CHAPTER III

EXPERIMENTAL METHODOLOGY

The research project was divided into two major sections

I. Studies to determine the reversible effect of the enhancers on the nasal membrane integrity following their removal from the rat nasal cavity, using *in situ* nasal perfusion technique.

a) Evaluation of membrane permeability to [D-Arg^2]–Kyotorphin in the presence and after removal of the enhancers.

b) Evaluation of LDH activity released from the nasal mucosa in the presence and after removal of the enhancers.

II. Study to confirm the efficacy of the enhancers selected from I in improving the nasal absorption of sCT in healthy volunteers.

MATERIALS:

Chitosan J (CS J) = Chitosan free amine, Kyowa Technos Co., Ltd., Japan.

Chitosan G (CS G) = Chitosan glutamate salt (Seacure G 210+), Lot No. 206-409-03, Pronova Biopolymer, Drammen, Norway.

DL-Lauroylcarnitine chloride, Lot No. 38F5049, Sigma Chemicals Co., St. Louis, USA.

Hydroxypropyl-β-cyclodextrin (HP-β-CD), Lot No. 01925 JN, Aldrich Chemical Company, Inc, USA.

Dimethyl-β-cyclodextrin (DM-β-CD), Lot No. 87H0529, Sigma Chemicals Co., St. Louis, USA.

[D-Arg^2]–Kyotorphin =L-Tyr-D-Arg (acetate salt), Lot No. 45H1537, Sigma Chemicals Co., St. Louis, USA.

L-Phenylalanine (L-2-Amino-3-Phenylpropanoic acid), Lot No. 104H2605, Sigma Chemicals Co., St. Louis, USA.

Miacalcic® (synthetic salmon calcitonin), Lot No. 037 MFD0697, Sandoz Pharma Ltd, Basle,Switzerland .

Acetonitrile HPLC grade, Farmitalia Carlo Erba, Milan, Italy.

Methanol HPLC grade, Lot No. 3041KPDE, Labscan Co.Ltd, Bangkok, Thailand.

Lactate dehydrogenase enzymatic assay kit, Sigma Diagnostics, St. Louis, USA.

Sodium chloride AR grade, Lot No. 479687, Farmitalia Carlo Erba, Milan, Italy.

Sodium hydroxide AR grade, Lot No. 19294D036, BDH Laboratories Supplies, Poole, England.

Sodium acetate trihydrate AR grade, Lot No. 306TA404695, E. Merck, Darmstadt, Germany.

Calcium standard, Lot No. 0691/100, Farmitalia Carlo Erba, Milan, Italy.

EQUIPMENT:

Franz Diffusion Cells and Diffusion Apparatus, Crown Glass Industries, California, USA.

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

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

Analytical Balance, Sartorius 1615 MP, Range 300 gm / 1 mg, Gottingen, Germany.

Thermostatted Circulating Water Bath, Heto InterMed, Heto Birkerod, Denmark.

Vortex Mixer, Model K-550-GE, Scientific Industries Inc., New York, USA.

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

Peristaltic pump, Minipuls 2 Gilson, Villier'Le. Bel, France.

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

High Performance Liquid Chromatograph (HPLC)

- Column : μBondapak C18, 10 μm, stainless steel column, 300 × 3.9 mm, Waters, MA, USA.
- HPLC pump : LC – 3A (Shimadzu®, Japan) equipped with variable wavelength UV detector (Model SPD-10A) and computer software (AcerMate 486 Model 7035B).

Atomic Absorption Spectrophotometer (AAS), SpectrAA Model 300P.

Part I. Studies to determine the reversible effect of the enhancers on the nasal membrane integrity following their removal from the rat nasal cavity, using *in situ* nasal perfusion technique.

Investigational Technique : *In situ* Rat Nasal Perfusion.

The *in situ* nasal perfusion in rat model was used as a primary method of investigation due to its simple experimental setup and ease of sample handling. It was first developed by Hirai et al (1981) and successfully used by many other researchers to screen for drugs with potential nasal absorption. With slight modification, Tengamnuay and Mitra (1990a) have found this technique to be very useful in evaluating the effect of nasal absorption enhancers such as bile salt and its mixed micelles with various fatty acids. The following is the detailed description of the perfusion procedure.

Male Sprague-Dawley rats weighing 250-300 g were obtained from National Laboratory Animal Centre, Mahidol University, Nakorn Pathom. After anesthetization by intraperitoneal injection of 45 mg/kg sodium pentobarbital., an incision was made at the neck of the animal to expose the trachea. A 4 cm-polyethylene tube was inserted about 1.5 cm deep into the trachea toward the lung to maintain respiration during the experiment. The esophagus was also cannulated with another similar polyethylene tube which was inserted toward the posterior part of the nasal cavity. This cannula served to introduce the perfusion solution into the nasal cavity. The nasopalatine was sealed with an adhesive agent (Elephant Glue®) to prevent drainage of the drug solution from the nasal cavity into the mouth. A funnel was then placed between the nose and the reservoir. Respective diagrams of the surgery and the setup of the *in situ* perfusion experiment are illustrated in Figure 4 and Figure 5.

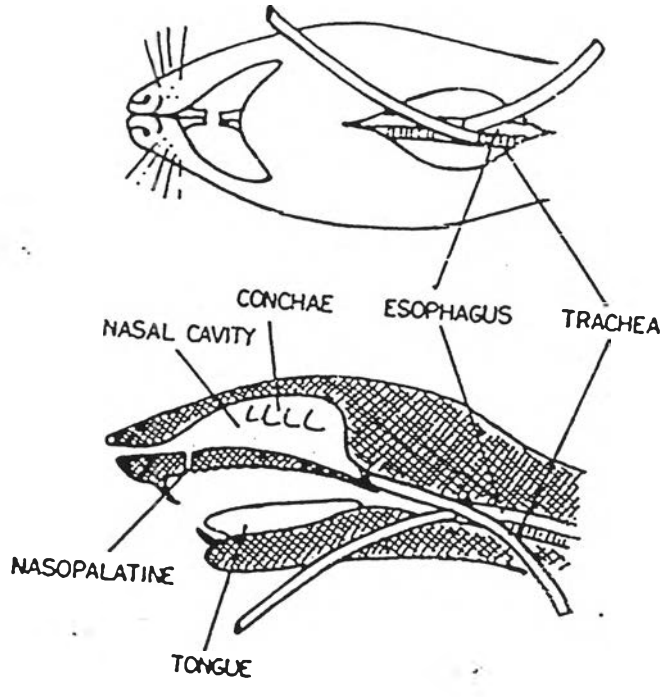


Figure 4 Diagram of the surgical procedure used in the *in situ* and *in vivo* nasal absorption studies.

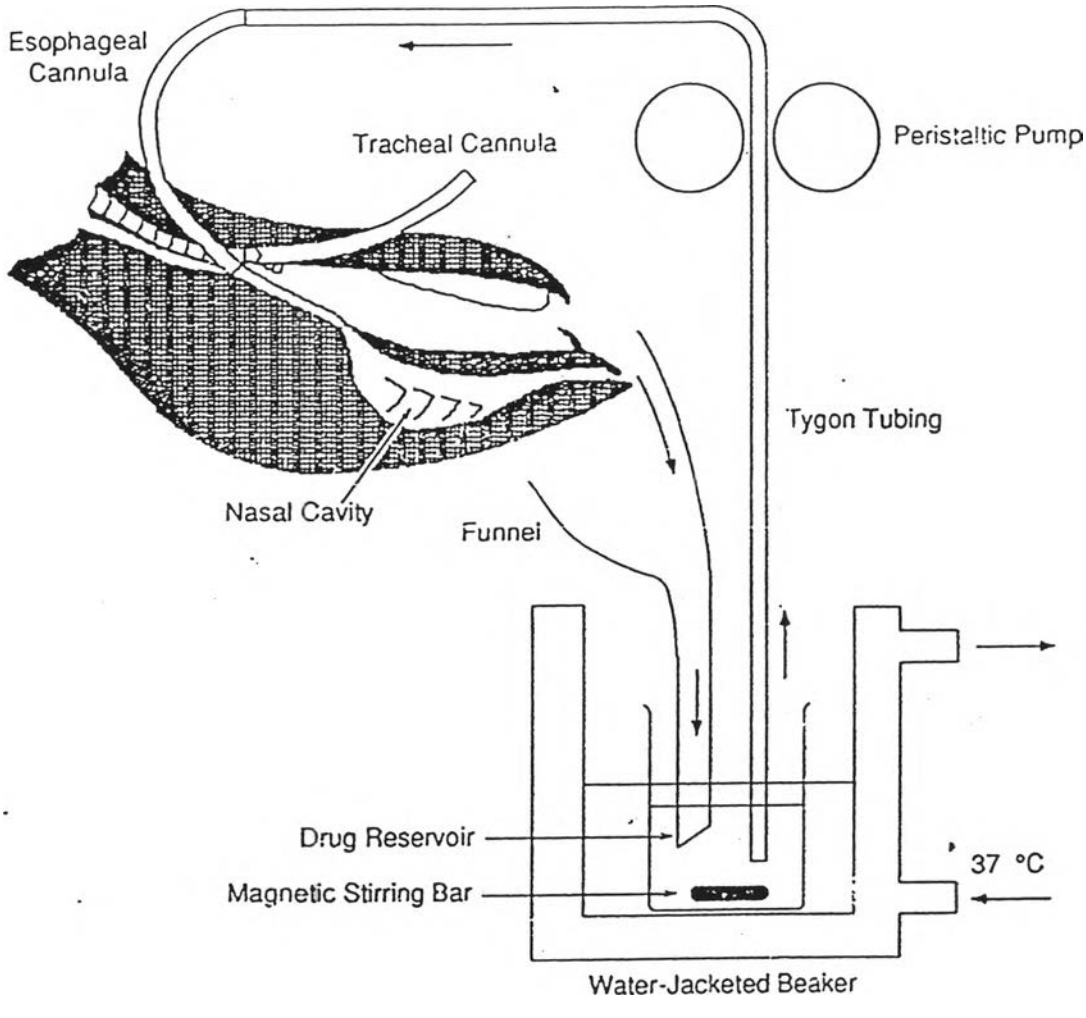


Figure 5 Schematic diagram of the *in situ* perfusion of the rat nasal cavity

Drug solution was placed in a reservoir beaker, which was water jacketed at 37±0.5°C via a circulating water bath. The solution was recirculated through the rat nasal cavity by means of a peristaltic pump. The rate of perfusion and the perfusate volume were set at 2.0 ml/min and 5.0 ml, respectively in all experiments. These are the values which gave optimal results during previous perfusion studies (Huang et al., 1985; Tengamnuay and Mitra, 1990a). The perfusate was constantly pumped from the reservoir through the larger, 20 cm-long polyethylene tubing the other end of which was connected to the esophageal cannula, flushed through the nasal cavity, and then returned to the reservoir via the nostrils. The concentration of the drug remaining in the perfusate was analyzed as a function of time to determine the extent of nasal absorption.

Preparation of Sample Solutions

L–Tyr–D–Arg ([D-Arg^2]–Kyotorphin) was selected as a model peptide in this study. It is an analog of L–Tyr–L–Arg or kyotorphin, a neural dipeptide which possesses opioid activity by stimulating the release of endogenous enkephalin. The reasons for choosing this dipeptide are due to its good stability in the nasal mucosa. It is not hydrolyzed by the nasal mucosal enzymes and at the same time is poorly absorbed from the perfusate (Tengamnuay and Mitra, 1990a). It thus serves as a suitable model dipeptide to study the effect of nasal absorption enhancers since the loss of the compound from the perfusate, would indicate the occurrence of nasal absorption.

One mM of [D–Arg^2]–kyotorphin stock solution was prepared by dissolving the dipeptide in isotonic saline (0.85 % NaCl) solution. The pH was adjusted to pH 6.0 by drop wise addition of either 1 N hydrochloric acid (HCl) or 1 N sodium

hydroxide solution (NaOH). Isotonicity was checked by osmometer (290–310 mosmole/Kg). If not yet isotonic, sodium chloride was then gradually added to solution until the isotonicity was obtained. The enhancer stock solutions were prepared at concentrations of 10.0 % for HP-β-CD, 0.2% for LCC and 2.5 % for DM-β-CD in isotonic saline solution prior to pH 6.0 adjustment.

For the two chitosans (CS J and CS G), stock solutions (0.2 and 1.0 % each) were prepared by dissolving each one in 0.5 N hydrochloric acid solution and allowing them to swell overnight and followed by pH and isotonic adjustment using 1 N NaOH and NaCl, respectively. The perfusing solutions were prepared by mixing each of the enhancer solutions with 1 mM [D-Arg^2]–kyotorphin solution at a 1:1 ratio. The final concentration of each enhancer in the perfusing solution was 0.1 and 0.5 % each for CS J and CS G, 5.0 % for HP-β-CD, 0.1% for LCC, and 1.25 % for DM-β-CD. Reasons for selecting these concentrations are given in Chapter IV.

Five ml of each enhancer with 0.5 mM [D-Arg^2]–kyotorphin solution was recirculated at 37°C. through the rat nasal cavity for 60 min at a flow rate of 2.0 ml/min. Aliquots (80 µl each) were taken at 15, 30, 45 and 60 min for subsequent analyses. At the end of perfusion, the nasal cavity was flushed for 10 min with isotonic saline pH 6.0 at the same flow rate to remove the enhancer. After that, the nasal mucosa was reperfused for further 60 min with 5.0 ml of only 0.5 mM [D-Arg^2]–kyotorphin in isotonic saline pH 6.0 (no enhancer) and aliquots were similarly taken at 15 min intervals.

The purpose of this part was to determine whether these enhancers could have a transient effect on the rat nasal mucosa permeability and integrity. Thus, aliquots of the perfusates were analyzed for a) the concentration of [D-Arg^2]–kyotorphin that

was absorbed during perfusion, and b) the content of lactate dehydrogenase (LDH) released from the nasal mucosa.

a) Evaluation of membrane permeability to [D-Arg^2]–Kyotorphin in the presence and after removal of the enhancers.

The concentration of [D-Arg^2]–Kyotorphin disappearing from the perfusate was used as a parameter indicating the extent of nasal absorption. The [D-Arg^2]–Kyotorphin solution without any enhancer showed very poor absorption from the nasal mucosa (Tengamnuay and Mitra, 1990a). However, increasing the nasal absorption of [D-Arg^2]–Kyotorphin by using an enhancer may cause the damage to the nasal mucosa. However, if an enhancer had only a transient effect, its removal from the nasal mucosa would be able to return the permeability close to its original state. Comparison of the extent of [D-Arg^2]–Kyotorphin absorption during the first hour of perfusion (in the presence of an enhancer) with that of the second hour (perfusion after removal the enhancer) would indicate the reversible effects of that particular enhancer.

Analytical Method

Drug concentration in the perfusate was quantitated by a reversed-phase high-performance liquid chromatography (HPLC) using a solvent delivery pump equipped with a variable wavelength UV detector set at 274 nm. Acetonitrile 1.0 % v/v in 0.01 M sodium acetate pH 4.0 was used as a mobile phase with a flow rate of 1.5 ml / min. Values of the peak areas or peak heights were obtained with an integrator. Twenty Five µl of the perfusate sample was mixed with 75 µl of the internal standard solution (5 mM of L-Phenylalanine in pH 6.0 isotonic saline), and injected to the

HPLC column (Injection volume was 25 µl). Standard curve was prepared by dissolving [D-Arg^2]–Kyotorphin in pH 6.0 isotonic saline at the concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mM. Each of the standard curves also contained respective type of the enhancer except for the group containing peptide alone without enhancer. Standard solutions were also mixed with the internal standard in a similar manner prior to HPLC injection. Each calibration curve was constructed by plotting the peak height ratios (or peak area ratios) of [D-Arg^2]–Kyotorphin to L-Phenylalanine versus the concentration of [D-Arg^2]–Kyotorphin and the straight line was obtained by linear regression.

The rate and extent of nasal absorption was determined from the semilogarithmic plots of percent [D-Arg^2]–Kyotorphin remaining in the perfusate versus time. The apparent first order absorption rate constants after nasal perfusion were calculated from the slopes of these plots whereas the percent [D-Arg^2]–Kyotorphin remaining at 60 min was used as a parameter indicating the extent of nasal absorption.

Statistical Analysis

Statistical evaluation of the data was made by Student's t-test, analysis of variance (ANOVA) and by multiple comparison of the means using Duncan's test at 5 % significance level where appropriate. The computation was performed using a statistical software package (SAS Inc.).

b) Evaluation of LDH activity released from the nasal mucosa in the presence and after removal of the enhancers.

The lactate dehydrogenase (LDH) is an intracellular enzyme which may have been released from the nasal epithelium due to changes in the membrane integrity caused by the enhancers. Therefore, any leakage of this enzyme into the nasal perfusates could be used as a specific biochemical indicator of the extent of membrane–irritating effect (Pujara et al., 1995). Furthermore, if an enhancer had only a transient effect, its removal from the nasal mucosa would be able to restore, in whole or in part, the membrane integrity to its original state. Comparison of the extent of LDH release during the first hour of perfusion (in the presence of the enhancer) with that of the second hour (perfusion after removal of the enhancer) would therefore provide some ideas as to the reversible effects of that particular enhancer.

Analytical method

Analyses were made immediately after completion of each perfusion experiment. The procedure was based on the spectrophotometric method of Wroblewski and LaDue (1955). The activity of LDH was measured by monitoring the rate at which the substrate, pyruvate, was reduced to lactate. The reduction was coupled with the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH), which was followed spectrophotometrically in terms of reduced absorbance at 340 nm.

$$\underset{(\text{High } A_{340})}{\text{Pyruvate} + \text{NADH}} \xleftrightarrow{\text{LDH}} \underset{(\text{Low } A_{340})}{\text{Lactate} + \text{NAD}}$$

Since NADH has a high absorbance at 340 nm compared to NAD, the reaction was measured in terms of the rate of decrease in absorbance at this wavelength.

Procedure

The LDH assay kit (Sigma Chemicals Co.) contained 20 preweighed vials of dried NADH (0.2 mg/vial), one bottle (100 ml) of 0.1 M potassium phosphate buffer, pH 7.5, and one bottle (100 ml) of 22.7 mM sodium pyruvate solution in 0.1 M phosphate buffer, pH 7.5.

1. Maintain cuvet compartment at 37°C.
2. Pipet directly into an NADH vial 2.85 ml phosphate buffer and 0.05 ml (50 μl) perfusate sample. Cap and mix well.
3. Leave the vial in a temperature-controlled water bath at 37°C for 20 min.
4. Add 0.1 ml sodium pyruvate solution. Cap and mix well by inversion and transfer to cuvet of 1-cm lightpath length.
5. Read and record absorbance (A) at 340 nm at 30-second intervals for 3 minutes vs water as reference.

Calculations

Select a period when the decrease in absorbance is linear with time. Calculate the ΔA per minute for this period.

$$\text{LDH activity in nasal perfusate (Units/ml)} = \frac{\Delta A \text{ per min} \times TCF}{0.001 \times 0.05 \times \text{Lightpath (cm)}}$$

Where : 0.001 = ΔA equivalent to 1 unit of LDH activity in a 3- ml volume With 1-cm lightpath at 37 °C

0.05 = Perfusate volume (ml) in cuvet

TCF = Temperature correction factor (0.51 at 37°C)

Temperature correction factions

If the temperature used in the enzyme reaction is 25°C, no temperature correction is needed. At other temperatures it is necessary to use a Temperature Correction Factor (TCF) in calculating the activity.

Cuvet Temperature (°C)	TCF	Cuvet Temperature (°C)	TCF
20	1.45	30	0.73
21	1.37	31	0.69
22	1.25	32	0.65
23	1.16	33	0.62
24	1.06	34	0.58
25	1.00	35	0.55
26	0.93	36	0.53
27	0.88	37	0.51
28	0.82	38	0.48
29	0.78	39	0.46

Thus, if a 1-cm lightpath is used, the above equation reduces to:

$$\text{LDH activity} = \Delta A \text{ per min} \times 20{,}000 \times \text{TCF}$$

Calibration

Values obtained by the procedure were reported in LDH units as described by Wroblewski and LaDue (1955), based on the absorptivity of NADH at 340 nm. The rate of decrease of the absolute absorbance was measured at this wavelength in a narrow-bandwidth spectrophotometer. The activity of LDH in the nasal perfusate was then expressed as units/ml/min, where 1 unit equals an absorbance decrease of 0.001 under the specified test conditions.

The maximum LDH activity that may be measured by this procedure is approximately 700 units/ml. Higher activity samples may be assayed by pre-dilution with phosphate buffer and multiply the results by the appropriate dilution factor.

The reaction can be set at any constant temperature between 20 - 39°C. If the reaction is run at 25°C, no correction factor is needed (TCF = 1). Otherwise, the result must be multiplied by an appropriate TCF value at a particular temperature.

Unit definitions

One unit of LDH activity will cause a decrease in A_{340} of 0.001 per minute at 25°C in a 3-ml reaction mixture in a cuvet of 1-cm lightpath. One international Unit (IU) of an enzyme is defined as that amount which will convert 1 μ.mol of substrate

per minute under the specified conditions of the procedure. The conventional units of LDH, as used in the described method, may be converted to IU by multiplying by 0.48 liter (Wroblewski and LaDue, 1955). For example, 100 LDH units per ml equals 48 IU per liter.

Part II. *In vivo* study of the efficacy of chitosan in improving nasal absorption of sCT in healthy volunteers.

Subjects

Eight healthy Thai male volunteers with the age ranging from 20 to 29 years, weight between 40 and 95 kilograms participated in this study. Prior to study, standard physical examination, and clinical laboratory test were carried out in all subjects. Subjects were excluded from the investigation if a history of drug abuse existed, if they were receiving chronic intranasal medication, or if any respiratory abnormalities, infections, or allergies were noted. They gave written informed consents before participating in the study and they were asked to take no medication, alcoholic preparations and cigarettes for at least one week preceeding the study and during the experimental period.

All subjects received each dose (300 I.U. sCT) in the morning after an overnight fast. No food or drink (other than water) was permitted until two hours after dosing.

Preparation of sample solutions

Two enhancers used in this part were CS G and DM-β-CD. Reasons for selecting these enhancers are given in chapter IV. The enhancer solutions were prepared at 1 % w/v for CS G and 2.5 % w/v for DM-β-CD in isotonic saline solution, with the pH adjusted to 6.0. The method of preparing the enhancer solutions was the same as in Parts I. The sample solution was prepared by mixing 200 µl of the enhancer solution with 175 µl of Miacalcic® (equivalent to 300 IU sCT). So the final sample solution (375 µl) contained 300 IU sCT in 0.53 % w/v CSG, and 300 IU sCT in 1.33 % w/v DM-β-CD and 300 IU sCT in isotonic saline (control group).

Experimental design

The study was conducted using a randomized block design in which the treatment sequence for the individual subject was completely randomized. Each subject received the drug according to the sequence shown in Table 2 with at least 1 week washout period between each administration.

Sample collection

Five ml of blood samples were collected from a forearm vein using a disposable syringe. They were immediately transferred to heparinized tubes. Blood samples were collected just before drug administration (time zero) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8 and 9 hr after dosing. The blood was immediately centrifuged at 2,500 rpm for 10 minutes and the plasma was separated and kept at -20°C until subsequent analysis.

Table 2 Treatment sequence for each subject in the randomized block design.

Subject No.	Order of Treatments		
	1st week	2nd week	3rd week
1	B	A	C
2	B	C	A
3	C	B	A
4	A	C	B
5	A	B	C
6	B	A	C
7	A	C	B
8	C	B	A

A = 300 IU sCT in isotonic saline

B = 300 IU sCT in 1.33%w/v DM-β-CD

C = 300 IU sCT in 0.53%w/v CS G

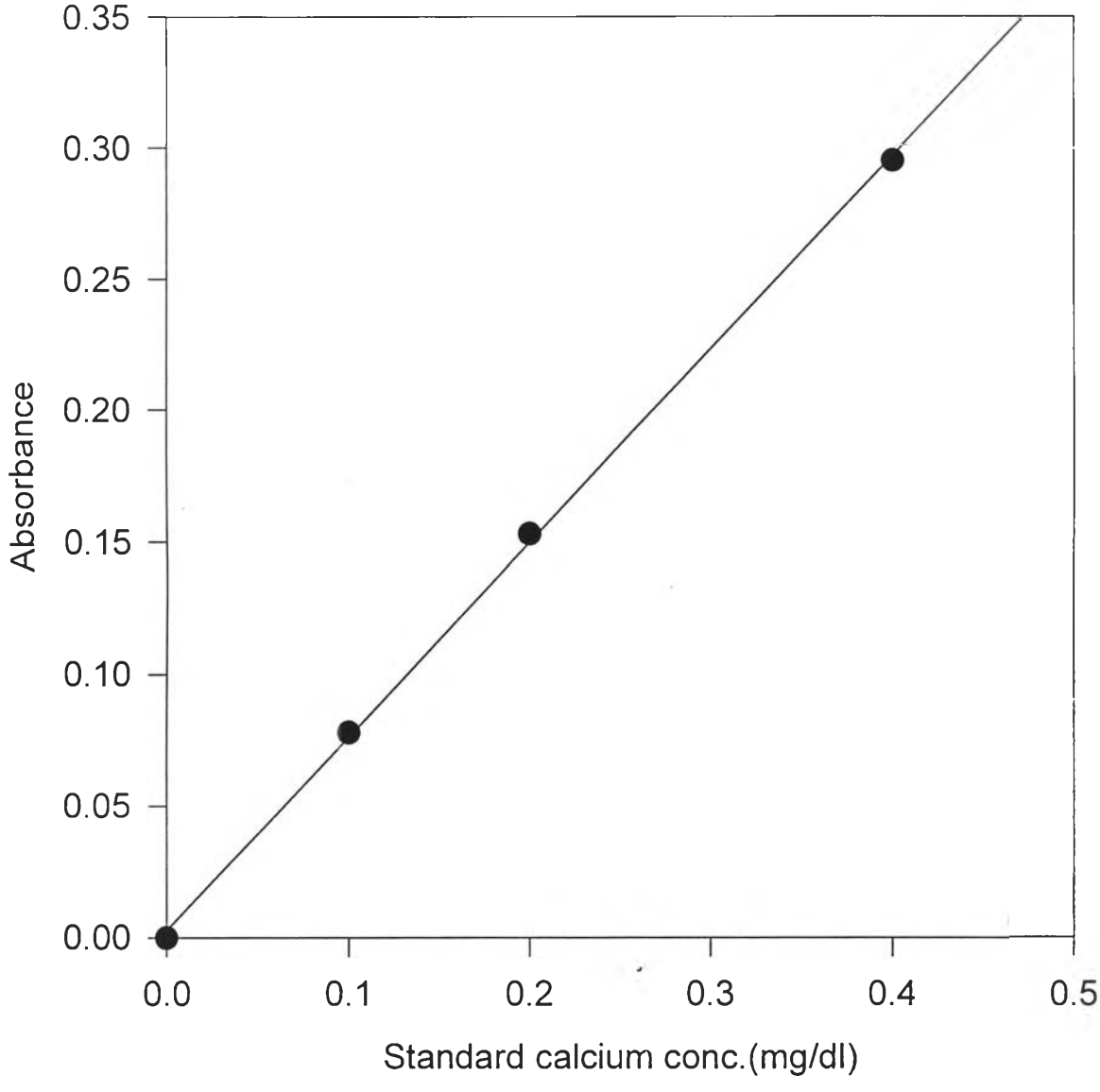
Analytical Method

The calcium contents in the plasma at various sampling points were measured by atomic absorption spectrophotometer (AAS) using the operating conditions as shown below in Table 3.

Table 3 Atomic Absorption Spectrophotometer Conditions

Element	**Calcium**
Wavelength (nm)	422.7
Slit width (μm)	0.5
Flame	Air-acetylene
Acetylene flow (L/min)	2.5
Air flow (L/min)	13.5

This procedure was based on the method of Zettner and Seligson (1964). One-Half percent w/v Lanthanum chloride (La_2Cl_3) in 0.1 mmol/l HCl was used as a carrier solution for removal of ion interference. Two hundred μl of each plasma sample was diluted with 5 ml of the carrier solution (26-fold diluted). Commercial calcium standard (Farmitalia Carlo Erba Ltd.) was used. Standard curve was prepared by dissolving calcium standard in the carrier solution at the concentration of 0.1, 0.2, 0.4 mg/dl. About 2 ml of the sample solution was injected into the nebuliser of an AAS (SpectrAA Model 300P). The calibration curve was constructed by plotting the absorbance of calcium versus the concentration of calcium standard and the straight line was obtained by linear regression (Figure 6).



$Y = 0.0028+0.7354x$ $r^2 = 0.9995$

Figure 6 Representative calibration curve of calcium content

The decrease in plasma calcium concentration versus time was used as a parameter indicating the extent of sCT nasal absorption. The percentage of calcium relative to the initial level at each time point after dosing was calculated and plotted against time. The area under this curve (AUC) during 0 to 9 hr was then determined for each subject. Increased sCT absorption was indicated by a decrease in blood calcium levels and thus a smaller AUC value.

Statistical Analysis

Statistical evaluation of the data was made by randomized block analysis of variance (ANOVA) and by subsequent multiple comparison of the means using Duncan's test at 5 % significance level where appropriate. The computation was performed using a statistical software package (SAS Inc.).