 ± 0.69	21.18 ± 0.89	22.14 ± 0.78	4,242.02 ± 130.85	0.28 ± 0.02

Data show mean ± S.D. (n = 3)

The value show the concentration of B-naphthylamine (mg/ml x 10^4)

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Table 17. Effects of various concentrations of CSG on LAP activity as determined from the formation of β-naphthylamine

B-naphthylamine (mg/ml x 10^4)	Time (min)									$AUC_{0\text{-}240min}$	k
	0	10	20	30	40	60	90	120	240	[mg/ml].min x 10^4	mg/ml/min x 10^4
Control	0.12 ± 0.02	3.07 ± 0.08	5.16 ± 0.43	6.85 ± 0.78	10.56 ± 0.81	14.73 ± 1.34	17.36 ± 0.93	20.01 ± 1.67	20.86 ± 1.26	3,951.23 ± 256.89	0.247 ± 0.01
Bestatin	0.78 ± 0.08	2.34 ± 0.23	4.11 ± 0.42	5.20 ± 0.73	5.53 ± 1.01	5.83 ± 0.98	5.66 ± 0.96	5.877 ± 0.76	5.82 ± 1.04	1,309.15 ± 47.65	0.124 ± 0.02
0.25% CSG	0.12 ± 0.02	2.736 ± 0.20	4.60 ± 0.42	6.59 ± 0.44	11.21 ± 0.94	17.35 ± 0.76	19.04 ± 1.29	20.18 ± 1.75	21.45 ± 1.29	4,113.22 ± 134.92	0.26 ± 0.02
0.50% CSG	0.10 ± 0.02	2.97 ± 0.57	4.23 ± 0.59	7.17 ± 0.72	9.89 ± 1.11	15.76 ± 1.81	20.24 ± 1.78	20.86 ± 1.92	22.06 ± 2.86	4,181.72 ± 295.70	0.24 ± 0.01
0.75% CSG	0.11 ± 0.01	3.67 ± 0.43	4.91 ± 0.41	7.28 ± 0.72	11.26 ± 1.09	17.91 ± 1.23	19.81 ± 1.48	20.07 ± 1.25	22.29 ± 1.73	4,212.53 ± 281.32	0.26 ± 0.03
1.00% CSG	0.12 ± 0.03	3.79 ± 0.38	4.07 ± 0.72	8.12 ± 0.78	11.55 ± 0.93	15.59 ± 1.16	19.78 ± 1.32	21.64 ± 1.55	22.23 ± 1.40	4,273.18 ± 81.82	0.27 ± 0.03
1.25% CSG	0.12 ± 0.02	4.62 ± 0.57	5.19 ± 0.65	7.85 ± 0.67	10.56 ± 0.92	19.60 ± 1.02	20.03 ± 1.26	21.42 ± 1.47	22.95 ± 1.66	4,415.18 ± 286.14	0.24 ± 0.02

Data show mean + S.D. (n = 3)

The value show the concentration of B-naphthylamine (mg/ml x 10^4)

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Figure 24. Formation of β-naphthylamine following incubation of L-Leu-β-NA with LAP in the presence of varying concentrations of CSJ. The reactions were carried out in 0.15 M phosphate buffer pH 6.0

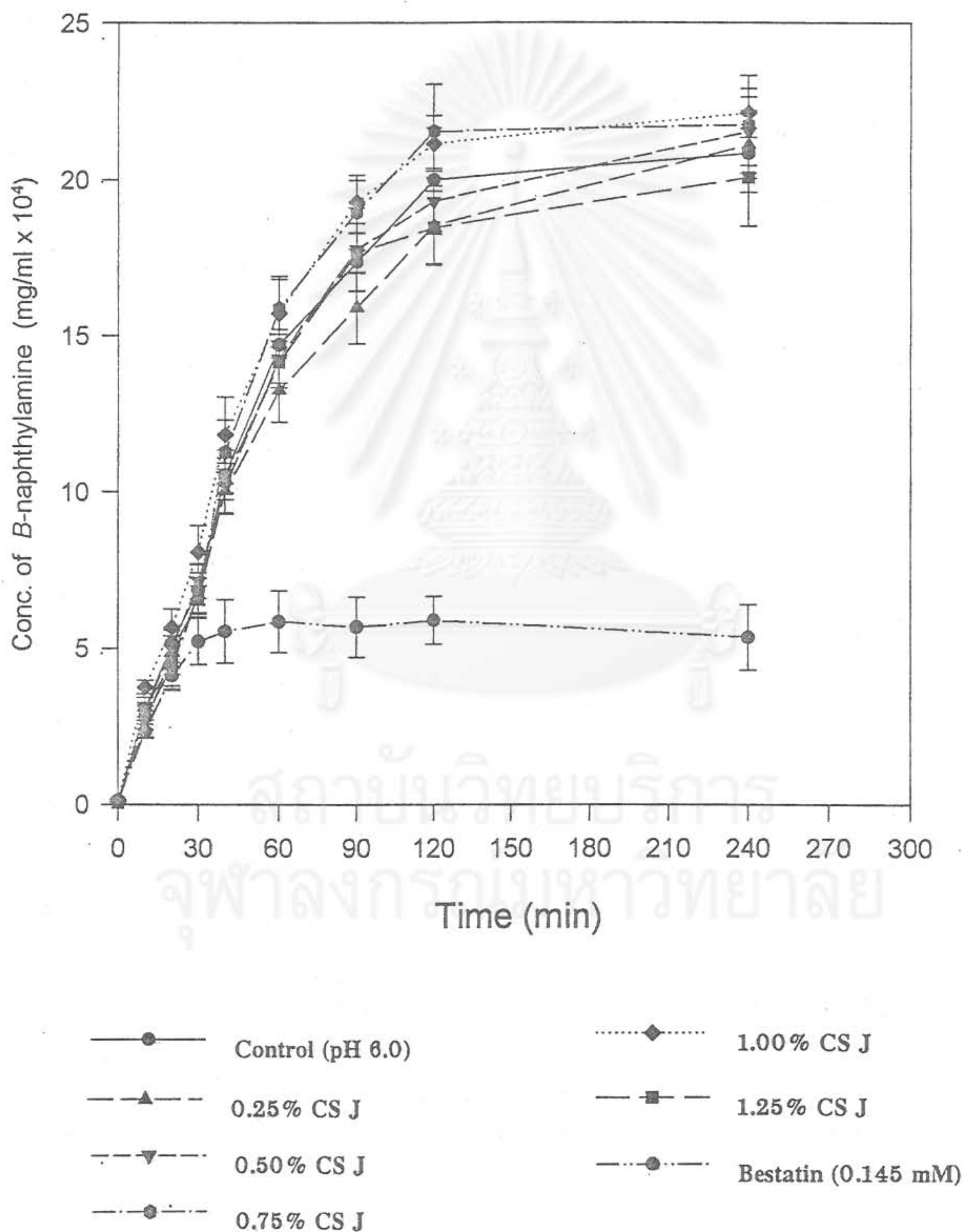
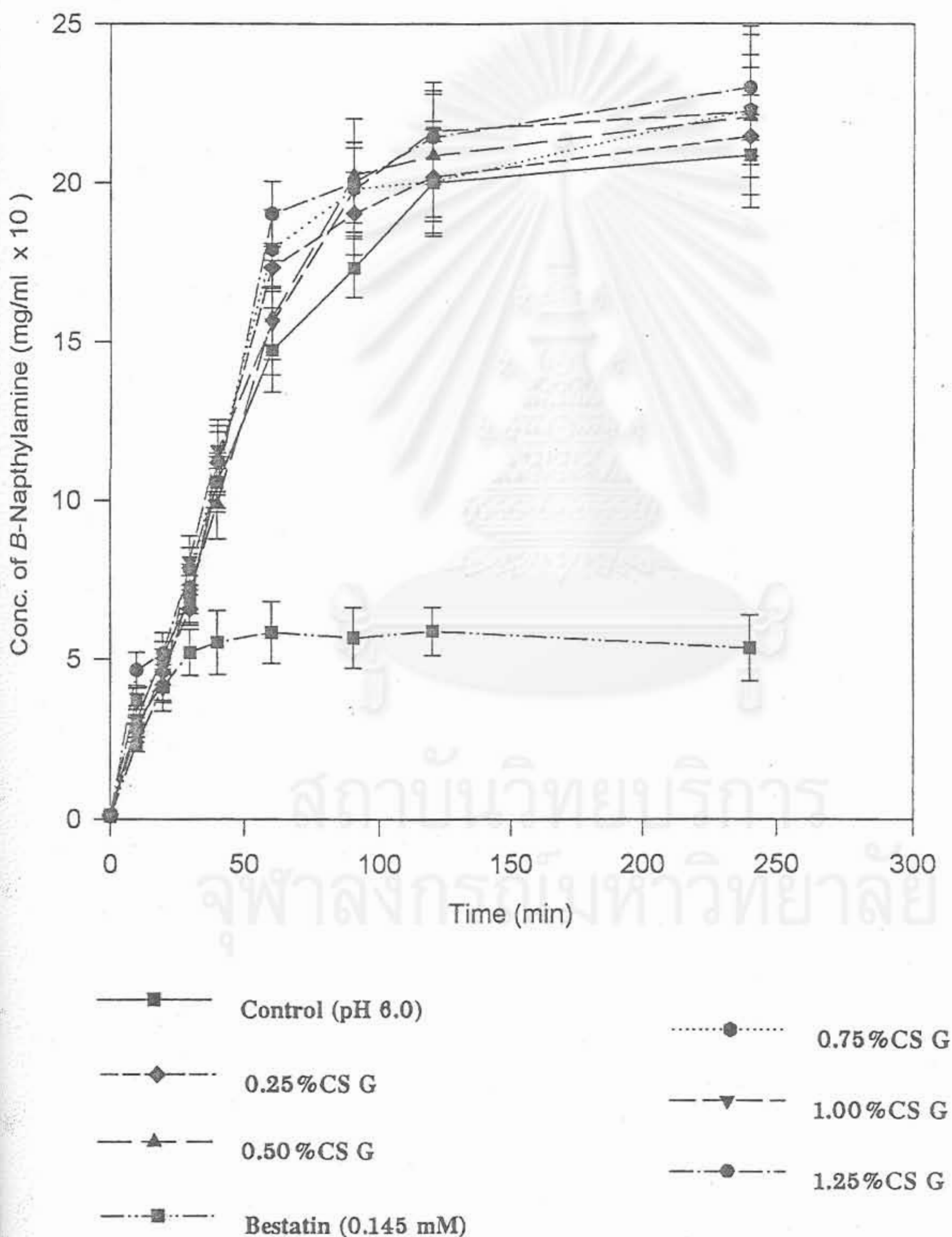


Figure 25. Formation of β-naphthylamine following incubation of L-Leu-β-NA with LAP in the presence of varying concentrations of CSG. The reactions were carried out in 0.15 M phosphate buffer pH 6.0



the LAP activity, causing practically no changes in the β-naphthylamine versus time profiles when compared to the control group (Figures 24 and 25).

On the other hand, the same figures show that bestatin (0.145 mM) strongly inhibited the LAP activity. The effect was highly significant from the control group (no inhibitor) with respect to both the AUC and k values ($p < 0.05$, Student's t-test). The average AUC was decreased by 3 folds whereas there was a 50 % reduction in the k values when bestatin was included in the incubation mixture (Table 16). Therefore, the result from this study demonstrated that both chitosans in the concentration range of 0.25 to 1.25 % did not possess any inhibitory effect on the *in vitro* activity of the enzyme LAP.

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CHAPTER V
CONCLUSION

The results of this first part can be summarized as follows :

1. The *in vivo* efficacy of two chitosans, CSJ and CSG, as a nasal absorption enhancer of sCT was assessed by measuring the hypocalcemic effect. The results demonstrated that both chitosans possess significant nasal absorption enhancing activity for a poorly absorbed drug like salmon calcitonin.

2. The enhancing effect of CSJ appears to depend on pH, with increasing adjuvant activity as the pH of the preparation is decreased. This is in accordance with the ability of the free amine chitosan to ionize, hydrate and dissolve better in the more acidic condition, which may lead to a more elongated shape and a better contact with the nasal epithelium. The pH for the optimum enhancing activity of CSJ was found to be pH 4.0. CSJ at pH 3.0 also exhibits good nasal absorption. However, the buffer used may be too acidic for the nasal mucosa and may have direct deleterious effects on the membrane.

3. The enhancing effect of CSG, on the other hand, appears to be optimum at higher pH. This could be due to the nature of CSG which exists as a soluble glutamate salt, a chemical form which may facilitate swelling and enable the chitosan molecule to maintain a high charge density, a property believed to be essential for its enhancing activity, even at high pH values. The optimum pH for CSG appears to be 6.0 since the highest absorption enhancement of sCT was observed at this pH. However, the reasons as to opposite ranking results to that of CSJ are not clearly known at present.

4. At their respective optimal pH (CSJ at pH 4.0 and CSG at pH 6.0), the enhancing activity was found to be concentration-dependent in the range of 0.25 to 1.0 % w/v. Above this concentration, the enhancing effect of both chitosans tends to become saturated since the nasal absorption of sCT at 1.25 % polymer concentration was not further increased. However, the two chitosans are already effective at a concentration as low as 0.25 % w/v.

5. The absorption enhancing activities of CSJ (1.0 %, pH 4.0) and CSG (1.0 %, pH 6.0) were compared to that of DMβCD and HPβCD (both at 5 %, pH 7.4). The extent of sCT nasal absorption based on the percent total decrease in plasma calcium (% D) was similar between CSJ and DMβCD (9.85 ± 1.89 % vs 9.68 ± 0.31 %). On the other hand, the plasma calcium lowering effect of CSG (8.43 ± 0.67 %) was significantly lower and equivalent to HPβCD (8.05 ± 0.46 %).

6. Using a specific radioimmunoassay method, the absolute nasal bioavailability of plasma sCT after comparison with intravenous administration was determined to be 2.45, 1.91 and 1.22 % for 1 % CSJ, 5 % DMβCD and the control group (intranasal sCT without enhancer), respectively. Although the values seem to be low when compared to the i.v. injection, the inclusion of 1 % CSJ resulted in a two-fold increase in the AUC_{0-180} of plasma sCT relative to that of the control group at the same nasal dose. This is equivalent to 201.18 % relative bioavailability. Addition of 5 % DMβCD also resulted in a significant increase in the AUC_{0-180}, with a relative bioavailability of 156.48 %. All the four enhancers demonstrated significant absorption enhancement of sCT ($p < 0.05$). However, 1 % CSJ and 5 % DMβCD appear to be the most effective.

7. Both chitosans did not possess any inhibitory effect on the *in vitro* activities of trypsin and leucine aminopeptidase, the two major nasal enzymes responsible for the local degradation of sCT in the nasal mucosa. As a result, the nasal absorption enhancement of chitosans may not involve protection of the peptide drug against proteolytic degradation by the nasal enzymes, but it may involve the direct effect of chitosans on the mucosal permeability.

8. In conclusion, the results from the *in vivo* absorption studies obtained here further confirm our previous findings based on the *in situ* perfusion that the cationic polysaccharide chitosans like CSJ and CSG demonstrate significant nasal absorption enhancing activities. The two polymers may have promising potential for use as a safe and effective absorption enhancer in the nasal formulations of sCT and other peptides.

RECOMMENDATION FOR FUTURE STUDIES

Future experiments should include studies to confirm the *in vivo* safety and efficacy of chitosans as nasal absorption enhancers in human volunteers. Complete evaluation of chitosan safety profiles should also be carried out regarding its ciliotoxicity, chronic toxicity at both the mucosal and the cellular levels, as well as its possible immunogenicity and mutagenicity. Successful application of chitosans as a safe and effective absorption enhancer in the nasal formulations will strongly depend on the results of these investigations.

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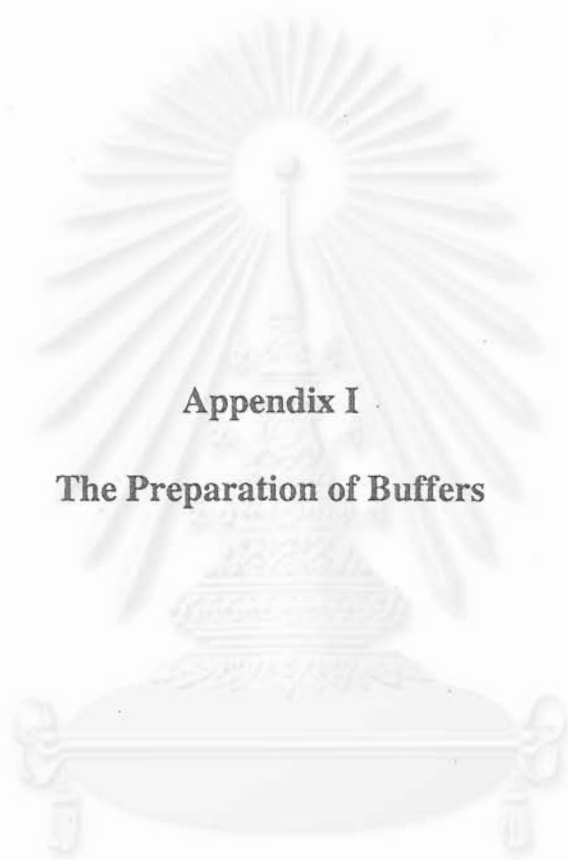


Appendix I

The Preparation of Buffers

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Buffer Preparations

IPB pH 3.0 (0.15 M):

85%H_3PO_4	0.56 ml
NaH_2PO_4	9.19 gm
NaCl	0.78 gm
H_2O qs.to	500 ml

IPB pH 4.0 (0.15M):

NaH_2PO_4	10.22 gm
85%H_3PO_4	40 μl
NaCl	0.42 gm
H_2O qs.to	500 ml

IPB pH 5.0 (0.15 M):

NaH_2PO_4	0.125 gm
Na_2HPO_4	10.28 gm
NaCl	0.59 gm
H_2O qs.to	500 ml

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

IPBpH 6.0 (0.15 M):

NaH_2PO_4	3.6 gm
Na_2HPO_4	0.47 gm
NaCl	2.6 gm
H_2O qs.to	500 ml

IPB pH 7.4 (0.15 M):

NaH_2PO_4	0.80	gm
Na_2HPO_4	3.79	gm
NaCl	2.24	gm
H_2O qs.to	500	ml

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix II

Individual Plasma Calcium Data (% of initial value) for baseline, control and $sCT_{treated}$ groups at various pH

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix II_a

Individual Plasma Calcium Data (% of initial value) for the baseline groups at pH 3.0 - 7.4

0.15 M Phosphate Buffer pH 3.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
1	100.00	100.52	101.23	100.91	97.71	93.32	90.34	92.63	96.45	97.33	22,910.79
2	100.00	104.33	105.11	101.26	100.61	96.29	93.85	95.63	93.23	94.83	23,080.41
3	100.00	98.45	101.00	98.75	96.58	92.61	91.77	93.49	92.21	94.40	22,568.96
4	100.00	103.66	100.58	102.66	100.63	97.44	93.44	90.22	94.22	95.34	22,891.07
5	100.00	102.01	98.15	97.33	94.77	93.54	93.00	94.59	92.80	91.89	22,606.00
Mean	**100.00**	**101.79**	**101.21**	**100.18**	**98.06**	**94.64**	**92.48**	**93.31**	**93.78**	**94.76**	**22,811.45**
± SD.	**0.00**	**2.13**	**2.24**	**1.90**	**2.29**	**1.88**	**1.28**	**1.85**	**1.49**	**1.75**	**194.71**

0.15 M Phosphate Buffer pH 4.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
1	100.00	104.81	103.91	104.21	102.31	101.99	99.02	100.52	98.93	97.86	24,079.62
2	100.00	105.22	101.23	102.34	101.17	100.52	100.11	97.05	96.83	98.15	23,743.52
3	100.00	101.82	99.02	100.12	98.62	100.06	98.74	96.96	98.44	97.72	23,653.80
4	100.00	100.47	102.76	103.22	101.50	101.56	100.26	98.45	100.64	101.90	24,158.96
5	100.00	98.60	100.60	98.01	100.22	98.64	97.56	96.72	97.65	95.23	23,436.00
Mean	**100.00**	**102.18**	**101.50**	**101.58**	**100.76**	**100.55**	**99.14**	**97.94**	**98.50**	**98.17**	**23,814.38**
± SD.	**0.00**	**2.53**	**1.70**	**2.24**	**1.26**	**1.18**	**0.99**	**1.43**	**1.29**	**2.14**	**269.47**

0.15 M Phosphate Buffer pH 5.0

Subject No.	Time (mins)										AUC$_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
1	100.00	103.91	105.41	102.83	104.42	103.62	100.51	98.74	95.52	97.29	23,886.18
2	100.00	106.11	103.60	104.45	102.34	100.12	102.93	100.56	96.15	98.84	24,026.44
3	100.00	101.11	102.15	100.34	105.70	101.62	97.33	96.55	100.52	100.23	23,964.66
4	100.00	100.81	101.89	100.09	102.07	100.40	101.16	102.82	100.89	98.73	24,245.13
5	100.00	98.68	100.59	101.15	100.93	103.00	100.65	101.44	98.70	100.83	24,124.36
Mean	**100.00**	**102.12**	**102.73**	**101.77**	**103.09**	**101.75**	**100.52**	**100.02**	**98.36**	**99.18**	**24,049.35**
± SD.	**0.00**	**2.60**	**1.65**	**1.65**	**1.72**	**1.38**	**1.81**	**2.18**	**2.20**	**1.24**	**125.12**

0.15 M Phosphate Buffer pH 6.0

Subject No.	Time (mins)										AUC$_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	% . min
1	100.00	103.46	103.45	102.46	103.26	100.86	101.67	102.20	100.34	100.07	24,335.55
2	100.00	104.12	102.25	103.52	104.23	101.93	102.87	100.31	98.11	101.77	24,250.30
3	100.00	103.13	102.41	103.45	100.56	101.45	101.11	101.58	100.25	100.54	24,270.34
4	100.00	101.51	100.58	98.78	100.32	98.46	100.56	98.19	98.31	100.26	23,816.72
5	100.00	102.25	101.43	100.20	98.74	97.55	96.55	97.26	97.10	98.53	23,513.42
Mean	**100.00**	**102.89**	**102.02**	**101.68**	**101.42**	**100.05**	**100.55**	**99.91**	**98.82**	**100.23**	**24,037.27**
± SD.	**0.00**	**0.92**	**0.97**	**1.88**	**2.02**	**1.73**	**2.14**	**1.91**	**1.27**	**1.04**	**319.92**

0.15 M Phosphate Buffer pH 7.4

Subject No.	Time (mins)										$AUC_{(0-240)min}$
	0	10	20	30	40	60	90	120	180	240	[% . min]
1	100.00	107.16	102.66	100.13	102.52	97.45	101.93	100.15	101.96	103.49	24,359.93
2	100.00	102.59	103.49	105.95	98.56	100.58	103.77	98.18	99.15	98.29	24,041.74
3	100.00	100.58	97.46	101.92	100.73	99.75	98.96	100.45	97.82	102.40	23,934.37
4	100.00	98.47	100.79	101.23	97.17	99.16	100.01	97.16	100.12	101.64	23,870.00
5	100.00	101.16	102.74	98.15	102.46	103.56	100.16	102.42	104.15	100.92	24,536.11
Mean	**100.00**	**101.99**	**101.43**	**101.47**	**100.29**	**100.10**	**100.97**	**99.67**	**100.64**	**101.34**	**24,148.43**
± SD.	**0.00**	**2.90**	**2.17**	**2.58**	**2.13**	**2.01**	**1.70**	**1.84**	**2.21**	**1.75**	**256.81**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix II_b

Individual Plasma Calcium Data (% of initial value) for the control groups (sCT_{alone} at pH 3.0 - 7.4)

sCT(in) pH = 3.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00				83.47	87.84	88.55	89.64	90.12	90.65	21,517.77	83.47	40.00	5.67
2	100.00	96.16	95.16	88.34	82.52	84.23	87.44	90.02	89.36	88.12	21,319.20	82.52	40.00	6.54
3	100.00	102.19	96.89	92.35	85.82	91.92	93.56	90.71	88.73	91.26	21,949.96	85.82	40.00	3.78
4	100.00	100.66	94.85	87.81	83.93	86.96	85.93	88.16	91.82	88.96	21,489.32	83.93	40.00	5.80
5	100.00	96.92	90.76	84.46	88.49	89.14	90.49	89.03	89.29	90.31	21,544.55	84.46	30.00	5.47
Mean	**100.00**	**98.98**	**94.41**	**88.24**	**84.85**	**88.02**	**89.19**	**89.51**	**89.86**	**89.86**	**21,564.16**	**84.04**	**38.00**	**5.45**
± SD.	**0.00**	**2.52**	**2.25**	**2.80**	**2.11**	**2.53**	**2.64**	**0.87**	**1.08**	**1.15**	**208.32**	**1.09**	**4.00**	**0.91**

sCT(in) pH = 4.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	101.22	99.67	100.62	96.80	89.38	92.50	94.68	94.26	93.91	22,709.97	89.38	60.00	4.64
2	100.00	100.79	98.57	98.53	92.83	87.58	94.81	91.42	93.83	92.72	22,430.29	87.58	60.00	5.81
3	100.00	98.43	95.95	97.73	93.49	85.93	90.74	93.71	89.41	94.12	22,099.16	85.93	60.00	7.20
4	100.00	103.82	100.47	102.82	97.49	90.92	86.73	88.43	94.85	90.69	22,299.32	86.73	90.00	6.36
5	100.00	102.13	101.34	98.49	95.83	91.20	91.27	95.66	95.93	93.82	22,850.14	91.20	60.00	4.05
Mean	**100.00**	**101.28**	**99.20**	**99.64**	**95.28**	**89.00**	**91.21**	**92.78**	**93.66**	**93.05**	**22,477.78**	**88.16**	**66.00**	**5.61**
± SD.	**0.00**	**1.76**	**1.86**	**1.86**	**1.83**	**2.01**	**2.64**	**2.59**	**2.24**	**1.28**	**272.04**	**1.90**	**12.00**	**1.14**

sCT(in) pH = 5.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	100.56	101.62	98.01	97.24	94.34	96.81	95.70	94,36	97.00	23,101.65	94.34	60.00	3.94
2	100.00	105.93	102.32	103.11	98.83	95.11	94.43	92.91	92.31	94.82	22,870.96	92.31	180.00	4.90
3	100.00	98.26	100.11	96.45	93.40	89.29	92.67	89.52	95.73	91.45	22,376.75	82.29	60.00	6.95
4	100.00	101.34	100.01	98.49	94.72	90.32	93.53	92.52	91.82	90.22	22,362.51	90.22	240.00	7.01
5	100.00	102.81	103.93	100.52	96.26	92.12	94.09	93.23	95.92	95.40	22,954.27	92.12	60.00	4.55
Mean	**100.00**	**101.78**	**101.60**	**99.32**	**96.09**	**92.24**	**94.30**	**92.78**	**94.03**	**93.78**	**22,733.23**	**90.26**	**120.00**	**5.47**
± SD.		**2.54**	**1.46**	**2.30**	**1.90**	**2.24**	**1.39**	**1.97**	**1.70**	**2.54**	**305.96**	**4.19**	**75.89**	**1.27**

sCT(in) pH = 6.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	101.20	100.56	98.70	95.79	93.16	93.91	96.23	95.87	95.97	23,049.33	93.16	60.00	4.11
2	100.00	105.56	102.97	97.51	94.58	92.81	95.80	94.25	91.11	92.53	22,657.13	91.11	180.00	5.74
3	100.00	97.16	101.15	99.02	96.88	93.53	97.75	95.63	90.37	93.71	22,733.56	90.37	180.00	5.42
4	100.00	100.23	98.81	96.34	94.66	90.92	93.17	90.42	95.63	92.06	22,510.05	90.42	120.00	6.35
5	100.00	102.82	99.22	100.62	97.35	95.64	93.27	96.83	94.33	94.81	23,036.91	93.27	90.00	4.16
Mean	**100.00**	**101.39**	**100.54**	**98.44**	**95.85**	**93.21**	**94.78**	**94.67**	**93.46**	**93.82**	**22,797.40**	**91.67**	**126.00**	**5.16**
± SD.	**0.00**	**2.78**	**1.48**	**1.44**	**1.12**	**1.51**	**1.76**	**2.29**	**2.30**	**1.44**	**213.15**	**1.29**	**48.00**	**0.89**

sCT(in) pH = 7.4

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	100.33	101.36	97.13	94.83	92.92	93.82	93.52	94.34	96.42	22,809.48	92.92	60.00	5.54
2	100.00	105.28	100.79	100.33	97.81	96.86	92.83	96.11	93.83	95.21	23,048.07	92.83	90.00	4.56
3	100.00	98.43	96.36	98.65	95.43	91.63	93.55	92.51	91.93	93.23	22,438.67	91.63	60.00	7.08
4	100.00	101.83	102.83	96.63	96.29	92.70	95.62	93.61	95.94	92.69	22,893.05	92.69	240.00	5.20
5	100.00	100.26	98.42	96.29	93.72	94.92	94.26	96.45	96.83	95.83	23,080.83	93.72	40.00	4.42
Mean	**100.00**	**101.22**	**99.95**	**97.80**	**95.62**	**93.80**	**94.02**	**94.44**	**94.57**	**94.68**	**22,854.02**	**92.76**	**98.00**	**5.36**
± SD.	**0.00**	**2.30**	**2.29**	**1.50**	**1.38**	**1.86**	**0.93**	**1.55**	**1.70**	**1.46**	**230.27**	**0.67**	**72.77**	**0.95**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix II$_c$

Individual Plasma Calcium Data (% of initial value) for the sCT$_{treated}$ groups (1.0 % CS J at pH 3.0 - 6.0)

1.0% CS J pH = 3.0

Subject No.	Time (mins)										AUC$_{(0-240)min}$	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	min)	
1	100.00	92.22	85.47	74.34	76.76	84.40	86.99	88.22	85.53	87.83	20,627.96	74.34	30.00	9.57
2	100.00	93.69	81.23	68.45	75.98	81.03	84.72	87.21	86.83	85.66	20,344.82	68.45	30.00	10.81
3	100.00	92.23	84.98	72.24	79.829	83.78	81.81	84.89	90.82	85.73	20,581.39	72.24	30.00	9.78
4	100.00	100.14	92.98	81.01	76.932	82.06	86.00	83.63	89.92	86.12	20,768.46	76.93	40.00	8.96
5	100.00	91.75	82.95	71.33	73.69	77.45	80.72	82.12	83.61	84.90	19,682.29	71.33	30.00	13.72
Mean	**100.00**	**94.01**	**85.52**	**73.47**	**76.64**	**81.74**	**84.05**	**85.21**	**87.34**	**86.05**	**20,400.98**	**72.66**	**32.00**	**10.57**
± SD.	**0.00**	**3.14**	**4.02**	**4.22**	**1.97**	**2.46**	**2.41**	**2.25**	**2.69**	**0.97**	**384.39**	**2.86**	**4.00**	**1.69**

1.0% CS J pH = 4.0

Subject No.	Time (mins)										AUC$_{(0-240)min}$	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	102.33	92.15	83.84	74.44	86.72	93.82	93.14	92.36	91.72	21,866.95	74.44	40.00	8.18
2	100.00	98.22	87.30	80.65	71.16	82.96	86.79	92.69	90.83	92.71	21,309.22	71.16	40.00	10.52
3	100.00	104.91	94.42	84.84	78.72	73.81	82.53	93.52	91.13	90.81	21,244.08	73.81	60.00	10.79
4	100.00	100.34	89.47	78.26	72.56	80.76	89.75	95.73	93.46	95.15	21,750.21	72.56	40.00	8.67
5	100.00	101.66	90.15	87.58	75.36	81.73	83.63	90.62	88.76	93.15	21,174.57	75.36	40.00	11.08
Mean	**100.00**	**101.49**	**90.70**	**83.03**	**74.45**	**81.19**	**87.30**	**93.14**	**91.31**	**92.71**	**21,469.01**	**73.47**	**44.00**	**9.85**
± SD.	**0.00**	**2.21**	**2.42**	**3.26**	**2.59**	**4.21**	**4.12**	**1.64**	**1.58**	**1.47**	**282.93**	**1.47**	**8.00**	**1.19**

1.0% CS J pH = 5.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	102.72	95.83	88.32	84.36	90.47	95.13	93.14	93.66	93.45	22,364.15	84.36	40.00	7.01
2	100.00	100.36	98.30	90.51	79.28	87.12	92.44	94.48	92.01	93.52	22,109.90	79.28	40.00	8.06
3	100.00	103.89	95.15	91.30	87.18	83.56	90.42	93.75	94.45	94.59	22,236.15	83.56	60.00	7.52
4	100.00	99.05	92.95	87.46	83.45	89.18	87.13	91.84	92.26	90.96	21,786.71	83.45	40.00	9.41
5	100.00	99.16	99.64	93.52	86.20	92.15	93.87	93.26	94.16	94.45	22,510.19	86.20	40.00	6.40
Mean	**100.00**	**101.04**	**96.37**	**90.22**	**84.09**	**88.49**	**91.80**	**93.29**	**93.31**	**93.39**	**22,201.42**	**83.37**	**44.00**	**7.68**
± SD.	**0.00**	**1.94**	**2.36**	**2.16**	**2.74**	**2.97**	**2.81**	**0.87**	**0.99**	**1.30**	**246.33**	**2.27**	**8.00**	**1.02**

1.0% CS J pH = 6.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	100.34	97.42	94.86	90.46	86.11	91.56	92.81	93.51	92.82	22,254.30	86.11	60.00	7.42
2	100.00	104.26	100.26	97.26	94.36	87.78	94.79	90.45	91.46	90.48	22,187.85	87.78	60.00	7.69
3	100.00	99.76	97.45	95.58	88.78	84.16	92.26	95.18	93.02	93.46	22,299.50	84.16	60.00	7.23
4	100.00	98.57	95.13	92.41	87.29	84.59	92.58	94.96	95.79	94.16	22,402.00	84.59	60.00	6.80
5	100.00	101.75	99.15	96.45	94.83	89.35	88.73	91.67	94.16	94.42	22,395.75	88.73	40.00	6.83
Mean	**100.00**	**100.94**	**97.88**	**95.31**	**91.14**	**86.40**	**91.98**	**93.01**	**93.59**	**93.07**	**22,307.88**	**86.27**	**56.00**	**7.19**
± SD.	**0.00**	**1.95**	**1.75**	**1.66**	**2.99**	**1.95**	**1.95**	**1.84**	**1.42**	**1.41**	**82.37**	**1.77**	**8.00**	**0.34**

Appendix II$_d$

Individual Plasma Calcium Data (% of initial value) for the sCT$_{treated}$ groups (1.0 % CS G at pH 3.0 - 6.0)

1.0% CS G pH = 3.0

Subject No.	Time (mins)										AUC$_{(0-240)min}$	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	92.42	85.47	82.36	86.12	85.89	88.23	89.79	89.07	88.11	21,225.20	82.36	30.00	6.95
2	100.00	97.56	94.11	85.19	79.46	82.35	84.18	88.59	90.28	89.11	21,121.30	79.46	40.00	7.41
3	100.00	96.92	89.71	84.40	91.02	84.04	85.42	87.45	88.46	89.33	21,161.95	84.04	60.00	7.23
4	100.00	94.39	83.74	81.05	84.96	87.17	87.76	87.38	89.85	87.92	21,148.50	81.05	30.00	7.29
5	100.00	94.12	85.70	83.29	87.12	90.56	86.68	85.49	88.26	89.59	21,125.65	83.29	30.00	7.39
Mean	**100.00**	**95.08**	**87.75**	**83.26**	**85.74**	**86.00**	**86.45**	**87.74**	**89.18**	**88.81**	**21,156.52**	**82.04**	**38.00**	**7.25**
± SD.	**0.00**	**1.90**	**3.74**	**1.46**	**3.74**	**2.80**	**1.49**	**1.43**	**0.78**	**0.67**	**37.43**	**1.63**	**11.66**	**0.17**

1.0% CS G pH = 4.0

Subject No.	Time (mins)										AUC$_{(0-240)min}$	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	102.22	96.58	92.44	89.23	85.83	93.66	90.89	92.20	94.52	22,163.75	85.83	60.00	6.93
2	100.00	100.94	94.79	88.80	85.45	93.72	93.23	94.96	93.52	89.46	22,337.15	85.45	40.00	6.20
3	100.00	98.15	95.01	89.54	86.34	88.31	90.10	93.75	92.59	91.26	22,045.00	86.34	40.00	7.43
4	100.00	100.61	97.22	94.49	82.66	86.86	89.36	90.43	92.72	94.85	21,993.45	82.66	40.00	7.65
5	100.00	97.82	91.48	85.16	83.68	85.34	92.79	89.82	95.95	92.88	21,997.30	83.68	40.00	7.63
Mean	**100.00**	**99.95**	**95.02**	**90.08**	**85.47**	**88.01**	**91.83**	**91.97**	**93.40**	**92.59**	**22,107.33**	**84.79**	**44.00**	**7.17**
± SD.	**0.00**	**1.69**	**1.99**	**3.20**	**2.28**	**3.03**	**1.75**	**2.01**	**1.35**	**2.02**	**130.35**	**1.39**	**8.00**	**0.55**

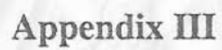
1.0% CS G pH = 5.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	101.56	100.24	96.65	85.86	80.75	86.42	92.62	95.23	96.85	22,171.29	80.75	60.00	7.81
2	100.00	98.24	95.95	84.65	80.55	86.92	92.57	95.81	93.64	95.97	22,255.16	80.55	40.00	7.46
3	100.00	97.81	94.75	87.91	82.01	89.23	95.50	95.92	94.86	93.10	22,431.55	82.01	40.00	6.73
4	100.00	100.37	98.19	93.39	81.31	85.46	92.42	94.52	95.49	94.14	22,354.56	81.31	40.00	7.05
5	100.00	101.00	96.85	90.09	83.12	88.44	93.12	95.15	93.57	92.47	22,312.85	83.12	40.00	7.22
Mean	**100.00**	**99.79**	**97.20**	**90.54**	**82.57**	**86.16**	**92.01**	**94.81**	**94.56**	**94.51**	**22,305.08**	**81.55**	**44.00**	**7.25**
± SD.		**1.50**	**1.89**	**4.18**	**1.85**	**3.00**	**3.00**	**1.20**	**0.80**	**1.67**	**88.20**	**0.94**	**8.00**	**0.37**

1.0% CS G pH = 6.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	104.98	101.53	95.46	84.46	81.45	85.55	89.26	92.49	94.16	21,779.86	81.45	60.00	9.39
2	100.00	102.64	98.15	92.79	80.51	84.57	92.21	93.82	94.98	92.07	22,206.94	80.51	40.00	7.61
3	100.00	98.19	89.49	84.92	78.08	83.96	87.47	91.62	95.32	96.48	22,102.10	78.08	40.00	8.05
4	100.00	100.63	96.26	87.96	80.35	87.65	94.45	92.41	93.45	91.93	21,856.99	80.35	40.00	9.07
5	100.00	95.84	92.45	83.15	76.48	82.56	89.90	94.85	97.56	95.15	22,099.04	76.48	40.00	8.06
Mean	**100.00**	**100.46**	**95.58**	**88.86**	**79.98**	**84.04**	**89.92**	**92.39**	**94.76**	**93.96**	**22,008.99**	**79.38**	**44.00**	**8.44**
± SD.	**0.00**	**3.22**	**4.23**	**4.65**	**2.70**	**2.11**	**3.19**	**1.93**	**1.74**	**1.76**	**162.21**	**1.82**	**8.00**	**0.68**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย



Appendix III

Individual Plasma Calcium Data (% of initial value) for sCT with chitosans at various concentrations

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix III_a

Individual Plasma Calcium Data (% of initial value) for sCT with CS J (pH 4.0) at various concentrations

0.25% CS J pH = 4.0

Subject No.	Time (mins)										AUC(0-240)min. [% . min]	Cmin [%]	Tmin (min)	%D
	0	10	20	30	40	60	90	120	180	240				
1	100.00	101.55	97.26	96.40	88.12	82.54	89.19	91.67	93.21	90.55	21,947.11	82.54	60.00	7.84
2	100.00	98.13	100.82	96.22	88.46	84.36	86.53	89.01	91.63	90.41	21,698.65	84.36	40.00	8.88
3	100.00	100.83	96.35	94.82	85.65	80.62	88.96	95.11	93.96	90.56	22,023.20	80.62	60.00	7.52
4	100.00	102.34	99.45	93.85	84.15	80.36	92.68	96.39	95.95	93.16	22,396.81	80.36	60.00	5.95
5	100.00	101.25	98.35	95.86	83.65	79.58	85.46	90.78	93.85	94.27	21,806.71	79.58	60.00	8.43
Mean	**100.00**	**100.82**	**98.45**	**95.43**	**86.01**	**81.49**	**88.56**	**92.59**	**93.72**	**91.79**	**21,974.50**	**81.49**	**56.00**	**7.72**
± SD.	**0.00**	**1.43**	**1.58**	**0.96**	**1.98**	**1.73**	**2.50**	**2.75**	**1.39**	**1.61**	**239.05**	**1.73**	**8.00**	**1.00**

0.50% CS J pH = 4.0

Subject No.	Time (mins)										AUC(0-240)min [% . min]	Cmin [%]	Tmin (min)	%D
	0	10	20	30	40	60	90	120	180	240				
1	100.00	98.34	95.12	86.52	79.20	84.06	88.21	92.63	94.66	94.72	21,924.71	84.06	60.00	7.93
2	100.00	102.96	100.73	96.47	87.16	80.71	85.62	91.88	95.15	93.62	22,047.71	80.71	40.00	7.42
3	100.00	100.50	97.13	88.16	77.51	82.62	86.26	94.95	92.75	90.12	21.715.28	82.62	60.00	8.81
4	100.00	104.96	101.46	94.19	81.16	86.62	82.18	90.55	90.45	91.89	21,613.08	82.18	90.00	9.24
5	100.00	100.15	98.16	84.49	80.12	83.65	83.17	95.15	91.46	93.45	21,688.73	83.17	90.00	8.93
Mean	**100.00**	**101.38**	**98.52**	**89.97**	**81.03**	**83.53**	**85.09**	**93.03**	**92.89**	**92.76**	**21,818.56**	**82.55**	**68.00**	**8.47**
± SD.	**0.00**	**2.32**	**2.33**	**4.59**	**3.29**	**1.93**	**2.17**	**1.78**	**1.80**	**1.60**	**175.25**	**1.11**	**19.39**	**0.68**

0.75% CS J pH = 4.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	105.44	99.26	97.29	85.08	79.49	82.41	88.16	93.50	94.25	21,631.66	79.49	60.00	9.17
2	100.00	102.00	102.26	99.56	82.12	87.62	84.72	86.65	91.19	93.15	21,647.72	80.82	40.00	9.10
3	100.00	98.45	94.70	85.78	77.06	82.45	87.62	91.34	95.86	92.69	21,777.16	77.06	40.00	8.55
4	100.00	104.86	100.05	95.85	84.12	72.11	84.76	91.49	95.78	95.68	22,060.04	72.11	60.00	7.37
5	100.00	98.46	95.86	90.63	74.14	83.16	83.71	91.67	93.45	91.45	21,542.06	74.14	40.00	9.54
Mean	**100.00**	**101.84**	**98.42**	**93.82**	**80.50**	**80.97**	**84.64**	**89.86**	**93.96**	**93.44**	**21,731.73**	**76.73**	**48.00**	**8.75**
± SD.	**0.00**	**3.00**	**2.78**	**4.98**	**4.22**	**5.14**	**1.72**	**2.06**	**1.74**	**1.43**	**180.50**	**3.24**	**9.80**	**0.76**

1.00% CS J pH = 4.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	102.33	92.15	83.84	74.44	86.72	93.82	93.14	92.36	91.72	21,866.95	74.44	40.00	8.18
2	100.00	98.22	87.30	80.65	71.16	82.96	86.79	92.69	90.83	92.71	21,309.22	71.16	40.00	10.52
3	100.00	104.91	94.42	84.84	78.72	73.81	82.53	93.52	91.13	90.81	21,244.08	73.81	60.00	10.79
4	100.00	100.34	89.47	78.26	72.56	80.76	89.75	95.73	93.46	95.15	21,750.21	72.56	40.00	8.67
5	100.00	101.66	90.15	87.58	75.36	81.73	83.63	90.62	88.76	93.15	21,174.57	75.36	40.00	11.08
Mean	**100.00**	**101.49**	**90.70**	**83.03**	**74.45**	**81.19**	**87.30**	**93.14**	**91.31**	**92.71**	**21,469.01**	**73.47**	**44.00**	**9.85**
± SD.	**0.00**	**2.21**	**2.42**	**3.26**	**2.59**	**4.21**	**4.12**	**1.64**	**1.58**	**1.47**	**282.93**	**1.47**	**8.00**	**1.19**

1.25% CS J pH = 4.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	96.22	92.54	85.40	76.91	83.41	87.21	94.04	91.13	93.88	21,712.58	76.91	60.00	8.83
2	100.00	96.87	98.49	87.20	75.16	83.16	79.68	82.50	90.42	90.09	20,862.77	75.16	40.00	12.39
3	100.00	102.79	98.45	92.85	74.26	82.02	80.64	90.79	93.46	92.10	21,580.21	74.26	40.00	9.38
4	100.00	100.03	97.46	94.90	76.10	85.75	82.16	95.45	91.02	94.46	21,863.89	76.10	60.00	8.19
5	100.00	100.76	97.76	89.22	75.78	81.06	85.02	83.69	89.19	89.16	20,983.32	75.78	40.00	11.89
Mean	**100.00**	**99.33**	**96.94**	**89.91**	**75.64**	**83.08**	**82.94**	**89.29**	**91.04**	**91.94**	**21,400.55**	**75.64**	**48.00**	**10.14**
± SD.	**0.00**	**2.46**	**2.24**	**3.51**	**0.89**	**1.58**	**2.80**	**5.30**	**1.39**	**2.06**	**401.90**	**0.89**	**9.80**	**1.69**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix III_b

Individual Plasma Calcium Data (% of initial value) for sCT with CS G (pH 6.0) at various concentrations

0.25% CS G pH = 6.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	100.23	98.41	96.82	89.76	85.22	92.74	98.62	94.94	95.76	22,730.97	85.22	60.00	5.43
2	100.00	96.79	94.79	90.60	84.46	88.79	93.76	94.89	95.76	95.82	22,510.78	83.46	40.00	6.35
3	100.00	103.69	100.14	97.47	91.86	89.16	87.86	89.75	95.75	93.63	22,348.38	87.86	90.00	7.03
4	100.00	98.45	97.68	95.47	89.05	83.94	91.49	95.49	95.15	96.49	22,495.18	82.24	60.00	6.42
5	100.00	101.86	102.49	98.75	92.85	85.42	89.83	94.46	94.76	95.62	22,559.04	85.42	60.00	6.15
Mean	**100.00**	**100.20**	**98.70**	**95.82**	**89.59**	**86.51**	**91.14**	**94.64**	**95.27**	**95.46**	**22,528.87**	**84.84**	**62.00**	**6.28**
± SD.	**0.00**	**2.43**	**2.56**	**2.82**	**2.91**	**2.08**	**2.10**	**2.85**	**0.41**	**0.96**	**123.12**	**1.91**	**16.00**	**0.52**

0.50% CS G pH = 6.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	97.88	95.69	91.72	82.57	89.51	93.45	96.85	94.33	95.66	22,520.82	82.57	40.00	6.31
2	100.00	100.46	96.17	89.35	84.32	88.59	95.49	92.19	90.85	94.19	22,129.43	84.32	40.00	7.94
3	100.00	102.49	97.46	91.20	83.41	91.60	95.05	94.19	94.83	92.15	22,496.35	83.41	40.00	6.41
4	100.00	98.19	100.79	95.15	81.25	89.49	93.23	95.69	92.73	93.45	22,366.72	81.25	40.00	6.95
5	100.00	104.48	97.15	92.48	86.45	84.16	91.92	95.75	95.23	93.58	22,428.64	84.16	60.00	6.69
Mean	**100.00**	**100.70**	**97.45**	**91.98**	**83.60**	**88.67**	**93.83**	**94.93**	**93.59**	**93.81**	**22,388.39**	**83.14**	**44.00**	**6.86**
± SD.	**0.00**	**2.52**	**1.79**	**1.89**	**1.75**	**2.46**	**1.30**	**1.61**	**1.61**	**1.14**	**140.24**	**1.13**	**8.00**	**0.58**

0.75% CS G pH = 6.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	96.58	95.66	92.14	82.56	84.75	93.72	91.67	93.23	94.71	22,197.69	82.56	40.00	7.65
2	100.00	100.90	97.49	95.75	80.81	83.75	90.15	92.97	95.49	94.96	22,212.97	80.81	40.00	7.59
3	100.00	103.46	101.49	96.90	84.79	82.48	89.45	95.49	94.81	90.19	22,226.78	82.48	60.00	7.53
4	100.00	99.76	96.66	88.85	79.93	89.48	92.78	93.65	96.46	96.73	22,476.64	79.93	40.00	6.49
5	100.00	101.23	95.88	90.81	83.65	90.49	88.46	93.33	92.50	95.45	22,163.15	83.65	40.00	7.80
Mean	**100.00**	**100.38**	**97.44**	**92.89**	**82.35**	**86.19**	**90.91**	**93.42**	**94.50**	**94.41**	**22,255.45**	**81.89**	**44.00**	**7.41**
± SD.	**0.00**	**2.25**	**2.13**	**3.01**	**1.78**	**3.20**	**2.01**	**1.23**	**1.45**	**2.22**	**112.61**	**1.33**	**8.00**	**0.47**

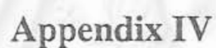
1.00% CS G pH = 6.0

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	104.98	101.53	95.46	84.46	81.45	85.55	89.26	92.49	94.16	21,779.86	81.45	60.00	9.39
2	100.00	102.64	98.15	92.79	80.51	84.57	92.21	93.82	94.98	92.07	22,206.94	80.51	40.00	7.61
3	100.00	98.19	89.49	84.92	78.08	83.96	87.47	91.62	95.32	96.48	22,102.10	78.08	40.00	8.05
4	100.00	100.63	96.26	87.96	80.35	87.65	94.45	92.41	93.45	91.93	21,856.99	80.35	40.00	9.07
5	100.00	95.84	92.45	83.15	76.48	82.56	89.90	94.85	97.56	95.15	22,099.04	76.48	40.00	8.06
Mean	**100.00**	**100.46**	**95.58**	**88.86**	**79.98**	**84.04**	**89.92**	**92.39**	**94.76**	**93.96**	**22,008.99**	**79.38**	**44.00**	**8.44**
± SD.	**0.00**	**3.22**	**4.23**	**4.65**	**2.70**	**2.11**	**3.19**	**1.93**	**1.74**	**1.76**	**162.21**	**1.82**	**8.00**	**0.68**

1.25% CS G pH = 6.0

Subject No.	Time (mins)										$AUC_{(0-240)min}$	C_{min}	T_{min}	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	102.46	97.86	88.90	79.03	86.78	89.98	91.76	94.55	92.88	22,014.68	79.03	40.00	8.41
2	100.00	97.00	92.23	87.92	80.24	85.57	91.47	94.89	94.76	92.61	22,092.36	80.24	40.00	8.09
3	100.00	102.57	98.32	95.83	84.51	81.66	88.34	91.66	93.45	93.01	21,948.48	81.66	60.00	8.69
4	100.00	98.04	94.23	86.63	80.31	88.95	93.47	90.12	94.36	93.50	22,043.22	80.31	40.00	8.30
5	100.00	101.42	99.75	93.95	82.45	89.30	92.43	92.76	93.01	92.33	22,218.20	82.45	40.00	7.57
Mean	**100.00**	**100.30**	**96.48**	**90.65**	**81.31**	**86.45**	**91.14**	**92.24**	**94.03**	**92.87**	**22,063.39**	**80.74**	**44.00**	**8.21**
± SD.	**0.00**	**2.33**	**2.80**	**3.59**	**1.94**	**2.76**	**1.81**	**1.57**	**0.68**	**0.39**	**90.32**	**1.19**	**8.00**	**0.38**

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย



Appendix IV

Individual Plasma Calcium Data (% of initial value) for sCT with 5% DM-β-CD and 5% HP-β-CD at pH 7.4

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix IV

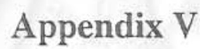
Individual Plasma Calcium Data (% of initial value) for sCT with 5% DM-*B*-CD and 5% HP-*B*-CD at pH 7.4

5% DM-*B*- CD pH = 7.4

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	100.14	94.85	85.75	76.29	83.02	87.87	90.90	93.92	94.28	21,717.15	76.29	40.00	10.07
2	100.00	106.17	101.56	96.83	81.15	79.43	83.78	88.78	94.55	93.90	21,747.22	79.43	60.00	9.94
3	100.00	98.16	96.76	86.77	79.22	85.75	90.20	95.81	91.82	91.62	21,923.97	79.22	40.00	9.21
4	100.00	101.22	95.25	86.16	77.12	81.52	89.49	91.45	94.49	94.23	21,855.81	77.12	40.00	9.49
5	100.00	100.81	98.19	89.59	75.49	87.56	94.20	92.48	90.15	90.15	21,807.91	75.49	40.00	9.69
Mean	**100.00**	**101.30**	**97.32**	**89.02**	**77.85**	**83.46**	**89.11**	**91.88**	**92.99**	**92.83**	**21,810.41**	**77.51**	**44.00**	**9.68**
± SD.	**0.00**	**2.65**	**2.43**	**4.13**	**2.06**	**2.91**	**3.38**	**2.30**	**1.73**	**1.66**	**74.37**	**1.57**	**8.00**	**0.31**

5% HP-*B-CD* pH = 7.4

Subject No.	Time (mins)										AUC(0-240)min	Cmin	Tmin	%D
	0	10	20	30	40	60	90	120	180	240	[% . min]	[%]	(min)	
1	100.00	98.22	94.04	89.26	83.40	85.18	89.24	92.82	94.30	93.19	22,324.14	83.40	40.00	7.55
2	100.00	103.50	100.91	96.16	85.69	83.78	89.86	90.96	96.87	94.60	22,003.53	83.78	60.00	8.88
3	100.00	102.59	97.43	93.49	81.49	87.62	90.26	93.15	94.29	92.62	22,183.18	81.49	40.00	8.14
4	100.00	101.89	98.18	95.36	84.26	89.16	93.16	90.43	93.53	93.45	22,226.35	84.26	40.00	7.96
5	100.00	100.34	96.45	90.30	81.22	86.79	91.95	92.83	95.97	94.26	22,281.29	81.22	40.00	7.73
Mean	**100.00**	**101.31**	**97.40**	**92.91**	**83.21**	**86.51**	**90.89**	**92.04**	**94.99**	**93.62**	**22,203.70**	**82.83**	**44.00**	**8.05**
± SD.	**0.00**	**1.86**	**2.24**	**2.72**	**1.68**	**1.87**	**1.45**	**1.11**	**1.23**	**0.72**	**110.93**	**1.24**	**8.00**	**0.46**



Appendix V

Individual Plasma sCT Concentration Data after Nasal Administration of sCT with or without Enhancers Compared with Intravenous Administration

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Appendix V

Individual plasma sCT concentration (pg/ml) of sCT with or without enhancers after intranasal and intravenous administration

sCT(in) + 1% CS J. pH = 4.0

Subject No.	Time (min)									$AUC_{(0-180)min}$	C_{max}	T_{max}
	0	5	10	15	30	40	60	120	180	[pg/ml].min	(pg/ml)	(min)
1	0.00	16.72	22.18	39.52	85.41	73.09	45.05	24.33	15.23	6,472.56	85.41	30.00
2	0.00	24.66	38.13	53.78	96.81	87.96	60.31	35.79	29.17	8,816.18	96.81	30.00
3	0.00	19.32	25.59	44.06	89.56	80.11	40.25	23.14	23.97	6,703.80	89.56	30.00
Mean	**0.00**	**20.23**	**28.63**	**45.79**	**90.59**	**80.39**	**48.54**	**27.75**	**22.79**	**7,330.85**	**90.59**	**30.00**
± SD.		**3.31**	**6.86**	**5.95**	**4.71**	**6.07**	**8.55**	**5.70**	**5.75**	**1,054.52**	**4.71**	**0.00**

sCT(in) + 5% DM-*B*-CD pH = 7.4

Subject No.	Time (min)									$AUC_{(0-180)min}$	C_{max}	T_{max}
	0	5	10	15	30	40	60	120	180	[pg/ml].min	(pg/ml)	(min)
1	0.00	19.65	26.40	30.13	74.68	51.90	30.60	16.06	18.26	4,978.95	74.68	30.00
2	0.00	15.12	22.34	28.76	69.94	48.03	34.18	21.24	15.32	5,170.80	69.94	30.00
3	0.00	31.08	38.98	45.16	80.85	60.23	45.34	30.40	20.10	6,956.58	80.85	30.00
Mean	**0.00**	**21.95**	**29.24**	**34.68**	**75.16**	**53.39**	**36.71**	**22.57**	**17.89**	**5,702.11**	**75.16**	**30.00**
± SD.		**6.72**	**7.08**	**7.43**	**4.47**	**5.09**	**6.28**	**5.93**	**1.97**	**890.49**	**4.47**	**0.00**

sCT(in) pH = 4.0

Subject No.	Time (min)									$AUC_{(0-180)min}$	C_{max}	T_{max}
	0	5	10	15	30	40	60	120	180	[pg/ml].min	(pg/ml)	(min)
1	0.00	0.00	12.42	15.61	21.51	36.68	21.92	14.82	18.46	3,357.08	36.68	40.00
2	0.00	0.00	18.96	24.67	35.16	43.42	27.08	21.05	15.45	4,242.00	43.42	40.00
3	0.00	0.00	14.35	17.48	23.29	32.50	19.63	15.20	20.34	3,332.58	32.50	40.00
Mean	**0.00**	**0.00**	**15.24**	**19.25**	**26.65**	**37.53**	**22.88**	**17.02**	**18.08**	**3643.88**	**37.53**	**40.00**
± SD.		**0.00**	**2.74**	**3.91**	**6.06**	**4.50**	**3.12**	**2.85**	**2.01**	**423.05**	**4.50**	**0.00**

sCT(iv) pH = 4.0

Subject No.	Time (min)									$AUC_{(0-180)min}$	C_{max}
	0	5	10	15	30	40	60	120	180	[pg/ml].min	(pg/ml)
1	65.93	46.16	35.21	27.09	24.43	20.36	23.65	18.23	25.22	4,249.85	65.93
2	84.75	59.44	47.52	36.73	32.20	27.18	25.12	20.75	17.08	4,686.25	84.75
3	78.73	56.31	43.74	28.34	25.56	22.54	19.47	24.91	20.59	4,528.89	78.73
Mean	**0.00**	**53.97**	**42.16**	**30.72**	**27.40**	**23.36**	**22.75**	**21.30**	**20.96**	**4,488.33**	**76.47**
± SD.		**5.67**	**5.15**	**4.28**	**3.43**	**2.84**	**2.40**	**2.75**	**3.33**	**180.45**	**7.85**

สถาบันวิทยบริการ
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