CHAPTER IV

RESULTS AND DISCUSSION

The results of the experiments are divided into two major sections but they are also interrelated. The data of the first part led to the development of the second part. Also, each part has its own results and discussions and subsequently, all the data will be summarized.

Part I: Studies to Determine the Optimum Enhancing Conditions for Chitosans

The purpose of this part of study was to investigate the optimum nasal absorption enhancing conditions for two types of chitosans, namely the free base (CS J) and the glutamate salt form (CS G).

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

From the preliminary study, the absorption spectra of the orthocresolphthalein complexone (OCPC) solution in the presence of 2.5 x 10^{-5} M calcium at pH 10.0 is shown in Figure 5 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 Morin (1974) and Mager et al. (1981) who reported the values of 740 and 525, respectively. As a result, the quantitative analysis of calcium study was performed by colorimetric measurements at 570 nm.

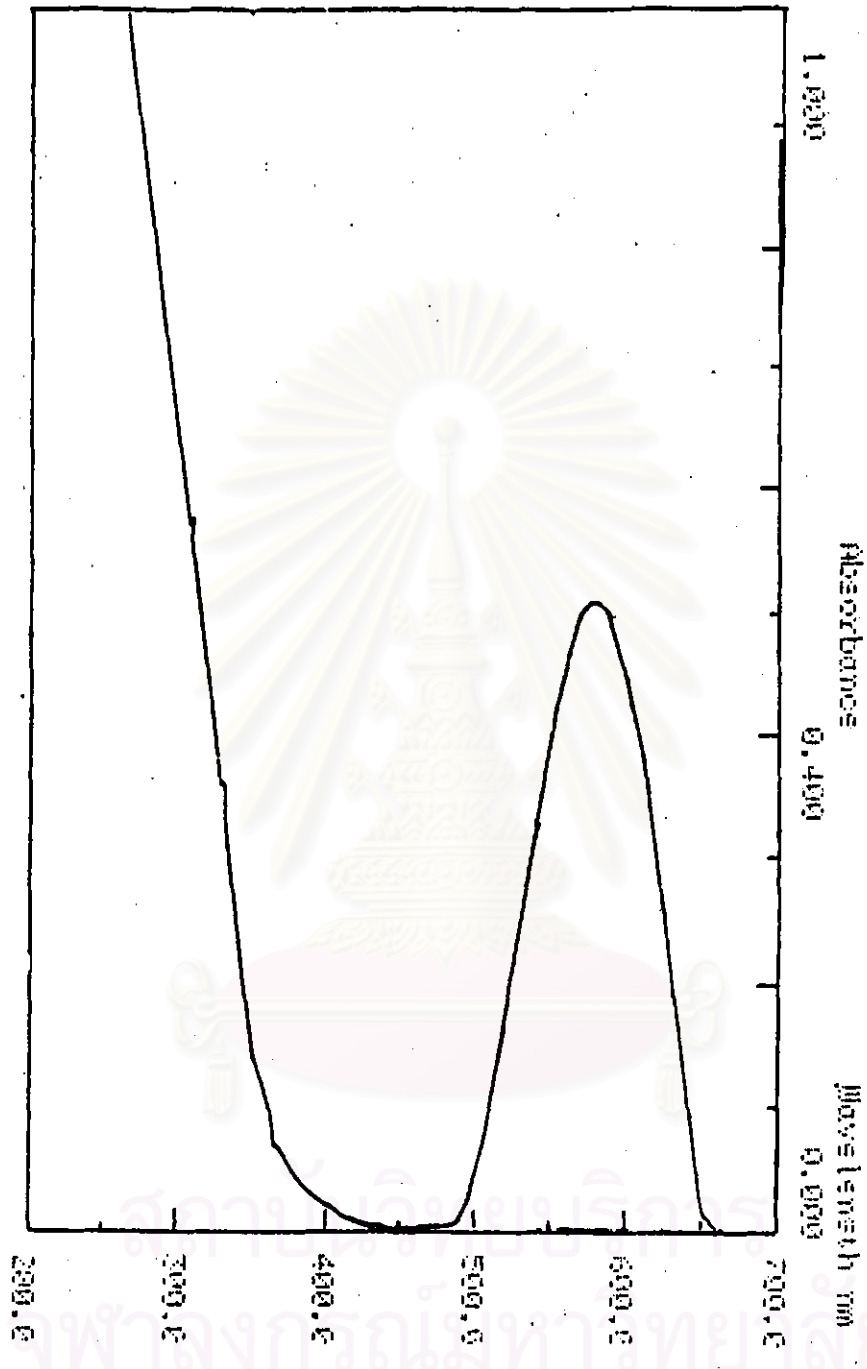


Figure 5 Absorption spectra of orthocresolphthalein complexone (OCPC) in the presence of calcium (2.5×10^{-5} M) at pH 10.0

Calibration curve of calcium assay.

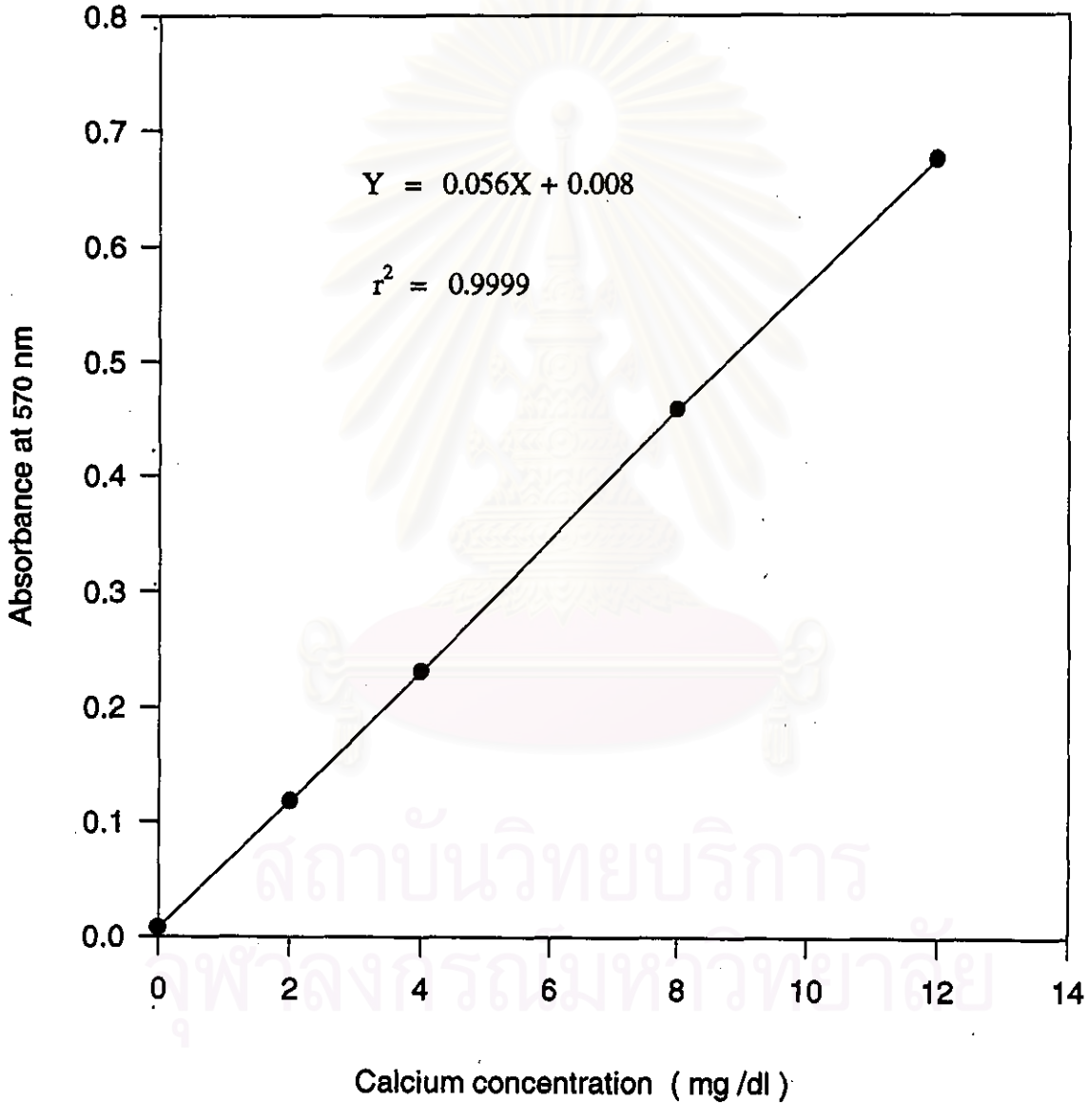
Figure 6 illustrates the representative calibration curve of calcium by making a plot of absorbance at 570 nm versus total calcium concentrations at pH 10.0. The standard solutions contained 0 to 150 mg of calcium per dl, representing 0 to 12 mg calcium per dl of blood serum under test conditions. These concentrations were used to construct calibration curves in all the experiments. The curves was linear but did not pass through the origin. The equation for this representative curve is expressed as $y = 0.056 x + 0.008$, 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.

In Vivo Nasal Absorption of Salmon Calcitonin with Chitosans

1.1 Determination of pH of optimum enhancing activities for various chitosans

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 CS J and CS G to dissolve in this pH range. Generally, chitosans can be dissolved in an acidic medium with a pH value not higher than 6.5 (Sanford et al., 1991). The pH of the rat nasal mucosa is reported to be 7.39 (Hirai et al., 1981) and the normal pH of the adult nasal secretions range approximately from 5.5 to 6.5

Figure 6 Representative calibration curve of orthocresolphthalein complexone (OCPC) color reagent containing with calcium at different concentration



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 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 CS J and G when the pH of their solutions was raised above 6.5, even with CS G, the "soluble salt" form of chitosan. 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 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 observe at about pH 3.3 (Lee et al., 1992). sCT encompasses several amino acids and functional groups that are known to cause degradation of peptides and proteins. For instance asparaginyl and/or aspartyl residue can undergo cyclization, deamidation and isomerization. The degradation reaction of sCT followed first-order kinetics and strongly dependent on pH in the pH range between 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.

However, the cyclodextrin derivatives (HP-β-CD and DM-β-CD) chosen as reference enhancers in this study were able to dissolve in physiological pH. Therefore, the absorption of sCT with the two cyclodextrins was studied at pH 7.4 due to its 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 % w/v of the two chitosans, were nasally administered by the methods as described in Chapter III. To prove that the nasally absorbed sCT was biologically active, measurements of the plasma calcium levels 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 buffers was to ensure that the decrease in the plasma calcium levels was due to the pharmacological effect of absorbed sCT and not due to the effects of the buffer itself, changes in the endogenous calcitonin level and other hormones, as well as the results of the surgical stresses and other experimental conditions. Preparation of 0.15 mM isotonic phosphate buffers (IPB) at various pH's was described in Appendix I.

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 (i) parathyroid hormone, (ii) calcitonin, and (iii) 1,25-dihydroxy-cholecalciferol. There three hormones regulate calcium by their actions on intestine, bone and kidney functions. (Robert et al., 1990). 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 minimum plasma calcium level (T_{min}). The value of %D was calculated for each rat from equation 6 as described in Chapter III and reflected the extent of hypocalcemic effect produced by the nasally absorbed sCT. The values of C_{min}, on the other hand, reflected the maximum hypocalcemic effect observed in each rat whereas T_{min} indicated the rate at which it occurred.

Tables 8 to 11 present the average plasma calicum level, expressed as percent of the initial level versus time during the 240 min 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_a to II_d. Summary of the average values of C_{min}, T_{min}, AUC and %D for the baseline (buffer only), the control (sCT alone), and the $sCT_{treated}$ (sCT plus chitosans) groups is provided in Table 12 and 13.

Figures 7-10 represent plots of the percent plasma calcium versus time for each of the control and $sCT_{treated}$ (sCT plus chitosans) 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 8, it can be seen that nasal administration of IPB pH 4.0 alone caused practically no changes in percent plasma calcium over the 240 min period. This indicated that neither the surgical procedure nor the buffer itself had an indirect effect on the plasma calcium during the experiment, resulting in a relatively stable baseline values of calcium level. At 240 min, the value was about 98.17 $\pm$ 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 8 except pH 3.0). It thus appeared that there was no buffer effect on the rat plasma calcium in this pH range (4.0 - 7.4) and that the experimental procedures (including the surgery) employed in this study 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 this peptide.

Results in Table 9 and Figure 7 revealed 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 $\pm$ 2.01 % and 66.00 $\pm$ 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 $\pm$ 1.14 % (Table 12). However, when 1 % w/v CS J 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 7). The percent decrease in plasma calcium (%D) was found to be 9.85 $\pm$ 1.19 %. The

Table 8 % Plasma calcium level (of initial value) of rats following nasal administration of 0.15 M isotonic phosphate 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

Table 9 % Plasma calcium level (of initial value) of rats following nasal administration of sCTalone (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

Table 10 % Plasma calcium level (of initial value) of rats following nasal administration of sCT (10 IU/Kg) with 1% CS J 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

Table 11 % Plasma calcium level (of initial value) of rats following nasal administration of sCT (10 IU/Kg) with 1% CS G at various pH

1% CS G	Time (min)										$AUC_{(0-240)min}$
	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

Table 12 Comparison of the total percent decrease in plasma calcium level following intranasal administration of baseline groups 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	pH 3.0	-	-	22,811.45 ± 194.71	-
		(baseline group)	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	pH 3.0	84.85 ± 2.11	38.00 ± 4.00	21,564.16 ± 208.32	5.45 ± 0.91
		(control group)	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 13 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)	$AUC_{0-240min}$ [% . 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 7 Percent of plasma calcium versus time after nasal administration of sCT with or without 1% w/v of chitosans and compared with basline group at pH 4.0. Each point represents mean ± S.D. (n = 5 rats/group)

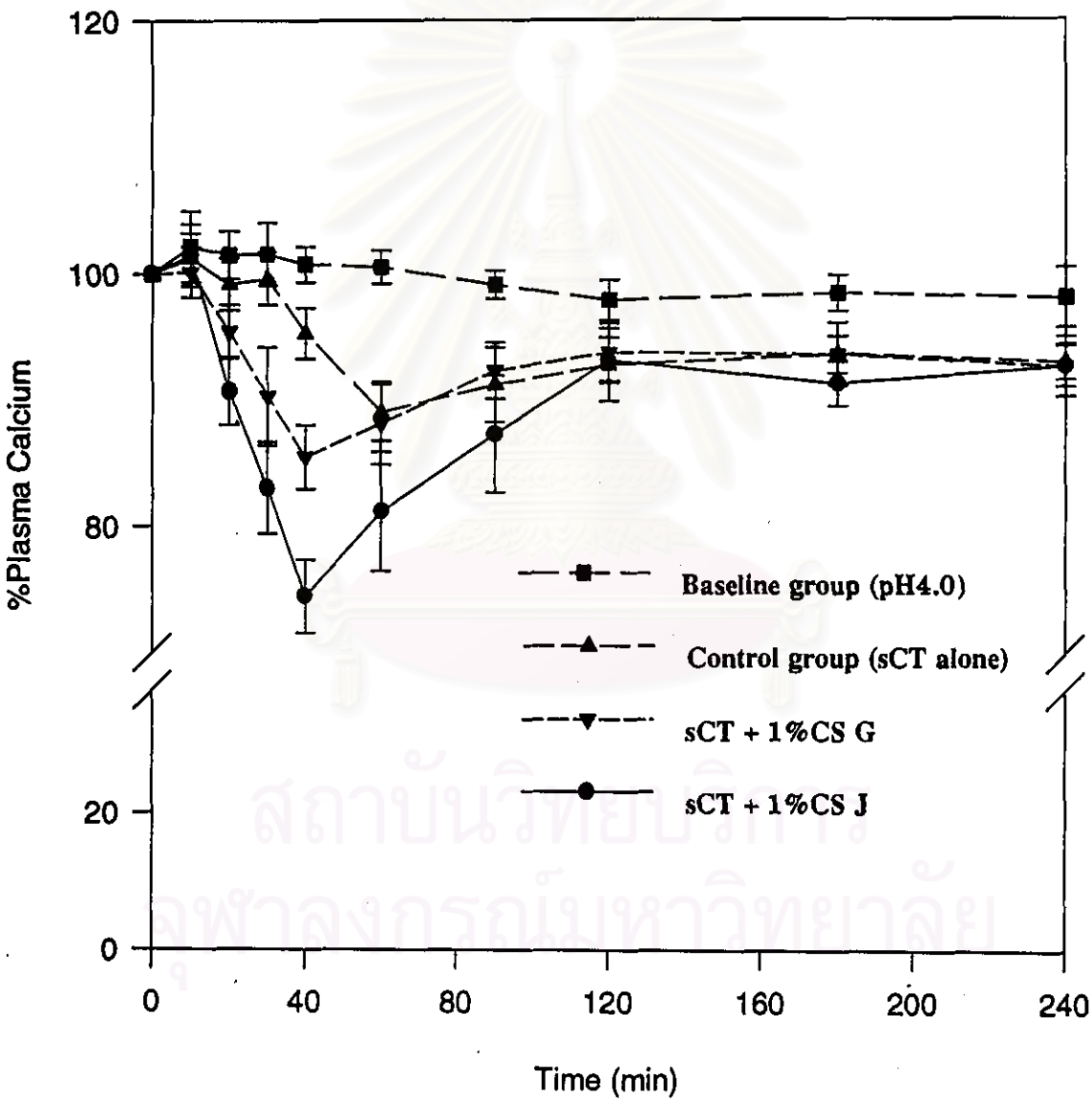


Figure 8 Percent of plasma calcium versus time after nasal administration of sCT with or without 1% w/v of chitosans and compared with baseline group at pH = 5.0. Each point represents mean ± S.D. (n = 5 rats/group)

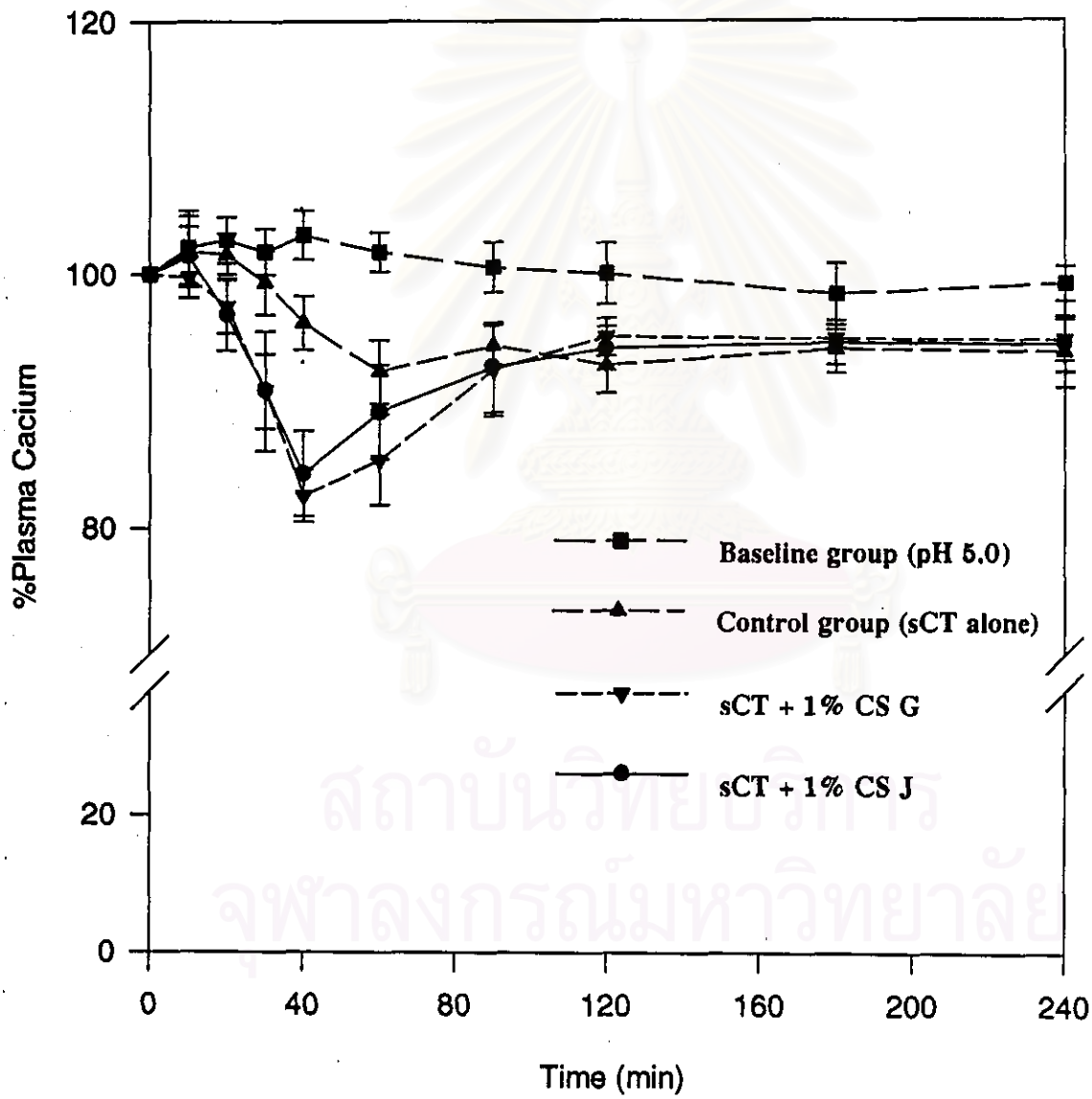


Figure 9 Percent of plasma calcium versus time after nasal administration of sCT with or without 1% w/v of chitosans and compared with baseline groupat pH = 6.0.

Each point represents mean ± S.D. (n = 5.0 rats/group)

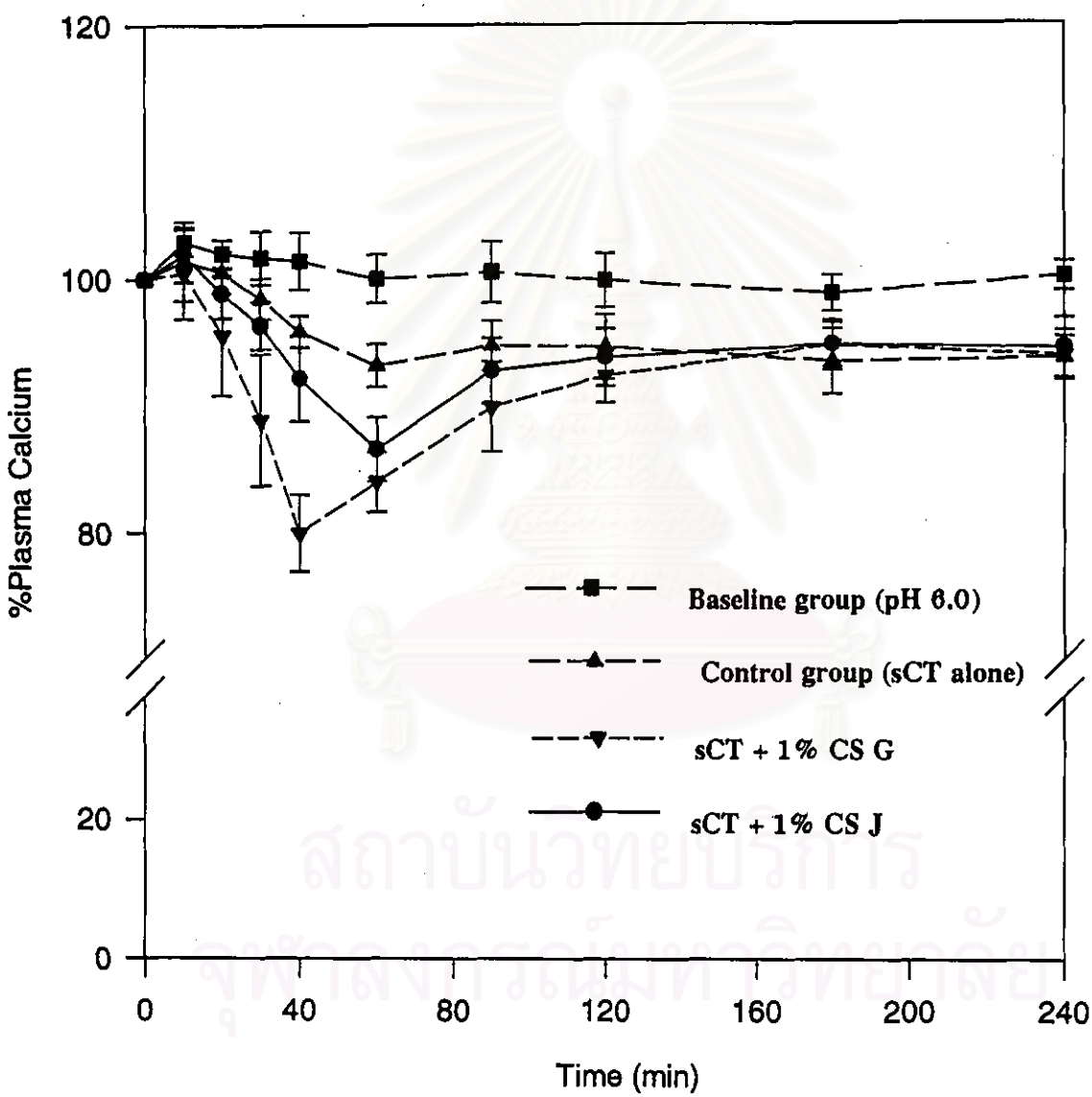
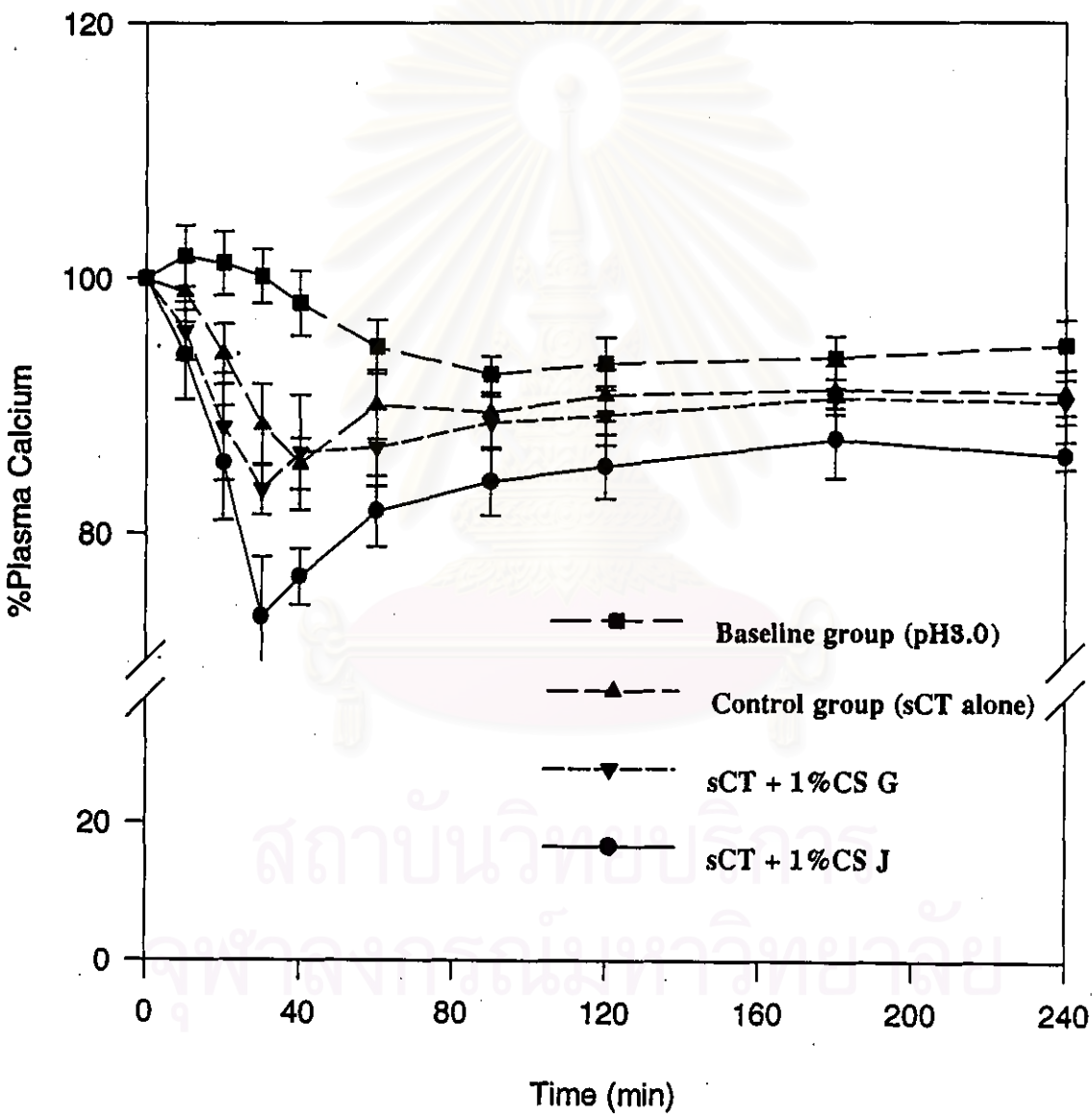


Figure 10 Percent of plasma calcium versus time after nasal administration of sCT with or without 1% w/v of chitosans and compared with baseline group at pH = 3.0.

Each point represents mean ± S.D. (n= 5 rats/group)



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 13). This was about 20 minutes faster than the control group. Addition of 1 % w/v CS G 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 CS G, as judged from the C_{min} value, was somewhat less than CS J (84.79 versus 73.47 %). However, the similar T_{min} values (44 min) indicated that both CS J and CS G enhanced 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 CS J, and sCT plus CS G, at 5 % significance level. The total percent decrease in plasma calcium (%D) was selected as the main parameter for statistical comparison since it 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 basal 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 was significant differences in %D among the three groups at pH 4.0 ($p < 0.05$) (Appendix VI_c). 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)	<	CS G	<	CS J
%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 group ($p < 0.05$). In addition, CS J was more effective than CS G 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 8 and Figure 8, 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 $\pm$ 1.24 %, indicating that there was no buffer effect nor fluctuation of the plasma calcium due to experimental stresses.

The nasal administration of sCT in this buffer (control group) resulted in the average C_{min} of only 92.04 $\pm$ 2.24 % which occurred very slowly at 120.00 $\pm$ 75.89 min (Table 12). The total percent decrease in plasma calcium was only 5.47 $\pm$ 1.27 %. However, when 1 % w/v of CS J or CS G 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 8). CS J rapidly caused maximum hypocalcemic effect at 44.00 $\pm$ 8.00 min with the average C_{min} value of 83.37 $\pm$ 2.27 %. The total percent decrease in plasma calcium (%D) was found to be 7.68 $\pm$ 1.02 %. CS G

also enhanced nasal absorption of sCT with similar rate and extent to that produced by CS J, 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 11). 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 (Appendix VI_d). 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)	<	CS G	<	CS J
%D	5.47		7.25		7.68 %

The line underneath the letters 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 CS J and CS G were still dissolved at this pH. As can be seen from the data in Table 8 and Figure 9, 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, thereby indicating the stability of the basal calcium level when no sCT was present. However, when sCT was nasally administered in this buffer, some absorption of the peptide was observed as demonstrated by a slow decrease in plasma calcium (Figure 9). The hypocalcemic effect

occurred very slowly, with average C_{min} of only 93.21 ± 1.51 % observed at 126.00 ± 48.00 min (Table 12). 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 CS J and CS G at this pH. CS J at 1 % w/v effectively increased 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 13). CS G appeared to be even more effective at this pH. The data in Table 11 revealed that 1 % w/v CS G induced rapid nasal absorption of sCT which resulted in a sharp drop of plasma calcium (Figure 9), 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 13). When the values of %D were analyzed by ANOVA at 5 % significance level, significant differences were found among the three groups ($p < 0.05$). Duncan's test was again applied to rank the enhancing effect with respect to %D (Appendix VI_e). The ranking results, in an increasing order, was

	Control (pH 6.0)	<	CS J	<	CS G
%D	5.16		7.19		8.44 %

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

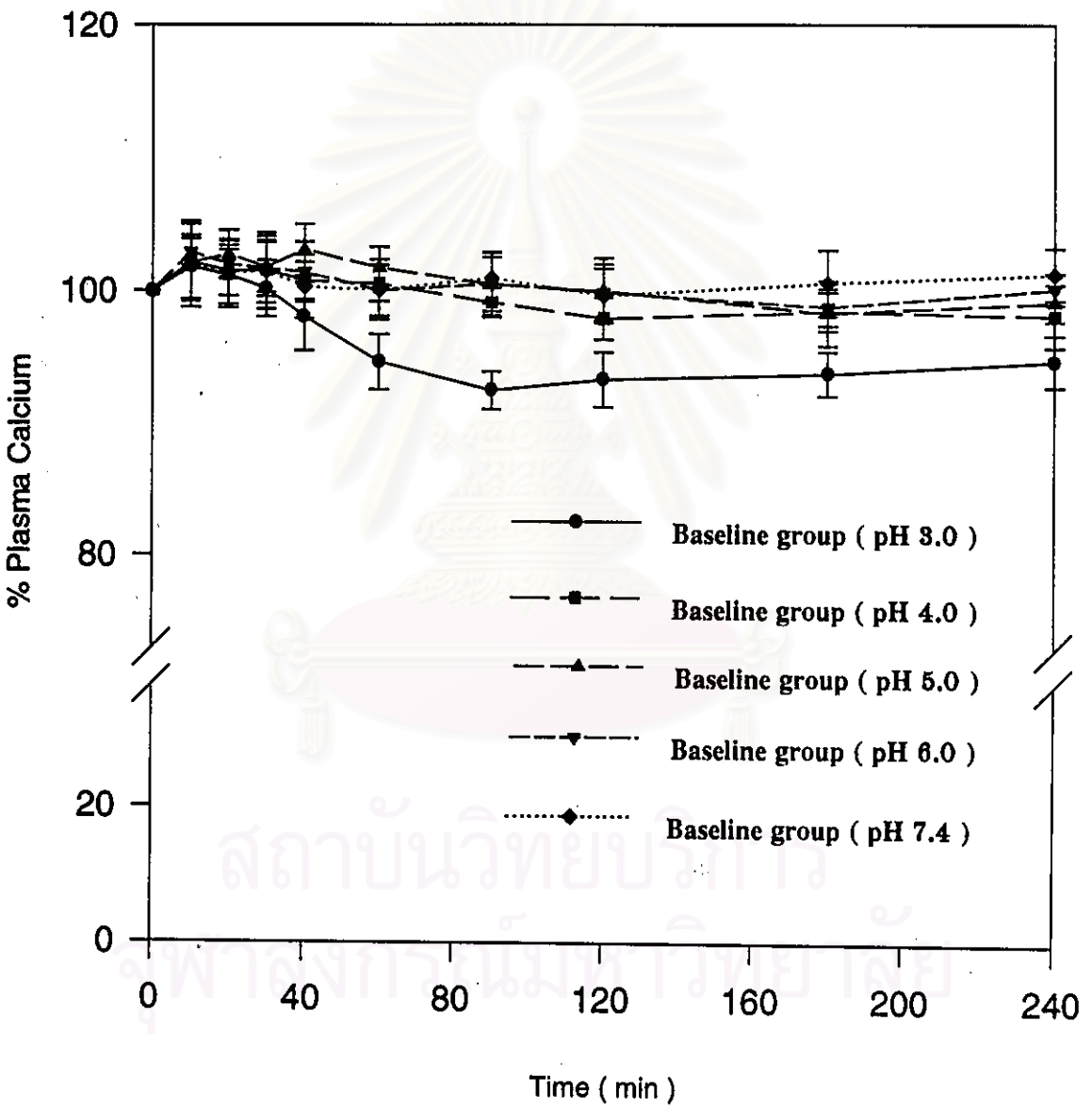
Because of the good stability of the sCT solution at acidic pH, its nasal absorption in the presence of chitosans was also studied at pH 3.0 for comparison. The results of these experiments are illustrated in Tables 8-11. It is interesting to note that nasal administration of IPB pH 3.0 alone did not result in a stable baseline calcium as opposed to other baseline groups (Table 8 and Figure 11). In fact, the plasma calcium appeared to decrease, although somewhat very slowly, reaching the minimum value of about 92.48 $\pm$ 1.28 % at 90 min and slightly increasing to 94.76 1.75 % at 240 min (Table 8). For comparison purpose, the AUC_{0-240} was used as a parameter to indicate the extent of plasma calcium fluctuation among the different baseline groups. ANOVA was then applied at 5 % significance level and the results 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 (Appendix $VIII_d$). 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)	22,811.4		23,814.38		24,037.27		24,049.35		24,148.43

The line underneath the letters indicated that the four baseline groups consisting of IPB pH 4.0, 5.0, 6.0, and 7.4 did not differ significantly in this parameter ($p > 0.05$) 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 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 as to the observation of the decrease in plasma

Figure 11 Comparison of percent plasma calcium following nasal administration of isotonic phosphate buffers (baseline groups) at differrent pH's

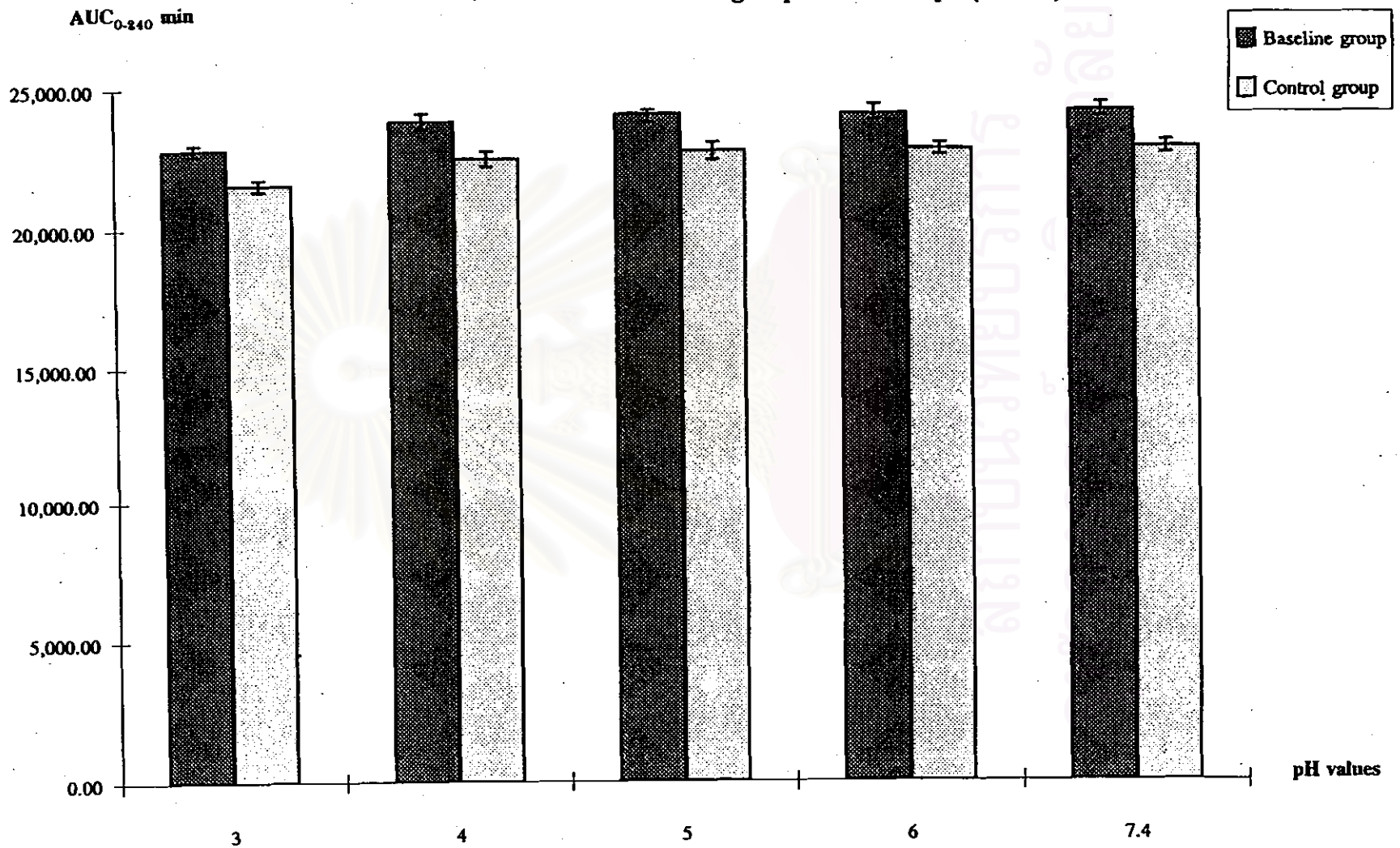
Each point represents mean ± S.D. (n = 5 rats/ group)



calcium after nasal administration of IPB at this pH were not known. It is possible that the acidity of this buffer may have caused some stress to the rats which resulted in changes in the levels of endogenous calcitonin or parathyroid hormone which play a key role in the regulation of calcium metabolism. Moreover, pH 3.0 may have induced the substantial absorption of phosphate ions from the buffer into systemic circulation. The increase of plasma phosphate ions might have influenced the calcium-phosphate mineral balance and resulted in the decrease 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 datas at pH 3.0 were excluded from the selection of optimum pH condition of each chitosans in order to minimize the confounding efffect from the buffer.

Although nasal administration of only IPB pH 3.0 did not result in stable baseline for plasma calcium, quantitation of the hypocalcemic extent exerted by nasal sCT was still possible by using the total percent decrease in plasma calcium (%D) as the main parameter since any possible buffer effect, if existed, had been accounted for in the calculation. For example, Figure 12 is a histogram comparing the $AUC_{0\text{-}240}$ 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 not always be the same, as was the case with pH 3.0, direct comparison of the AUC values among different

Figure 12 Comparison of AUC_{0-240} after nasal administration of baseline and control groups at various pH(4.0-7.4)



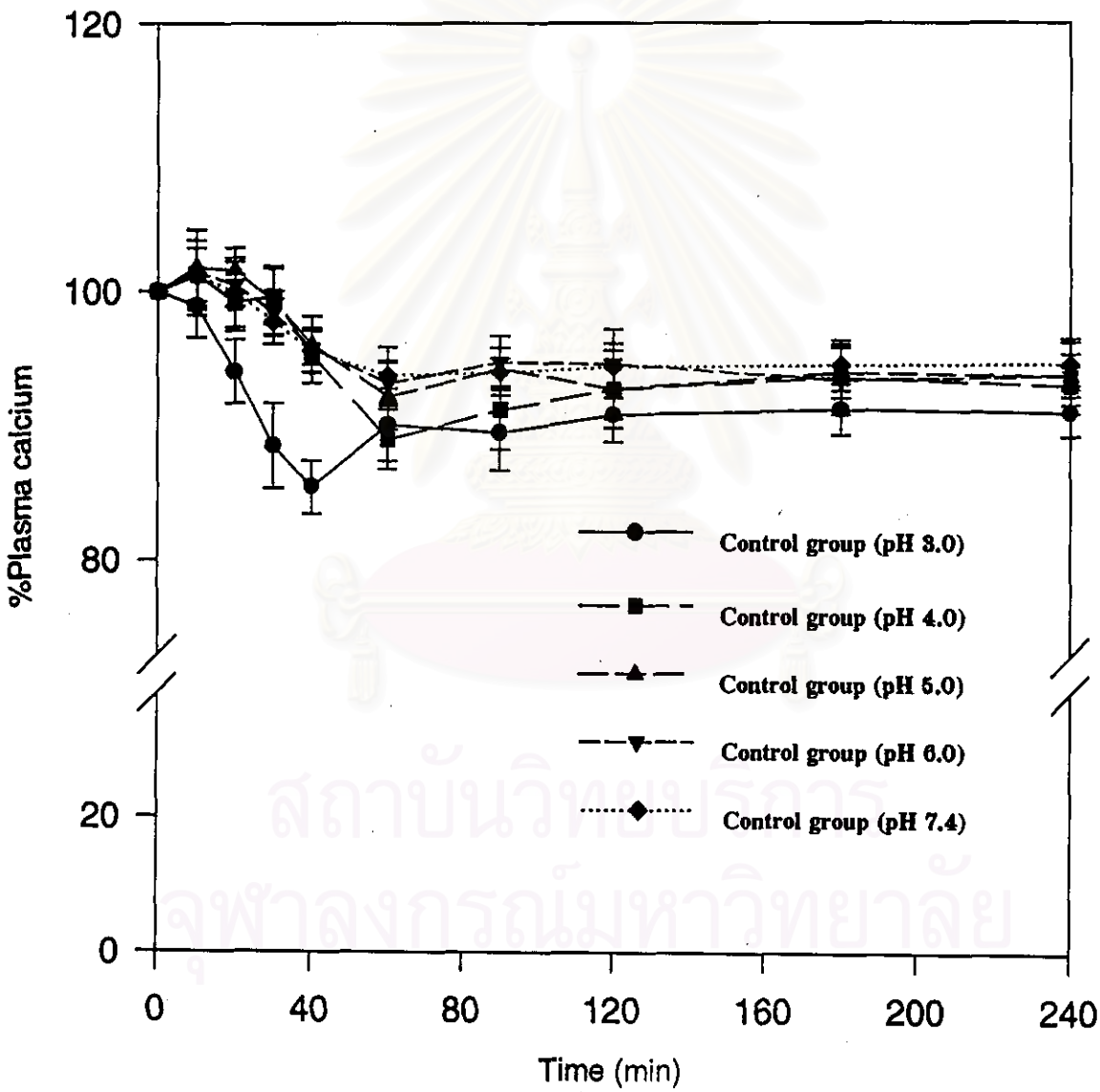
sCT-treated groups could be confounded by the different baseline values. Therefore, comparison based on the %D appeared to be more appropriate since the individual AUC value of the sCT-treated rat must be subtracted from the average AUC value of the corresponding baseline group in order to obtain the actual decrease in plasma calcium induced by sCT (equation 6). From Figure 13, the percent of plasma calcium of control groups at pH. 3.0 seem to be markedly absorbed than another pH When ANOVA was applied to analyzed %D at 5 % significance level , however, it is obvious that there did not significant difference ($p > 0.05$) in this parameter among the five groups (Appendix $VIII_a$)

As seen from Table 12, 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 $\pm$ 2.11 %, 38.00 $\pm$ 4.00 min, and 5.45 $\pm$ 0.91 %, respectively. As expected, inclusion of either 1 % w/v of CS J or CS G in sCT nasal formulations further increased its absorption (Figure 10 and Table 13). At pH 3.0 CS J induced the highest rate and extent of hypocalcemic effect, with the respective C_{min}, T_{min}, and %D of 72.66 $\pm$ 2.86 %, 32.40 $\pm$ 4.00 min, and 10.57 $\pm$ 1.69 % (Table 13). CS G also rapidly enhanced nasal sCT absorption at this pH, with the relatively similar T_{min} of 38.00 $\pm$ 11.66 min. However, the extent of hypocalcemic effect was somewhat smaller than CS J, with the average C_{min} and %D of 82.04 $\pm$ 1.63 % and 7.25 $\pm$ 0.17 %, respectively (Table 13).

When ANOVA was applied at 5 % level, significant difference was found in the values of %D among the three groups ($p < 0.05$). Duncan's test

Figure 13 Percent of plasma calium versus time after nasal administration of sCT without enhancers at various pH (4.0-7.4).

Each point represents mean ± S.D. (n = 5 rats/group)



was further applied and the ranking result (Appendix VI_a), in an increasing order was

	Control (pH 3.0)	<	CS G	<	CS J
%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 CS J was the most effective absorption enhancer of sCT ($p < 0.05$). CS G, although exerted the enhancing effect which was significantly smaller than CS J, 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 seemed to suggest that the adjuvant activity of CS J may be higher in a more acidic pH. On the other hand, the ranking results at pH 6.0 indicated that CS G tended to be more effective at higher pH value. The average %D of the three sCT-treated groups (control, CS J, and CS G) at various pH's are summarized graphically for overall comparison in Figure 14.

Data in Table 12 also indicated 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 ($p > 0.05$, Appendix IX). 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 7.4 to 84.8 % at pH 3.0. This indicated that, even in the absence of 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

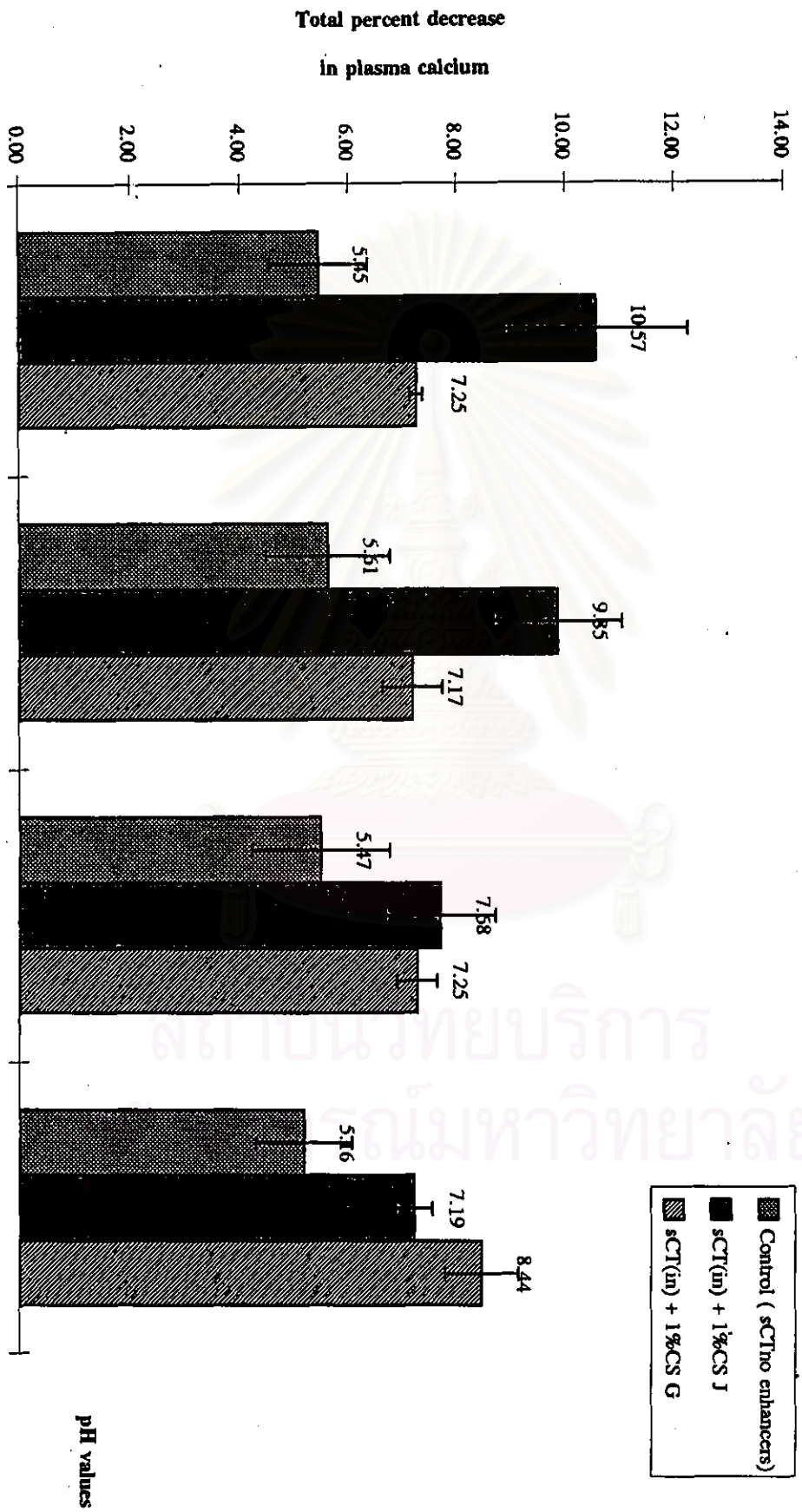


Figure 14 Comparison of the total percent decrease in plasma calcium (%D) after nasal administration of sCT with or without 1%w/v chitosans (CS J and CS G) at various pH

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 that of Sahamethapat (1997). Using the in situ perfusion of the rat nasal cavity, she 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. She attributed the absorption at pH 3.0 to be due 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, she found that IPB pH 3.0 induced the most pronounced leakage of these membrane components when compared to IPB pH 4.0, 5.0, 6.0, and 7.4 which produced only minimal release (Sahamethapat, 1997).

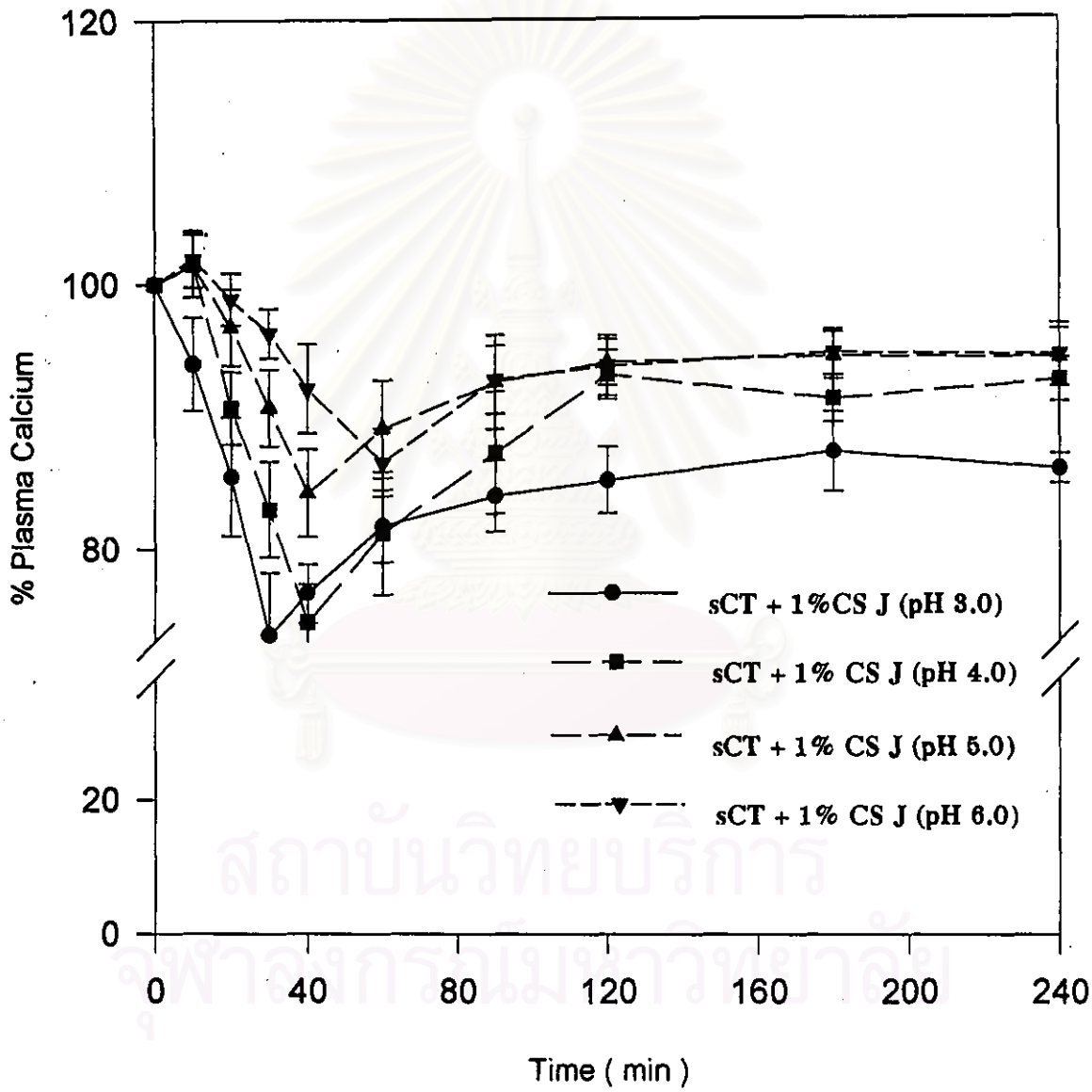
Similar to the results of Sahamethapat (1997), the more rapid nasal absorption of sCT observed with CS J and CS G at the most acidic pH in this study (pH 3.0) may not be due solely to the effect of the enhancers, but may be due to the deleterious effect of this buffer. Fluctuating plasma calcium baseline also reflected the extent of stress 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 since 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 15 shows the plots of percent plasma calcium versus time after nasal administration of sCT with 1 % w/v CS J in IPB of various pH's. From this Figure and the data in Table 13, it appears that the enhancing effect of CS J 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 $\pm$ 1.69, 9.85 $\pm$ 1.19, 7.68 $\pm$ 1.02, and 7.19 $\pm$ 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 CS J at the more acidic pH. As previously discussed, however, the high acidity of solution containing sCT and CS J 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 (Appendix VII_a)

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

Figure 15 Comparison of percent of plasma calcium of sCT with 1% CS J at various pH (4.0-6.0)

Each point represents mean ± S.D. (n = 5 rats/ group)

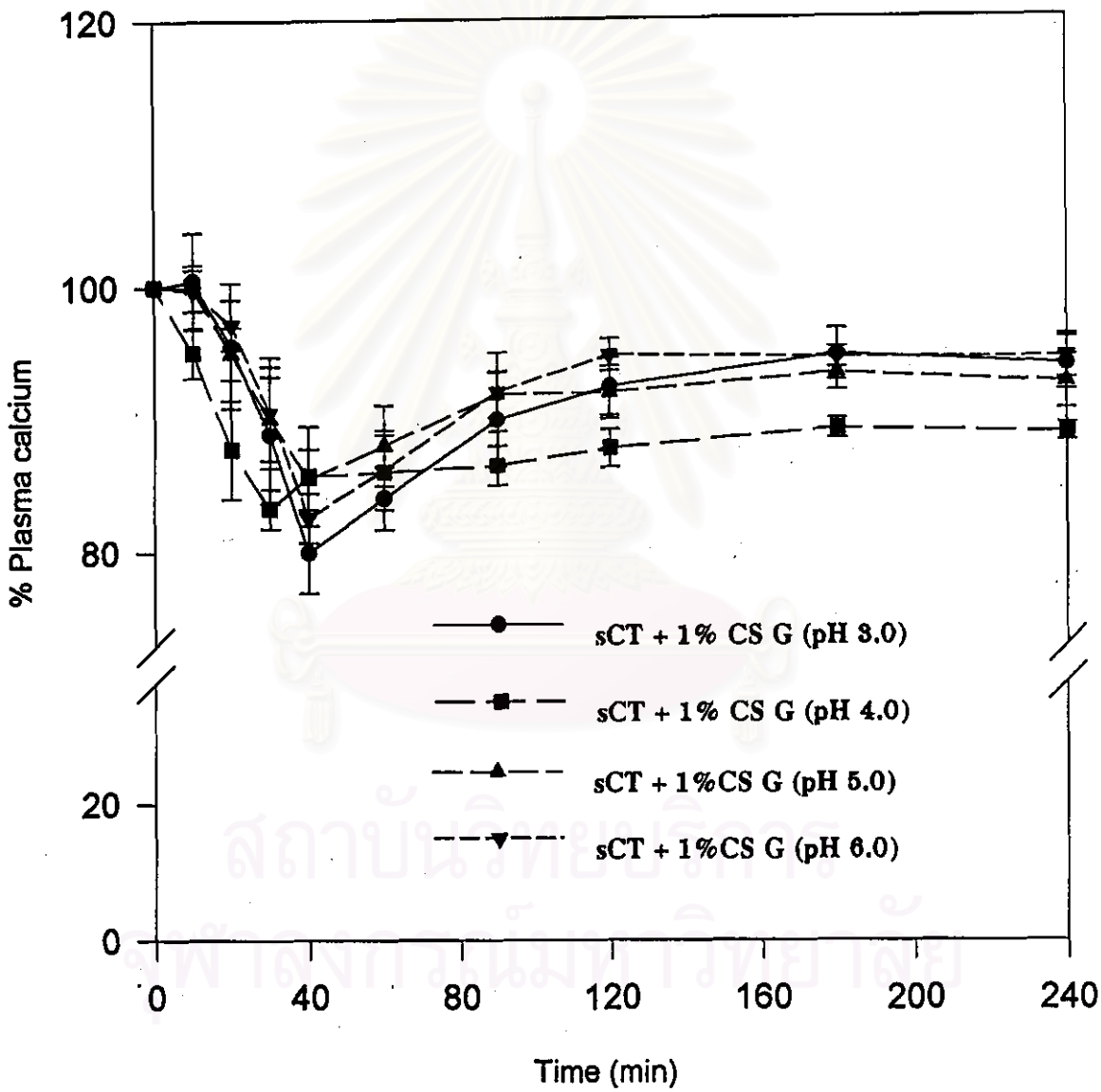


Duncan's test thus indicated that, at the same concentration, CS J was significantly more effective at pH 4.0 than at pH 5.0 and 6.0 ($p < 0.05$). On the other hand, the effect of CS J at pH 5.0 and 6.0 was comparable ($p > 0.05$). Thus, CS J appeared to be most effective at pH 4.0. The general tendency is that the free amine CS J showed increasing adjuvant activity when the pH of the solution was lowered and that its enhancing activity appeared to decrease at higher pH. 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 agreed well with that of Sahamethapat (1997) who reported that CS J was most effective in enhancing the nasal absorption of L-Tyr-D-Arg when the in situ nasal perfusion of this dipeptide with 0.5 % w/v CS J was conducted in IPB pH 4.0 in comparison with pH 5.0 and 6.0 ($p < 0.05$).

ANOVA was also applied to the values of %D comparing the enhancing effect of CS G at pH 4.0, 5.0, and 6.0. (The data at pH 3.0 was excluded by the same reasons). Figure 16 is the plots of percent plasma calcium versus time after nasal administration of sCT in the presence of 1 % w/v CS G at various pH's. It can be seen from this figure that the glutamate salt CS G tended to be more effective when the pH of the solution was

Figure 16 Percent of plasma calcium level after nasal administration of sCT with or without 1% CS G at various pH (4.0-6.0)

Each point represents mean ± S.D. (n = 5 rats/group)



increased as opposed to CS J. ANOVA results revealed 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 (Appendix VII_b)

$$\underline{\text{pH 4.0} < \text{pH 5.0}} < \text{pH 6.0}$$

%D	7.17	7.25	8.44 %

Thus, CS G 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 underline ($p > 0.05$). Sahamethapat (1997) reported that the effect of CS G in enhancing the nasal absorption of L-Tyr-D-Arg was independent of pH in the range of 4.0 to 6.0 ($p > 0.05$). However, the ranking was found to be the same as the result observed here, i.e, the absorption of the dipeptide in the presence of 0.5 % w/v CS G was higher at pH 6.0 and tended to decrease as the pH was lowered. It is interesting that the ranking order of CS G with respect to the pH effect was opposite to that of CS J. The reason for such difference could be partly explained by the difference in the chemical form of the two chitosans. CS J 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, CS G 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 CS G may be able to retain its absorption enhancing activity at a pH which

is less than acidic. It is possible that CS G 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 13). The true explanations as to the reverse order observed with CS G 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 CS G used in this study was different from the previously reported results. This lot of CS G was found to be unable to swell or dissolve in water as opposed to the results of Lehr et al (1992) despite coming from the same manufacturer. It is possible that lot-to-lot variation in the production of CS G may have contributed to the observed discrepancies. Apparently, more studies with the new lot of CS G are needed to clarify this observation. Summary in Figure 12 and 13 show comparison of percent total decrease in plasma calcium after nasal administration of sCT with or without 1 % w/v chitosans. The result show the different tendency of each chitosans to enhance absorption of sCT. For CS J is effective in acidic pH while CS G effective in more alkaline pH.

Based on the currently available data, the pH which gave the most enhancing activities of CS J and CS G appeared to be pH 4.0 and 6.0, respectively. For this reason, CS J at pH 4.0 and CS G at pH 6.0 were selected for the next studies.

1.2 Determination of concentration of optimum enhancing activities for various chitosans

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

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 canbe acheived by inclusion complex formation. The cyclodextrins complexation enables the development of formulations for drugs that are difficult to formulate and deliver with the existing pharmaceutical excipients, which is reflected in the increasing numbers of pharmaceutical products being placed on the market as cyclodextrins-based formulations (Irie et al.,1997). Of the cyclodextrins, DM-β-CD was found to be dramatically enhance the permeability of the larger molecular weight of

peptide and protein drugs whereas HP-β-CD had no statistically significant effect on the permeability of any of the peptide and protein drugs. However, it also cause extensive removal of membrane bound 5′ ND and a time dependent linear release of LDH. HP-β-CD in contrast showed only moderate protein absorption promotion activities and released minimal amounts of 5′ ND and LDH. For this reason , DM-β-CD was choosen to represent the potent enhancer and HP-β-CD was represent the mild or safety enhancer for this study.

The experiments were set similarly to the previous section (1.1), but the concentration of CS J and CS G were varied from 0.25% to 1.25% w/v. The sCT solution was administered at the dose 10 IU/Kg. The experiments of CS J were set at pH 4.0 and those of CS G were at pH 6.0. When concentrations of 0.25, 0.50, 0.75 1.00 and 1.25% chitosans were administered to rats in combination with sCT (10 IU/kg), a concentration-related absorption of sCT was found as expressed by the corresponding decreases in plasma calcium. (Table 13, and Figures 16, 17) Table 13 shows the percent decrement of plasma calcium (%D) of sCT using CS J as an enhancer at various concentrations at pH 4.0. The individual data are provided in Appendix VIIa. The values of % D are 7.72 ± 1.00 %, 8.47 ± 0.68 %, 8.75 ± 0.76 %, 9.85 ± 1.19 %, and 10.14 ± 1.69 % compared with the value of the control group (0 % CS J) at the same pH (5.61 ± 1.14 %). The lowest percent decrement of plasma calcium was obtained for concentrations of CS J of 1% w/v and higher. At 0.25% w/v, the enhancing effect of CS J was already significant when compared to the control group receiving only sCT alone at the same pH ($p < 0.05$, ANOVA test on %D, Appendix VIIa). ANOVA at 5 % significance level was

Table 14 % Plasma calcium level (of initial value) of rats following nasal administration of sCT (10 IU/Kg) with 1% CS J (pH 4.0) at various concentration

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

Table 15 % Plasma calcium level (of initial value) of rats following nasal administration of sCT (10 IU/Kg) with 1% CS G (pH 6.0) at various concentration

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

Table 16 Comparison of the total percent decrease in plasma calcium level following intranasal administration of sCT with or without chitosans at various concentrations

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)

Figure 17 **Percent of plasma calcium versus time after nasal administration of sCT with or without CS J (pH 4.0) at various concentrations in rats**

Each point represents mean ± S.D. (n = 5 rats/group)

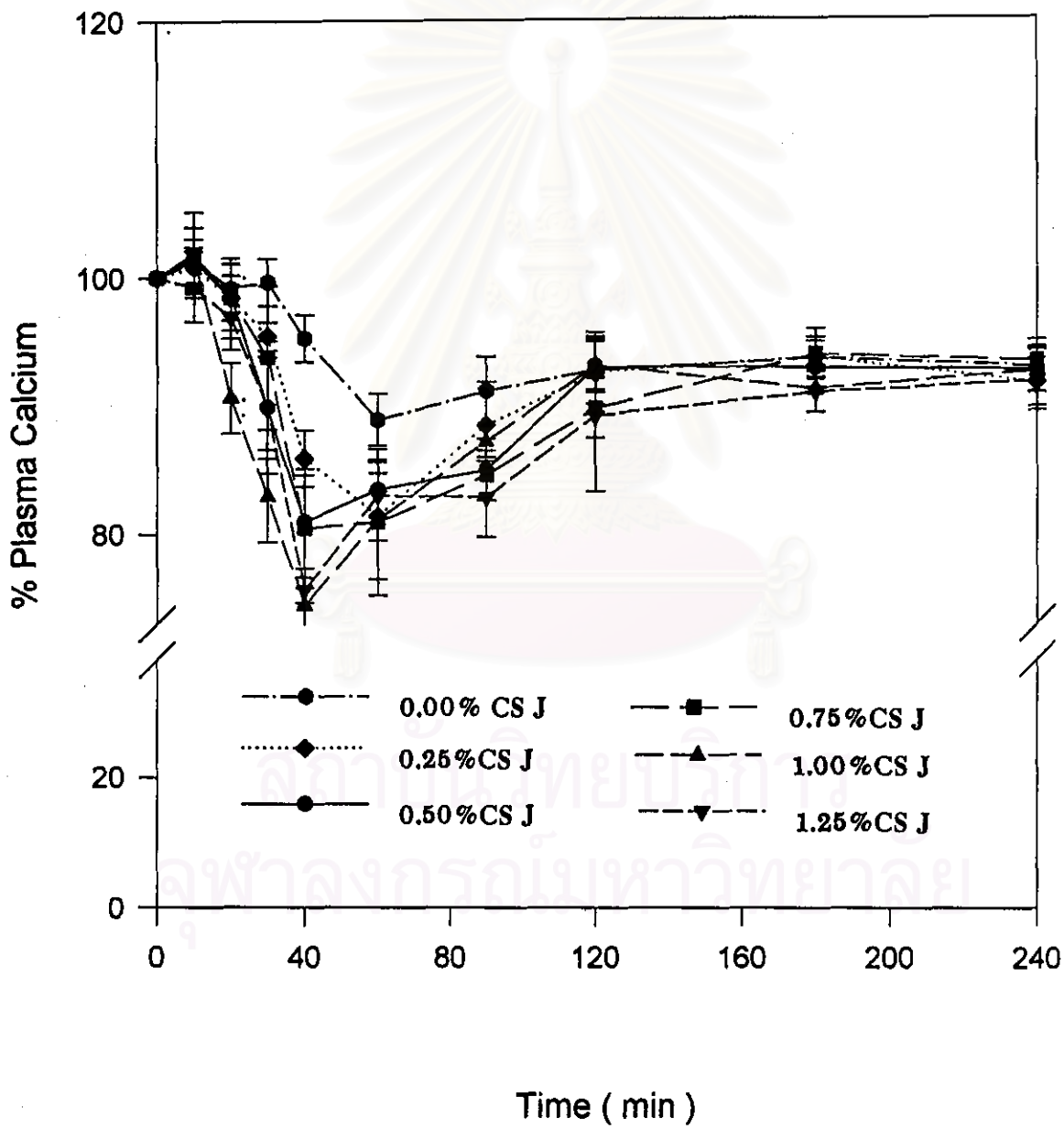
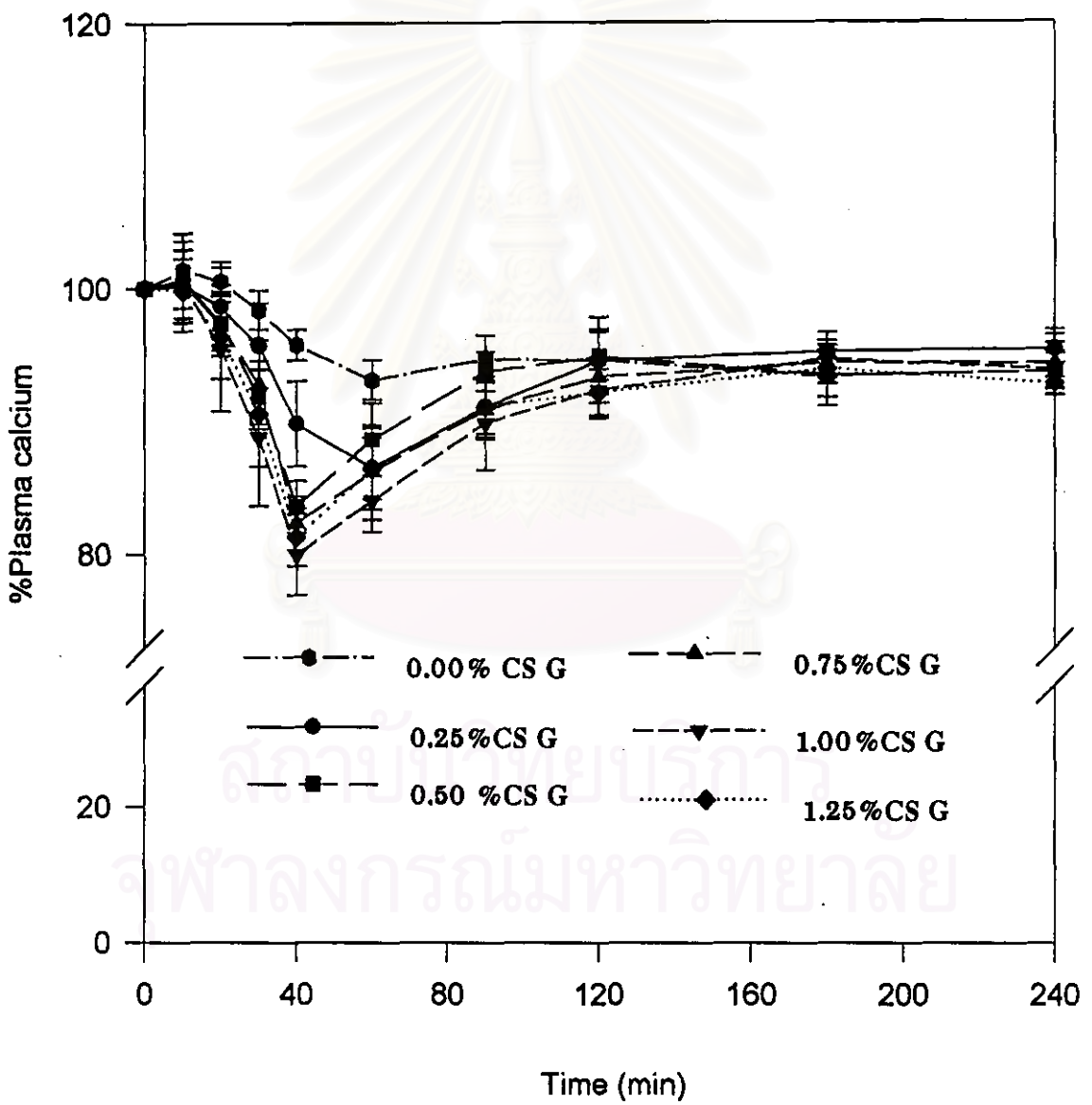


Figure 18 Percent of plasma calcium versus time after nasal administration of sCT with or without CS G (pH 6.0) at various concentrations in rats

Each point represents mean ± S.D. (n = 5 rats/group)



applied to classify which concentration of CS J was suitable for further studies. It is obvious that there was a significant difference in the percent decrement of plasma calcium among different concentration of CS J at pH 4.0 ($p < 0.05$). A Duncan's test was further applied to these data at the same significance level in order to rank this difference (Appendix IX_a). The ranking result, in an increasing order, was

CS J : control (pH4.0) < 0.25% < 0.50%< 0.75% < 1.00%< 1.25% w/v

% D		5.61	7.72	8.47	8.75	9.85	10.14 %

The lines underneath the letters signify that there were no significant differences between chitosans on each line ($p > 0.05$) Duncan's test results indicate that the above six groups can be roughly divided into three groups. At 1.00 and 1.25% w/v, CS J appeared to be the most effective ($p < 0.05$) whereas the control group was least effective. On the other hand, CS J at 0.25 to 0.75% w/v appeared to have intermediate effect at pH 4.0. Figure 16 is the plots of percent of plasma calcium versus time for different concentrations of CS J. Data in Table 15 and Figure 16 indicated that the enhancing activity of CS J was concentration-dependent. As the concentration was increased from 0.25 % to 1.25 % w/v, the values of % D increased from 7.72 ± 1.00 % to 10.14 ± 1.69 %. It is also interesting to note that CS J was already effective even at a concentration as low as 0.25 % when compared with the control group ($p < 0.05$).

Similar results were obtained with CS G after nasal administration at its optimal pH. Data in Table 15 and Figure 17 revealed that the

enhancing activity of CS G at pH 6.0 was also concentration-dependent. The mean ± SD of %D, as shown in Table 15, are 5.16 ± 0.89 %, 6.28 ± 0.52 %, 6.86 ± 0.58 %, 7.41 ± 0.47 %, 8.44 ± 0.68 %, and 8.21 ± 0.38 % when the concentration of CS G was 0, 0.25, 0.50, 0.75, 1.00 and 1.25 % w/v, respectively. The enhancing effect was noticeable even at 0.25 % w/v, the lowest concentration studied when compared with the control group. One-way analysis of variance (ANOVA) was then applied to the data at 5 % significance level. It is obvious that there was a significant difference in the percent total decrease in plasma calcium among the various concentrations of CS G ($p < 0.05$, Appendix VIIb). A Duncan's 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

CS G : control (pH6.0) < 0.25% < 0.50%< 0.75% < 1.25%< 1.00% w/v

% D	5.16	6.28	6.86	7.41	8.21	8.44 %

The lines underneath the letters signify that there were no significant differences between chitosans on each line ($p > 0.05$). However, Duncan's test considered the problem when the study is to compare several treatments. The results indicated that the above six groups can be roughly divided into sevseral groups. At 1.00% w/v, CS G appeared to be the most effective ($p < 0.05$) whereas the control group was least effective. Roughly screening, CS G at 0.25 to 0.75% w/v appeared to have intermediate effect at pH 6.0. In addition, CS G at 1.25% w/v seemed to produce less hypocalcemic effect at than at 1% concentration. This implied that the effect CS G appeared to be saturable, reaching maximum enhancement at concentration about 1% w/v. Duncan

test results with CS J also implied the same trend, i.e. pre-enhancer concentration at 1.25% was not superior to concentration at 1% w/v ($p < 0.05$).

Thus, the enhancing effect of both CS J and CS G was concentration-dependent with the maximum activity observed at 1.0 % w/v. Selection of the optimum concentration of these chitosans for the next studies was subsequently based on comparison of their enhancing activity with that of 5% w/v DM-β-CD and HP-β-CD. The sCT nasal absorption using DM-β-CD and HP-β-CD as adjuvants was conducted at physiological pH (pH 7.4). The data of both cyclodextrins and their baseline group are given in Table 1 and 15. Their plasma calcium profiles are also illustrated in Figure 18 which includes the profile of both cyclodextrins at 5% w/v in IPB pH 7.4 in comparison with the control and the baseline groups. As expected, baseline of plasma calcium after nasal administration with only IPB pH 7.4 was relatively unchanged from the initial value.Addition of 5% w/v DM-β-CD or HP-β-CD resulted in significant absorption ($p < 0.05$, ANOVA test on %D) when compared with the control group (Appendix IX). The average value of %D for DM-β-CD was 9.68 ± 0.31 % and 8.05 ± 0.46 %for HP-β-CD (Table 15). Consequently, comparative study was made among these adjuvants, i.e. CS J, CS G, DM-β-CD and HP-β-CD. Figure 19 is the overall histogram showing the average percent decrement of plasma calcium after administration of sCT with various concentrations of CS J and CS G in comparison with 5% w/v DM-β-CD and 5% w/v HP-β-CD at their respective optimal pH conditions.

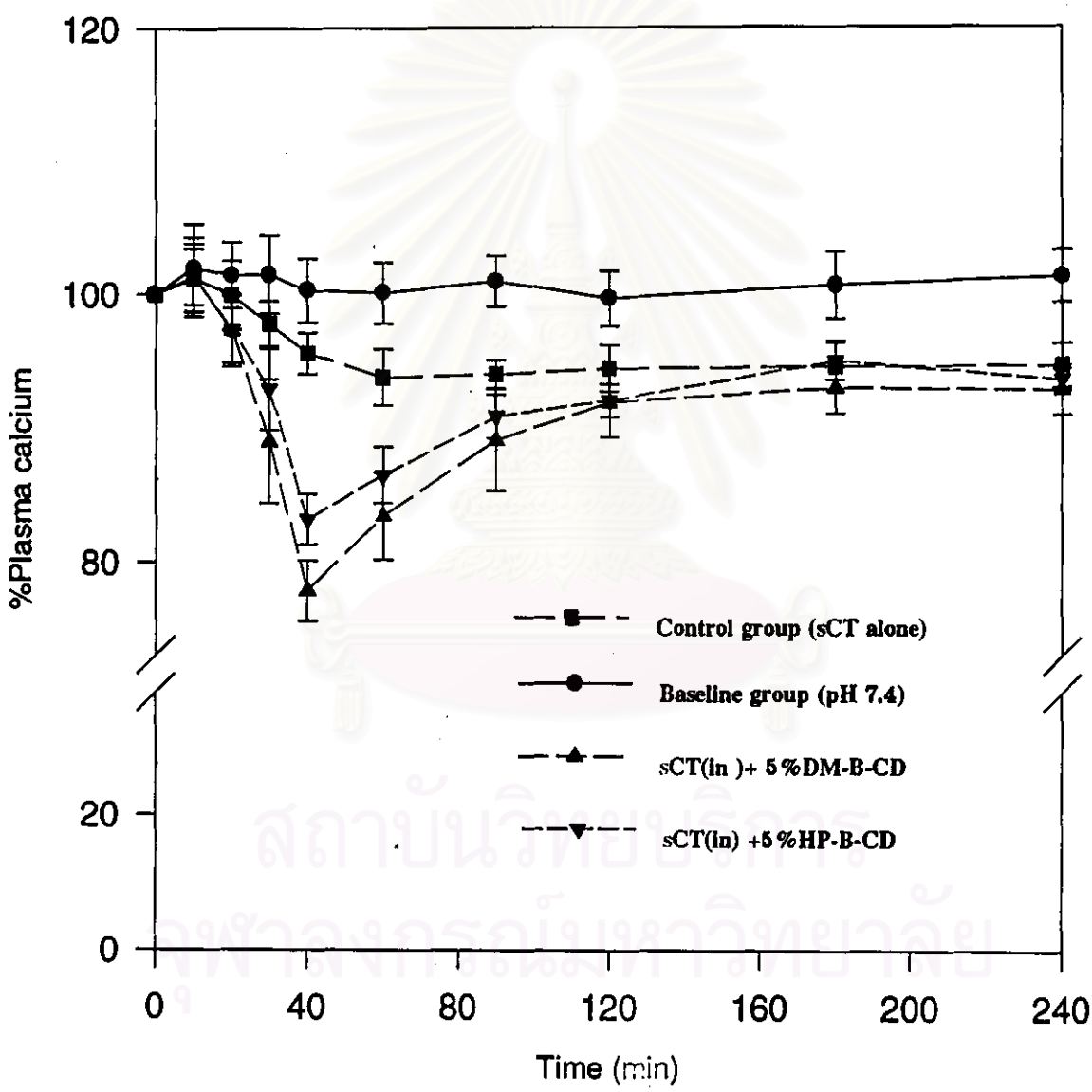
Table 17 % Plasma calcium level (of initial value) of rats following nasal administration of sCT (10 IU/Kg) with 5% w/v of DM-*B*-CD and HP-*B*-CD CS G at pH 7.4

CD (pH 7.4)	Time (min)										$AUC_{(0\text{-}240)min}$
	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

Figure 19 Percent of plasma calcium versus time after nasal administration of sCT with or without 5% cyclodextrins compared with the baseline group
Each point represents mean + S.D. (n = 5 rats/group)



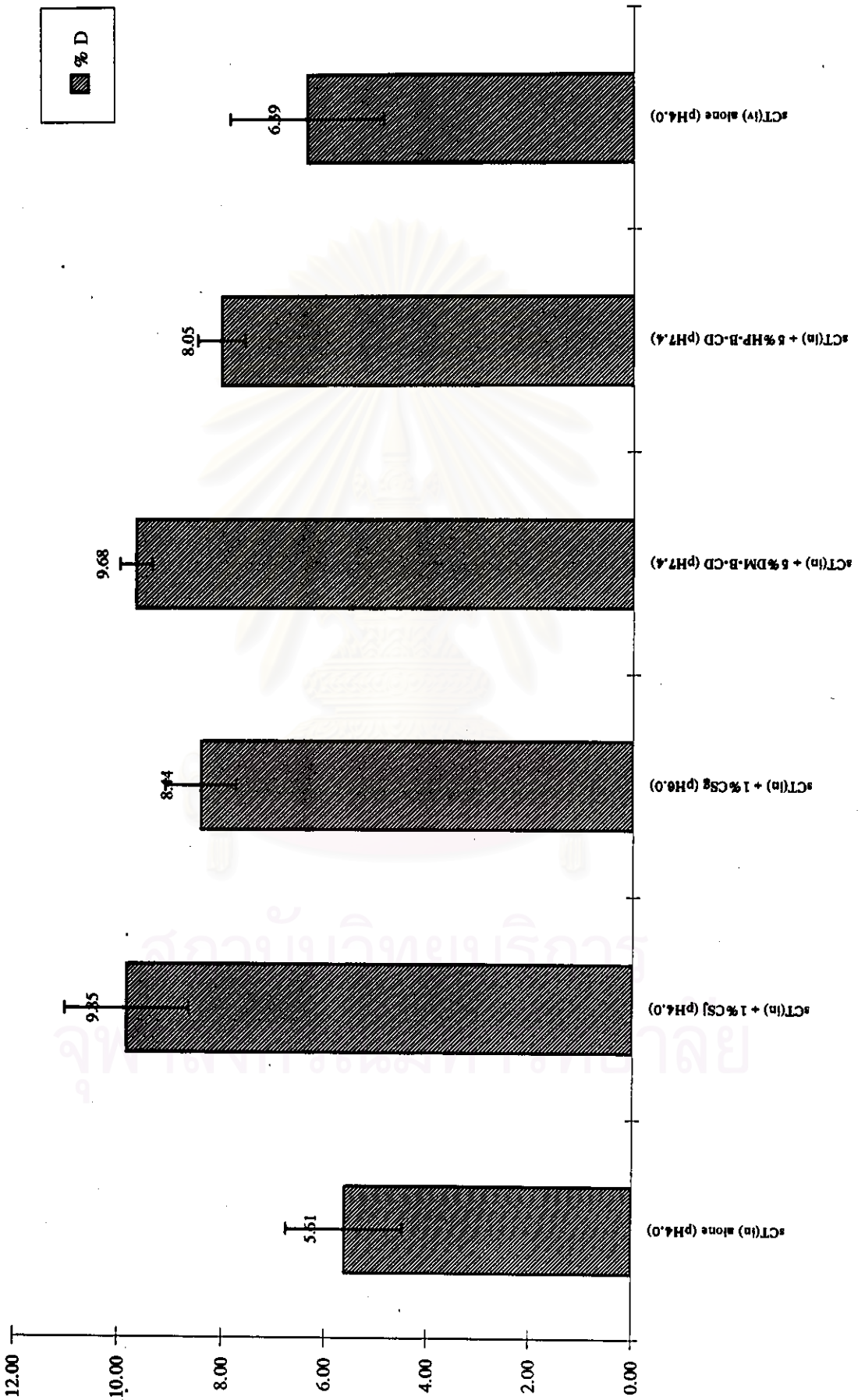


Figure 20 Comparison of the average total percent decrease in plasma calcium after administration of sCT with or without enhancers

ANOVA was then applied to compare the effect of 1% CS J (pH 4.0) and 1% CS G (pH 6.0) to that of reference enhancers. The parameter tested was the percent total decrease in plasma calcium during 240 min (% D). These values were tested among the four groups; i.e., 1.0 % w/v of CS J at pH 4.0, and 1.0 % w/v of CS G at pH 6.0, 5.0 % w/v of DM-β-CD and HP-β-CD at pH 7.4. All of these were studied at 5 % significance level. From ANOVA table (Appendix X), it is obvious that there was a significant difference among these groups ($p < 0.05$). When multiple range test (Duncan's test) was further applied to the data at the same level. The ranking order of the %D was

HP-β-CD pH 7.4	<	CS G pH 6.0	<	DM-β-CD pH 7.4	<	CS J pH 4.0
% D	8.05		8.44		9.68	9.85 %

The line underneath signifies that there were no significant differences among these groups on the same line ($p > 0.05$). Duncan's test results indicated that the four enhancers can be divided into two groups. The first group, containing 5% DM-β-CD at pH 7.4 and 1% CS J pH 4.0, is more effective than the other group (5% HP-β-CD pH 7.4 and 1% CS G pH 6.0). CS J appeared to give highest enhancing effect whereas HP-β-CD was the least effective. Moreover, the effect of 1% w/v CS J at pH 4.0 was already equivalent to that of 5% w/v DM-β-CD pH 7.4 ($p > 0.05$). Therfore, CS J may have a promising potential as a potent nasal absorption enhancer of sCT provided that more information is available regarding its absolute bioavailability and toxicity.

In conclusion, based on its effect on plasma calcium, the nasal absorption of sCT (10 IU/Kg) without additive in rats was very low as is apparent from the small effect observed on plasma calcium. Similar results have been reported by Morimoto et al (1995) who found that nasally administered sCT had an effect on plasma calcium in rats of only 3 % of the effect after intravenous administration. The nasal absorption of sCT in rats could be improved by coadministration with an absorption-enhancing agent like chitosan. 1% w/v CS J at pH 4.0 was found to be most effective in enhancing the nasal absorption of this peptide.

1.3 Plasma sCT Determination

To prove that sCT is absorbed intranasally into circulation, measurements of the plasma sCT levels following nasal administration were made and compared to the intravenous administration. CS J at 1.0% (pH 4.0) and DM-β-CD at 5% (pH 7.4) were selected for study in this part of experiments due to their similarly potent 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 21 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 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 22 represents plasma sCT concentration-time profiles following nasal administration of sCT (10 IU/Kg) with and without adjuvants, along with data from 0.15 IU/Kg intravenous administration. The individual plasma sCT data are given in Appendix V together with the AUC, C_{max} , T_{max} values. From data in Table 18, 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 profile of sCT may be different among various routes of administration. The nasal administration of sCT

Figure 21 **Representative standard binding curve of plasma sCT (pg/ml) concentration**

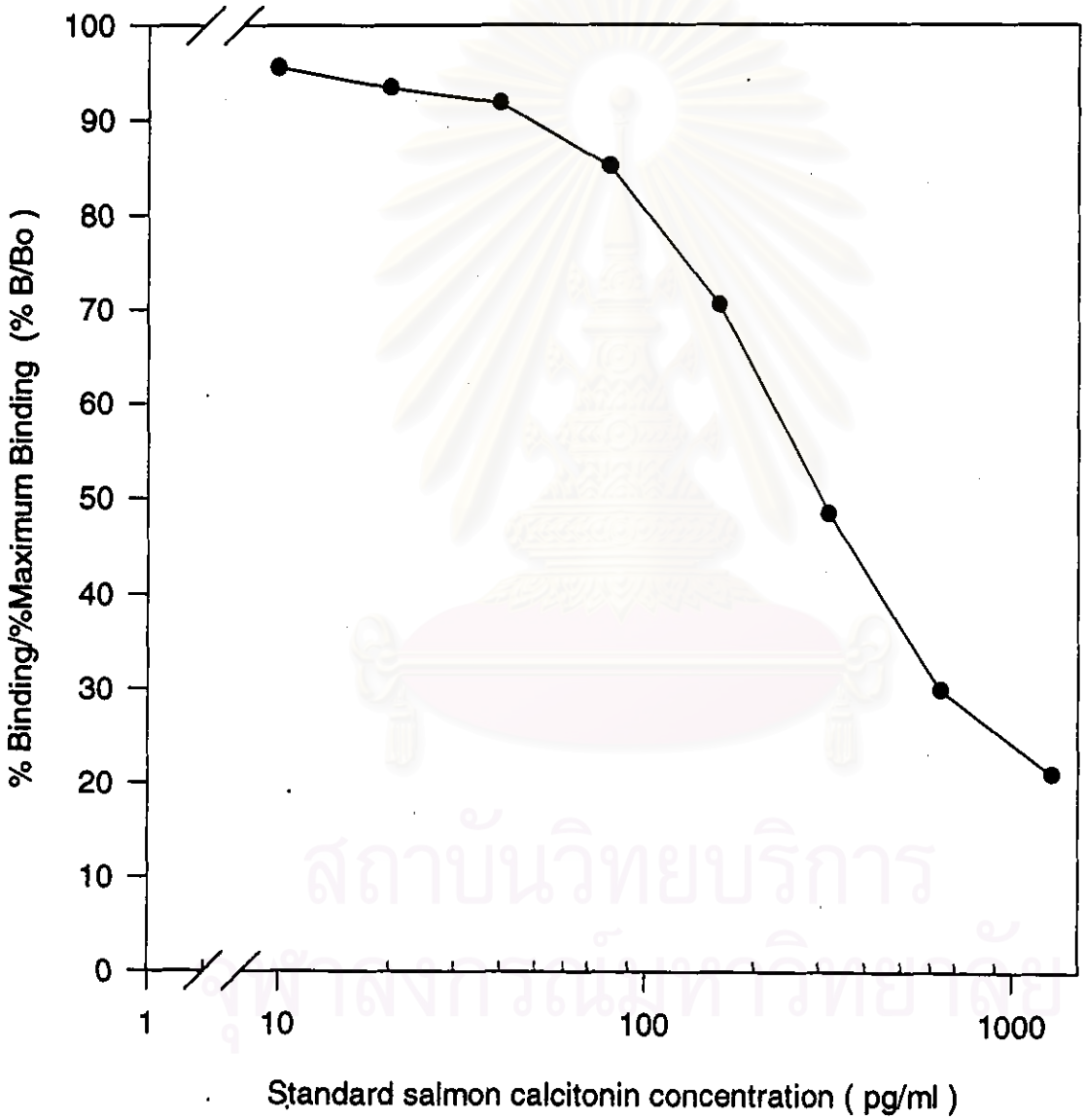


Table 18 Plasma sCT concentration of rats following intravenous and intranasal administration of sCT with or without enhancers

Condition	Time (min)									$AUC_{(0\text{-}180)min}$
	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

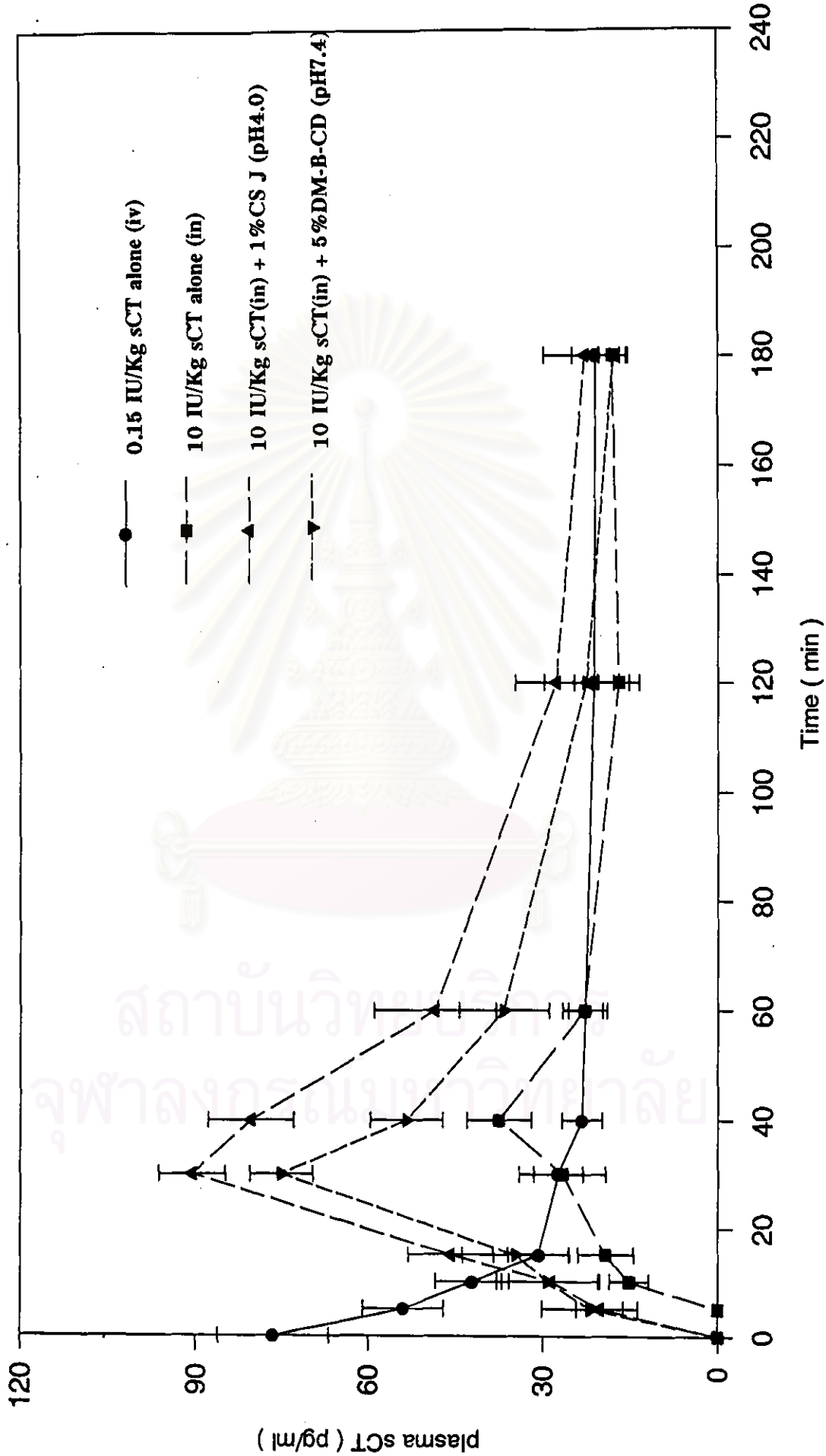


Figure 22 Plasma sCT concentration time-profiles following intranasal administration of sCT with or without adjuvants compared with intravenous administration

(10 IU/Kg) dissolved in 0.15 M isotonic phosphate buffer pH 4.0 without enhancers resulted in the very low plasma concentration of sCT and hence the lowering of the plasma calcium level was minimal (%D, 5.61 ± 1.14 %). The obtained pharmacokinetic parameters were listed in Table 19.The maximum plasma sCT level (C_{max}) remained very low about 37.53 ± 4.50 pg/ml at 40 min. The bioavailability calculated based on the AUC value relative to that of 0.15 IU/Kg (i.v.) of 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 slightly absorbed, with nasal bioavailability of only 1.16 %. Therefore, the inclusion of absorption enhancers appears necessary in the nasal formulations of sCT.

When sCT was intranasally administered at a dose of 10 IU/Kg in the presence of 1% CS J (pH 4.0), the absorption was considerably enhanced. As seen from Figure 22, sCT was rapidly absorbed into systemic circulation, reaching a maximum plasma level of 90.59 ± 4.71 pg/ml at 30 min, compared with the control group. In addition, nasal administration of the same dose of sCT with 5% DM-β-CD resulted in the peak plasma sCT concentration of 75.16 ± 4.47 pg/ml observed at 30 min. After 40 min following nasal administration of sCT 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). It is interesting to note that the peak plasma level of intranasal sCT with CS J was greater than that with DM-β-CD and the marked difference in this parameter implied that CS J was highly effective in rapidly delivering sCT across the nasal mucosa. The area under the plasma sCT curve during the 180 min

Table 19 Pharmacokinetic parameters of sCTwith or without enhancers after intranasal administration compared with intravenous 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

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

period (AUC_{0-180}) produced by CS J (7,330.85 ± 1,054.52 pg/ml.min) was also greater than that of DM-β-CD (5,702.11 ± 890.49 pg/ml.min). ANOVA was applied to the values of AUC_{0-180} (Appendix XI) comparing between the three groups i.e. control (nasal only), sCT plus CS J, sCT plus DM-β-CD at 5% significance level. Although CS J appeared to enhance sCT absorption to a greater extent than DM-β-CD, as seen by comparing their plasma sCT AUC_{0-180} values, this was not significant ($p > 0.05$, Appendix XII). However, both CS J and DM-β-CD were significantly effective over the control group ($p < 0.05$). The absolute bioavailability of intranasal sCT was also calculated for each $sCT_{treated}$ group by comparing the AUC_{0-180} values to that after an intravenous injection (equation 8). As shown in Table 19, the absolute nasal bioavailability, as indicated by the percent absorbed, was found to be 2.45 % and 1.91 % when 10 IU/Kg of sCT was administered with the CS J and DM-β-CD, respectively. On the other hand, the absolute bioavailability for the nasal control group was only 1.22 %. Although the absolute nasal bioavailability seemed to be low when compared to the intravenous administration, the inclusion of 1 % w/v CS J resulted in two-fold increase in the AUC_{0-180} of plasma sCT relative to that of the control group (intranasal administration of sCT without enhancers). This was equivalent to 201.18 % relative bioavailability when compared to the control group at the same nasal dose (10 IU/Kg). Addition of 5% w/v DM-β-CD also led to the relative nasal bioavailability of 156.8% or 1.56 fold increase in absorption over the control group. Table 20 compares the percent relative pharmacological availability (% PA_{rel}) with the percent relative nasal bioavailability (% F_{rel}). The values of % PA were calculated from the equation ($\%D_{enhancer}$ / $\%D_{control}$) x 100 %, where ($\%D_{enhancer}$ is the average total percent decrease in plasma calcium

Table 20 Comparison of the relative pharmacological availability and nasal bioavailability of sCT with or without enhancers

Adjuvants	$\%PA_{rel}$	$\%F_{rel}$
sCT alone (pH 4.0)	100.00	100.00
sCT + 1% CS J (pH 4.0)	175.00	201.18
sCT + 5% DM-B-CD (pH 7.4)	171.97	156.48

$$\%PA_{rel} = \frac{\% D_{enhancer}}{\% D_{control}}$$

where $\%PA_{rel}$ = Percent of the relative pharmacological availability

$\% D_{enhancer}$ = The average percent total decrease in plasma calcium during 240 min period after nasal administration of sCT with an enhancer

$\% D_{control}$ = The average percent total decrease in plasma calcium during 240 min period after nasal administration of sCT alone

during the 240 min period after nasal administration of sCT with an enhancer and $\%D_{control}$ is that after nasal administration of sCT alone at the same dose (10 IU/Kg). Data from this table demonstrate that the values of % PA_{rel} for control group, sCT plus 1% CS J and 5% DM-β-CD are 100%, 175% and 171.97% ,respectively in good agreement with % F_{rel} of them (100%, 201.18% and 156.48%).

The result from the in vivo experiment substantiated that chitosan, especially CS J, was effective in enhancing the nasal absorption of peptides such as sCT into the blood. Although not significant, it is interesting to note that the effect of 1% CS J appeared to be greater than 5% DM-β-CD in enhancing sCT nasal absorption based on the AUC_{0-180} values. These results also agreed with the plasma calcium data in that the maximum hypocalcemic effect, as judged from C_{min}, was more pronounced and observed at an earlier time (T_{min}) for CS J than for the cyclodextrin derivatives ($p < 0.05$, Duncan's test in Appendix X_b and X_c).The average value of %D also tended to be higher for CS J (9.85%) than for DM-β-CD (9.68%) but it was not significant at 5% level. The slightly better enhancing activity of CS J over DM-β-CD could be partly explained by the difference in pH conditions between the two enhancers (pH 4.0 for CS J and pH 7.4 for DM-β-CD). Figure 13 indicates that the more acidic (pH 3.0 and 4.0) tends to facilitate nasal absorption of sCT as demonstrate by the lower C_{min} and T_{min} values even in the absence of any enhancer ($p < 0.05$, Appendix $VIII_b$ and $VIII_c$), thus suggesting a rather minor effect due to the pH difference.

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 (Table 12 and Figure 13). Nevertheless, whether such mechanisms did exist, the effect was very small and need more studies to confirm the above explanations. Studies with HP-β-CD and DM-β-CD 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 Sahamethapat (1997) 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 that of Sahamethapat who reported that the promoting effects of 1% CS J (pH 4.0) and 1% CS G (pH 6.0) were significantly better than 5% HP-β-CD 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 ($p > 0.05$, Sahamethapat, 1997). 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

All the results from this experiments indicated that chitosans could remarkably increase the absorption of the polypeptide like sCT across the rat nasal mucosa. All types of the investigated chitosans gave these enhancing effects when compared with their control groups. The

enhancing effects of chitosans occurred according to the proposed combination mechanisms of the 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 interaction between the positively charge amino group of the chitosan 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, 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). Chitosan thus affected the permeability of the tight junctions, thereby increasing the drug absorption across the epithelium.

Illum et al (1994) proposed that the cationic nature of chitosan could have a transient effect on the gating properties the tight junctions. Because of their positive charge, cationic macromolecules such as protamine, polylysine and chitosan can interact with the anionic components (sialic acid) of the glycoproteins on the surface of the epithelial cells (Artursson et al, 1994). These researchers have proposed that chitosan might be able to displace cations from electronegatives sites (such as tight junctions) on a membrane which require coordination with cations (such as calcium) for dimentional 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 chitosan such as inhibition of the proteolytic enzymes in the nasal cavity. More studies need to be carried out regarding the possible mechanisms of absorption enhancement caused by chitosans, particularly their inhibition effect on the 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)

Before examining the enzymatic activity, it was necessary to setting the optimal conditions for substrate hydrolysis such as the wavelength of maximum absorption wavelength, pH of preparation, and concentration of substrate.

Preliminary Study

The absorption spectra of BAPA at concentration of 2×10^{-5} M in 0.15 M phosphate buffer pH 6.0 are shown in Figure 23, as well as the spectrum of p-nitroaniline at concentration of 6×10^{-5} M determined under identical conditions. The anilides possess maxima at 315 nm with an extinction coefficient of 382.29. p-Nitroaniline has a maximum at 380 nm (extinction coefficient of 662.29). The spectrum of the latter remains unchanged between pH 4.0 and 9.0. Because the two curves still overlap at 380 nm, the extent of hydrolysis of BAPA was determined by measurement of p-nitroaniline at 410 nm, at which the extinction coefficient was 624.77 and no contribution to the over-all absorbance by the anilides was observed. Therefore, the optimum wavelength was found to be at 410 nm for this study.

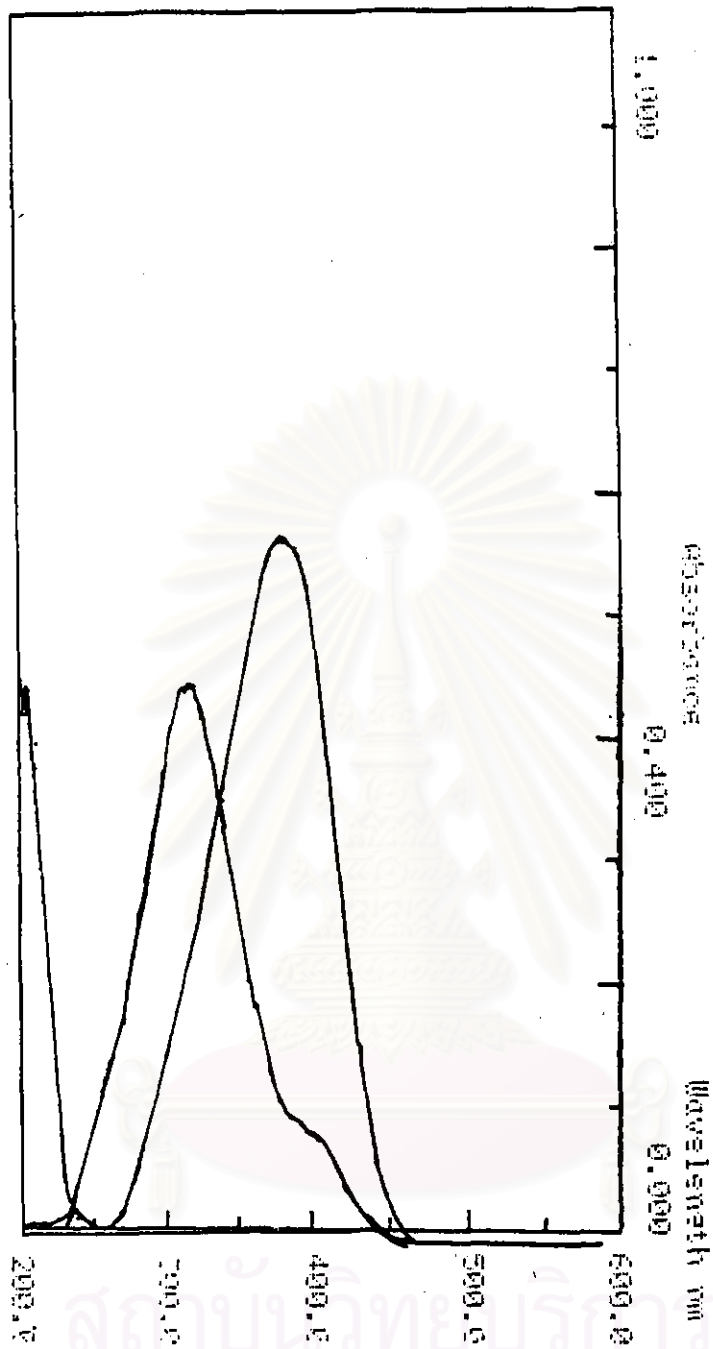


Figure 23 Absorption spectra of the BAPA (2.0×10^{-5} M) and p-nitroaniline (6.0×10^{-4} M) in phosphate buffer at pH 6.0

Effect of pH on Trypsin Activity

Determination of the pH optima 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 effect of pH on the degradation activity of trypsin was investigated by varying the pH of the preparations from 4.0 to 9.0. 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 analysed as described in the analytical method (experiment 2.1 in Chapter III).

The pH activity curves for tryptic hydrolysis of BAPA were obtained by plotting the concentration of p-nitroaniline produced versus time. The plots are shown in Figure 24 whereas the individual data show in Appendix $XIII_a$. This figure indicated that the activity of trypsin was strongly pH-dependent and reduced in the more acidic pH, particularly at pH 4.0. Figure 25 is a plot between concentration of metabolite formed at 240 min and pH. The curve shows that the maximum activity occured in an alkaline pH (mean pH 8.0). At pH 9.0, the activity seemed to decline. At alkaline pH, however, chitosans show poor swelling abilities, which restrict their use as an absorption enhancer. As a result, the optimal activity for these studies were performed at a slightly acid pH value (pH

Figure 24 Plots of p-nitroaniline concentration released as a function of time at various pH

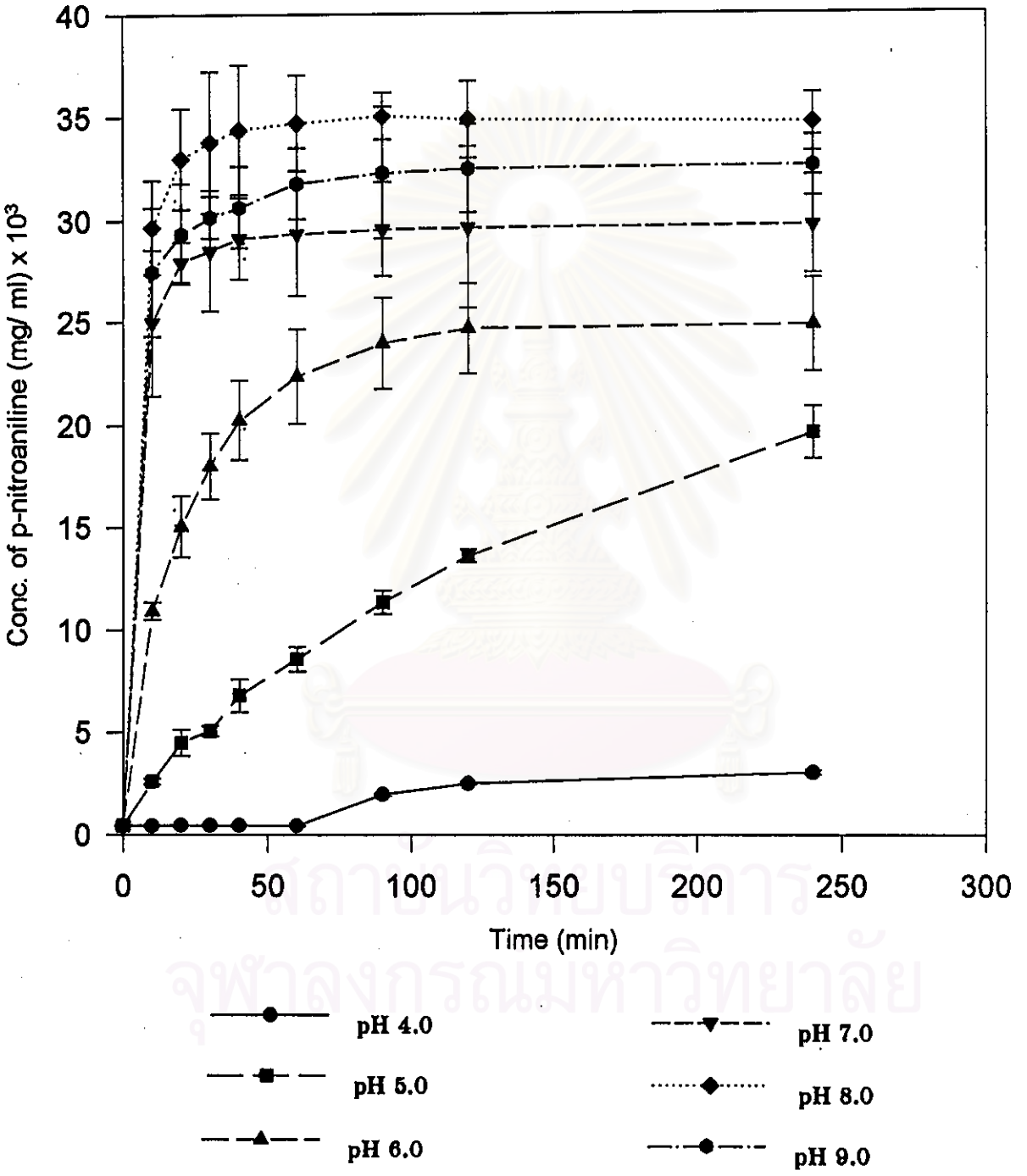
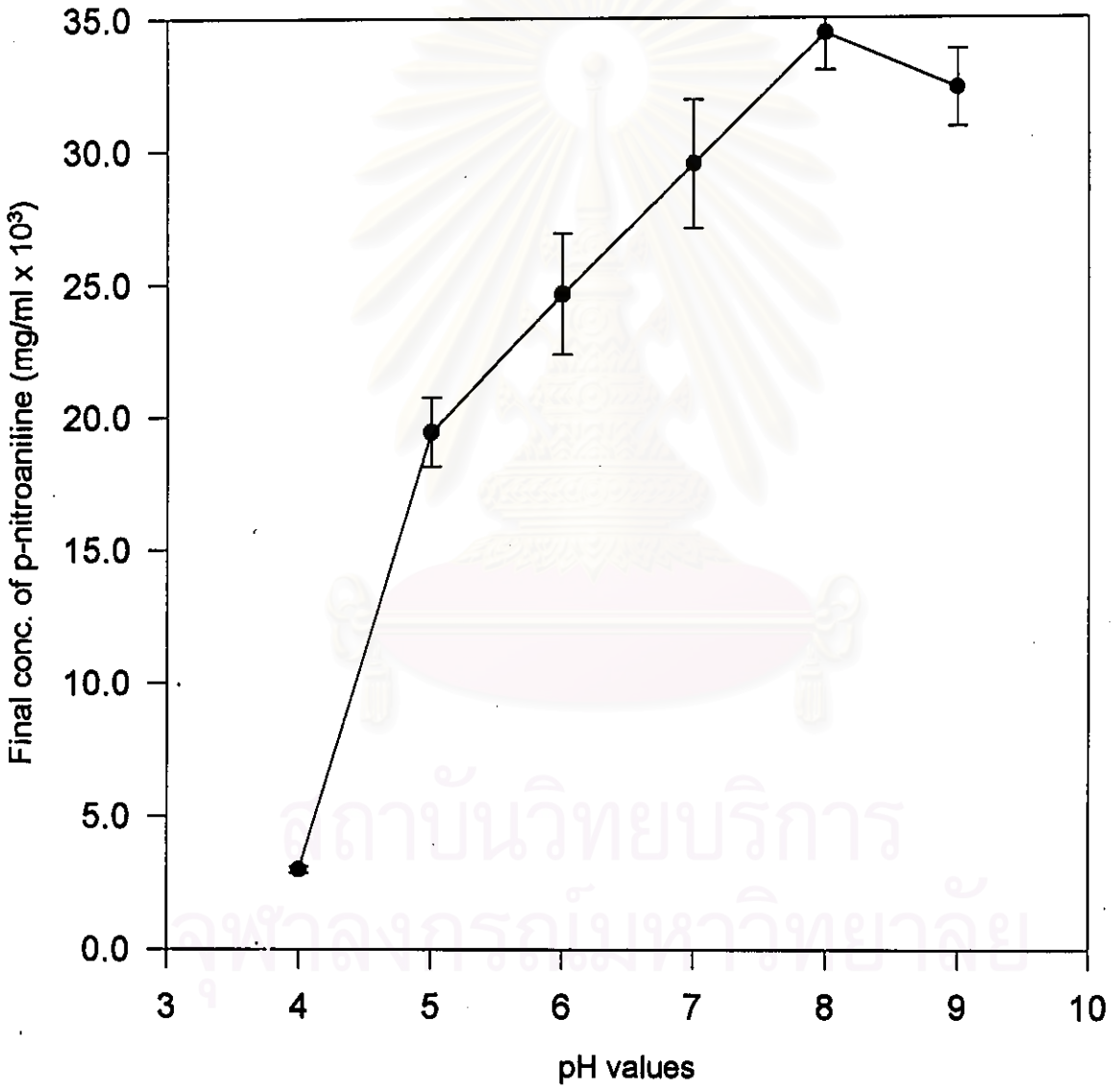


Figure 25 The pH-activity curves for the tryptic hydorlysis of BAPA (1 mM) incubation with trypsin (0.2 mg/ml) : Plot of the final conc. of p-nitroaniline at 240 min versus pH values



6.0) at which chitosans can still be dissolved in the preparation. In addition, this pH is also close to the pH optima of the rat nasal mucosa.

Effect of Substrate Concentration on Trypsin Activity

At the optimum pH (pH 6.0), the substrate concentrations in the range of 0.25 mM to 1.5 mM were investigated to determine the optimum substrate concentration. This was necessary to provide meaningful comparison of the enzyme activities. The degradation experiments was started by adding trypsin (0.2 mg/ml) to the different concentrations of substrate. The preparations were incubated at 37° c for 240 min and periodically sampled for analysis as described in the analytical method. As shown in Figure 26, the final concentration at 240 min of p-nitroaniline was directly proportional to the substrate concentration and plateaued off at about of 1.0 mM. The individual data were shown in Appendix XII_b So, the optimum substrate concentration in this experiment was 1.0 mM, which was shown to provide sufficient substrate for maximal hydrolytic activity of trypsin.

Determination of Trypsin Activity

The calibration curve of p-nitroaniline is shown in Figure 27. This curve was plotted between the concentration of p-nitroaniline and the absorbance at 410 nm. It was linear and regression coefficient (r^2) was 0.9977. The linear regression equation for this curve was $y = 0.066x + 0.006$, where y = absorbance and x = concentration in mg/ml x 10^{-3}. The inhibitory effect of chitosans on the tryptic activity was studied at various concentrations. Figures 28 and 29 are the plots of p-nitroaniline concentration versus time showing the formation of p-nitroaniline

Figure 26 Formation of p-nitroaniline as a function of substrate concentration following substrate incubation with trypsin (0.2 mg/ml) at 240 min (pH 6.0).

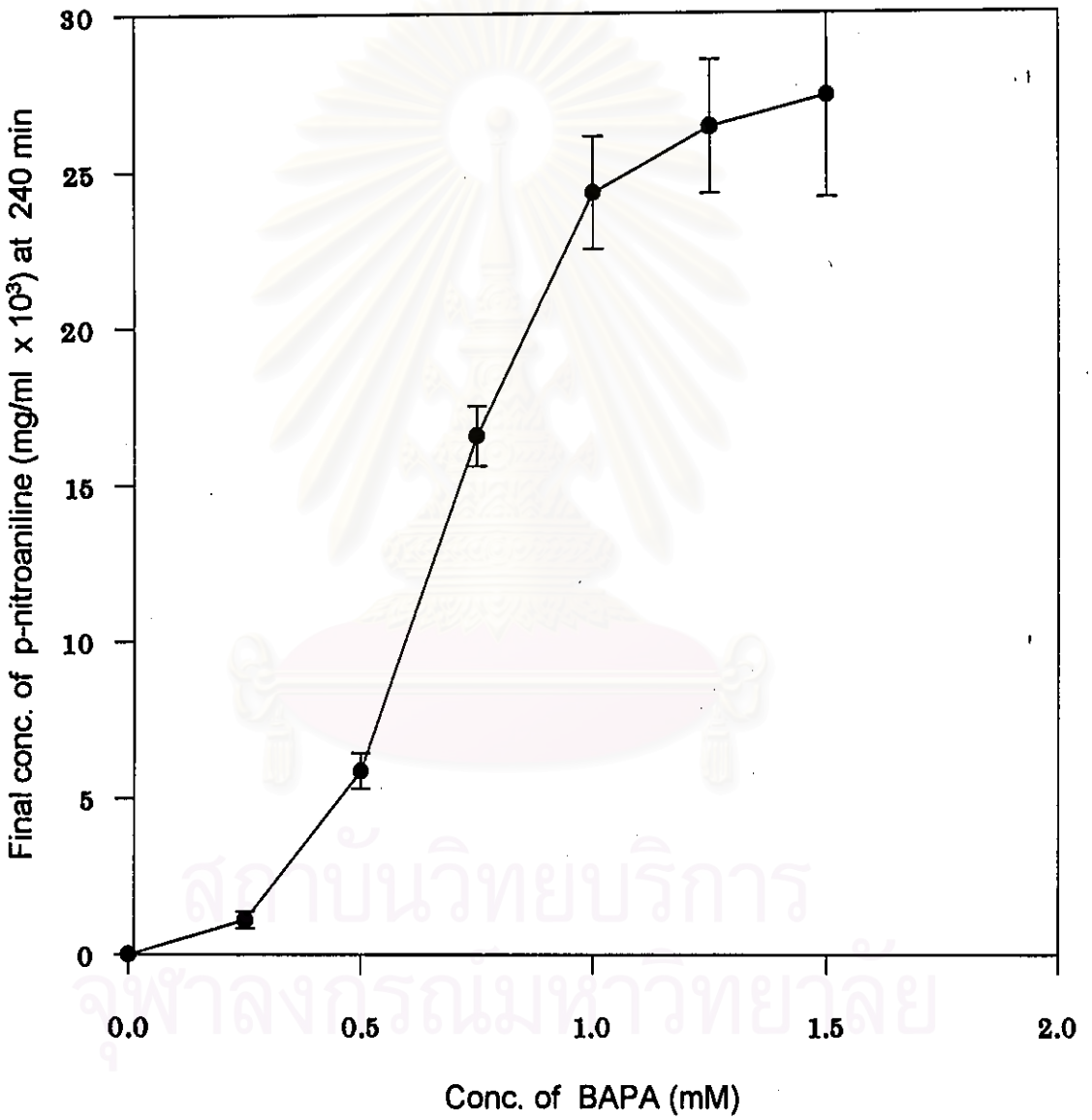


Figure 27 Representative calibration curve of p-nitroaniline in 0.15 M phosphate buffer (pH 6.0)

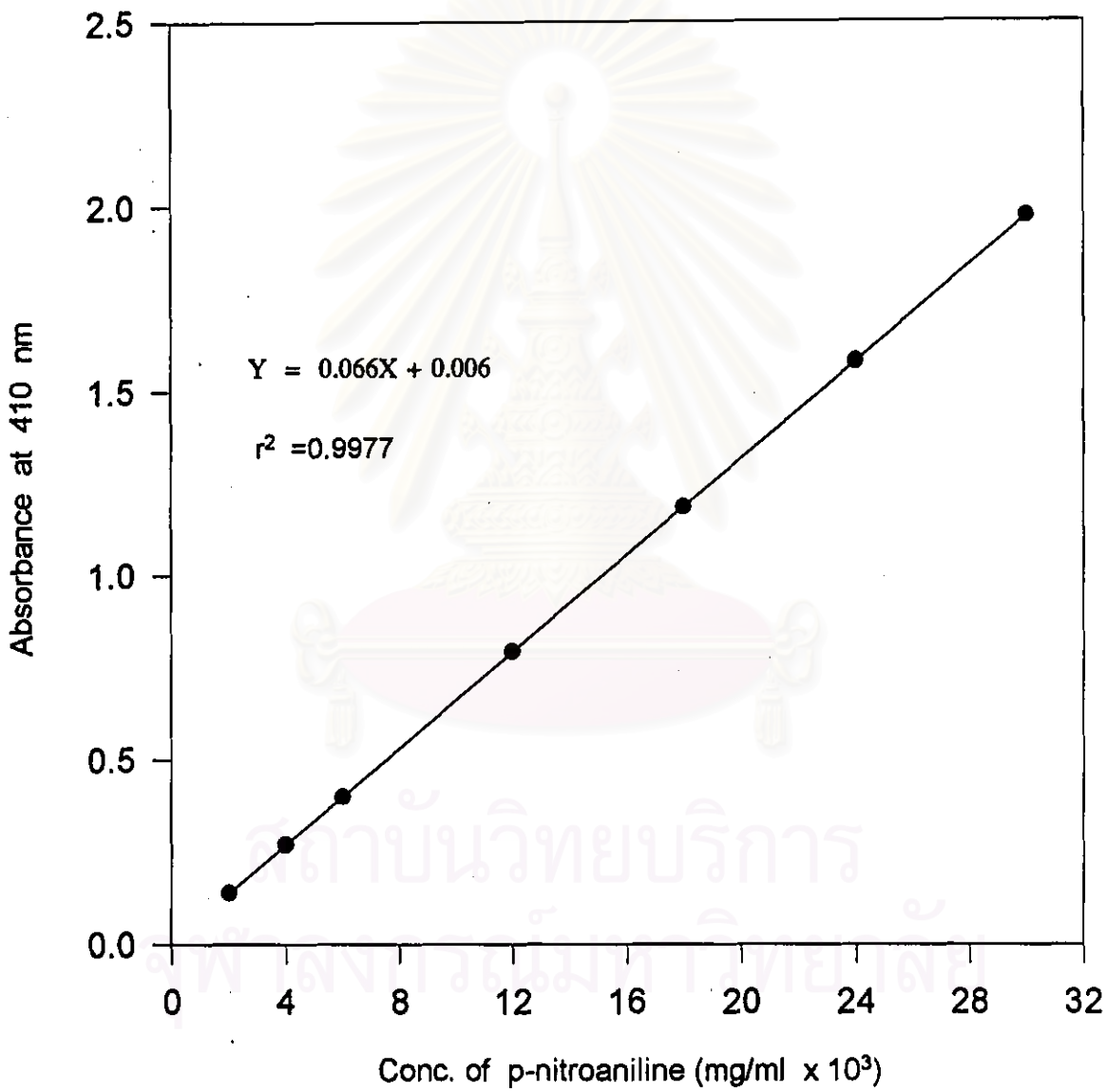


Table 21 Comparison of inhibitory effect of CS J at various concentrations (pH 6.0) on trypsin activity

[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)

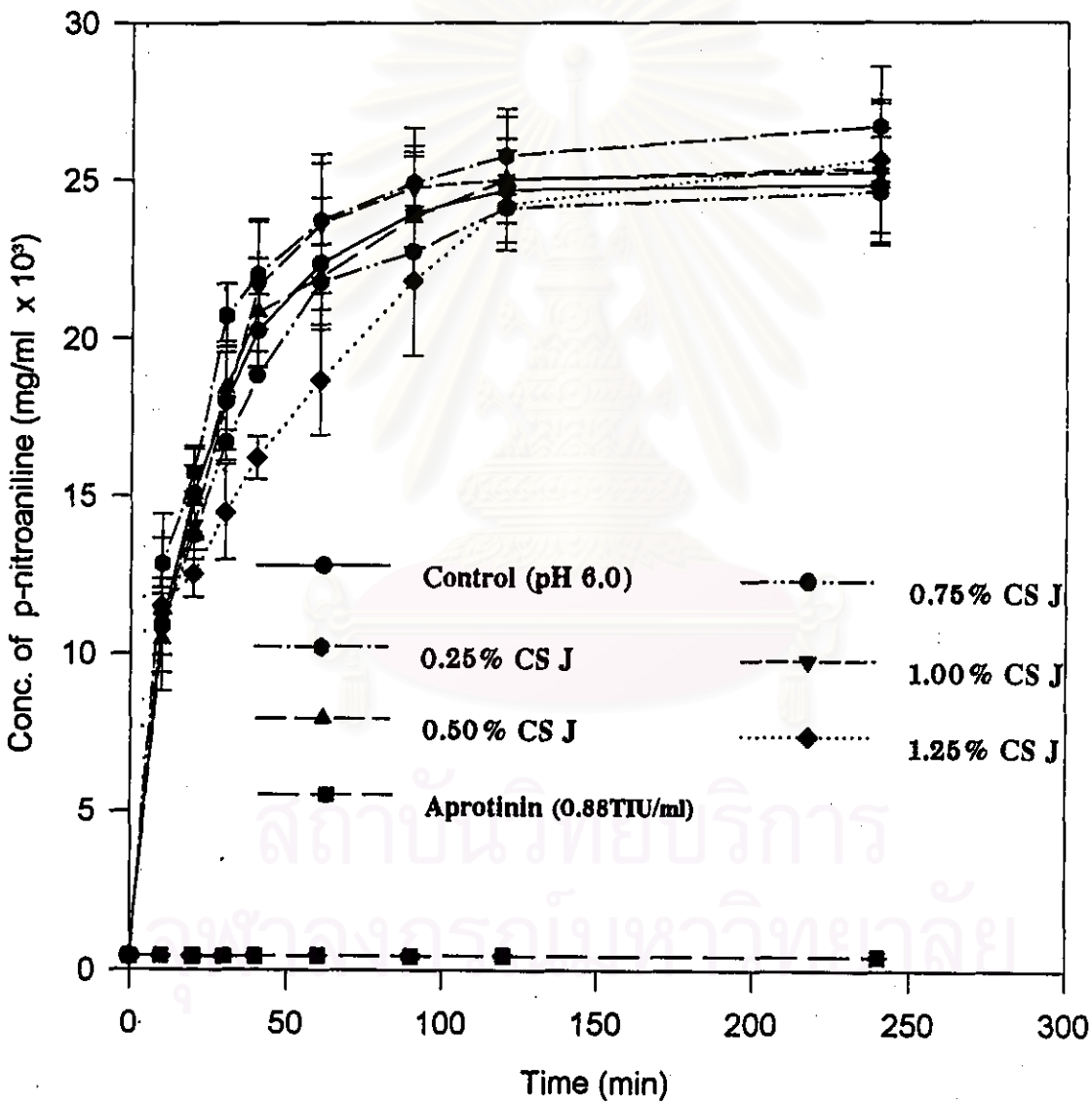
Table 22 Comparison of inhibitory effect of CS G at various concentration (pH 6.0) on trypsin activity

[p-nitroaniline]	Time (min)									$AUC_{0\text{-}240min}$	k
(mg/ml x 10^3)	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)

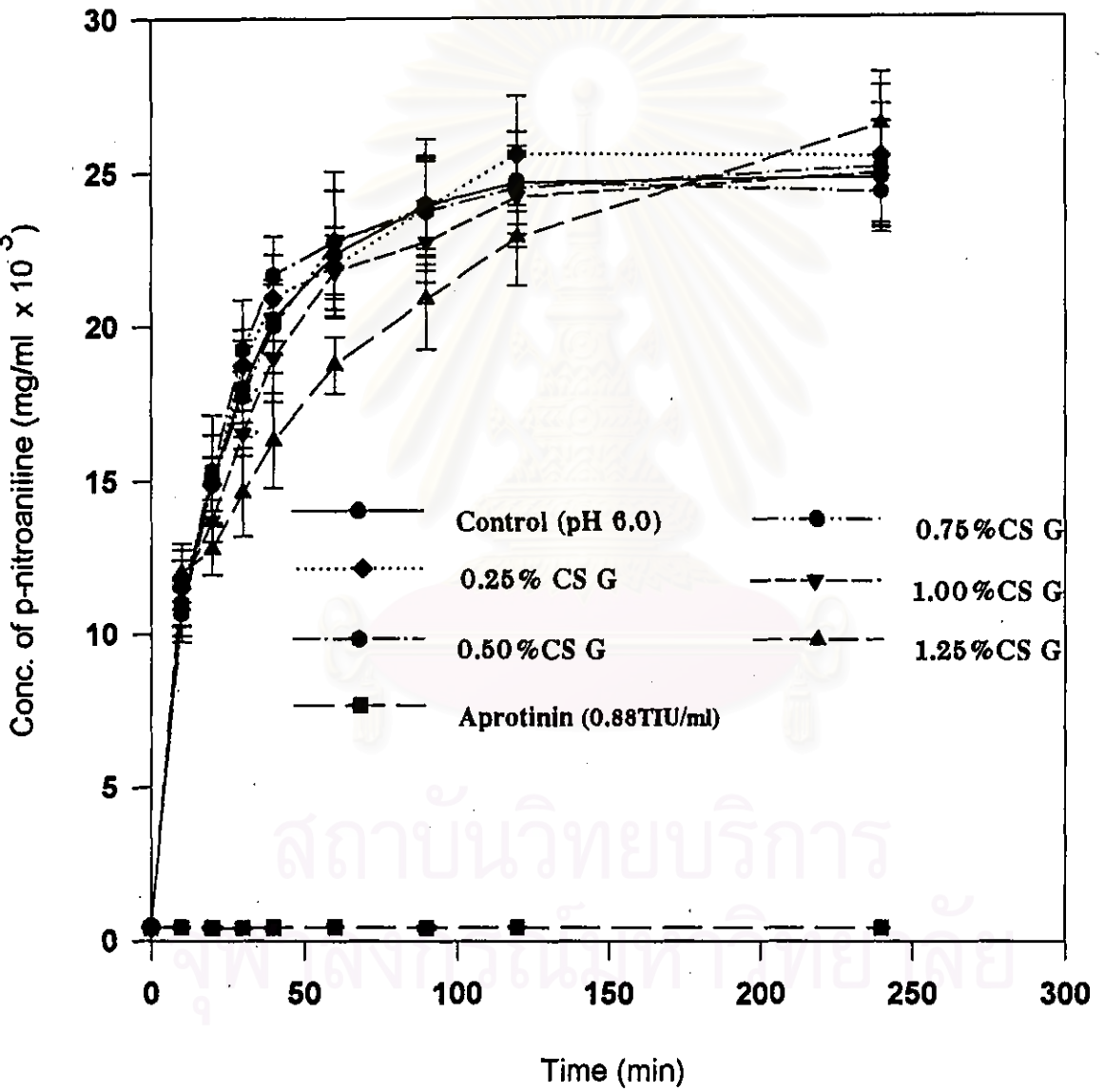
The value show the concentration of p-nitroaniline (x 10^3 mg/ml)

Figure 28 Formation of p-nitroaniline following incubation of BAPA with trypsin (with or without CS J) at various concentrations. The reaction were carried out in 0.15 M phosphate buffer pH 6.0



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

Figure 29 Formation of p-nitroaniline following incubation of BAPA and trypsin with or without CS G at various concentrations. The reactions were carried out in 0.15 M phosphate buffer pH 6.0.



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

following incubation of BAPA and trypsin with and without CS J and CS G, respectively. The average values are tabulated in Tables 21 and 22 whereas the individual data are provided in Appendix XIV. Analysis of the data from these tables indicated that both CS J and CS G , at all concentrations, did not induce any significant reduction in the extent of p-nitroaniline formation in com

parison with the control ($p > 0.05$, ANOVA on AUC in appendix XV_a and XV_b). The results revealed non-linear metabolite concentration versus time profiles similar to the control group. However, data in Tables 21 and 22 showed that, depending on concentrations, both CS J and CS G had similarly effects on the rate of tryptic hydrolysis as judged from the k values, the initial rate of product formation.

At concentrations between 0.25 and 1.0% of both CS J and CS G, the rate of hydrolysis did not appear to differ from the control group (0.47 $\pm$ 0.03 mg/ml/min x 10^{-3}), with the k values ranging from 0.43 $\pm$ 0.05 and 0.51 $\pm$ 0.04 mg/ml/min x 10^{-3}, (Tables 21 and 22). In contrast, the highest concentration used in this study (1.25%) tended to slightly decrease the hydrolysis rate with the k values of 0.35 $\pm$ 0.04 and 0.34 $\pm$ 0.05 mg/ml/min x 10^{-3} for CS J and CS G, respectively (Table 20 and 21). ANOVA was not performed on the k values since each k value was obtained from the initial slope by linear regression over the first six timepoints. Variation in the number of timepoints selected for slope calculation (3, 4.5, or 6 points) may have yield different statistical results. The slight decrease in the hydrolysis rate observed with 1.25% CS J and 1.25% CS G may be due to the formation of the gel matrix at this relatively high polymer 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 decrease in trypsin activity from the control buffer group, as judged from the AUC values ($p < 0.05$, Student's t-test, Appendix XV_c). By comparison with aprotinin, it is evident that chitosans in the concentration range of 0.25-1.0% hardly possess any inhibitory effect on the in vitro activities 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. Actually, Lueβen (1997) even found that chitosans (0.4 - 2.0%) were able to increase the tryptic hydrolysis rate of substrate BAEE at pH 5.6. However, the authors did not give any explanations regarding the possible activating effect of chitosans but their results strongly supported that chitosans possess no trypsin-inhibiting activity.

2.2 Degradation Studies with Leucine Aminopeptidase

Preliminary Study

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 absorption spectra of β-napthylamine (after diazotization and coupling with sodium nitrite and naphthylethylene diamine) at concentration of 5 x 10^{-4} mg/ml and pH 6.0 are shown in Figure 30. The maximum absorption was detected at the wavelength of 580 nm with an extinction coefficient of 1,131.6. The spectra remained unchanged between pH 4.0 and 9.0. Moreover, the other reagents present in the reaction mixture did not absorb nor interfere at this wavelength (Appendix XX). Thus, the quantitative analysis of LAP activity by colorimetric measurements was performed at the wavelength of 580 nm and final solution of azo dye was blue.

Effect of pH on Leucine Aminopeptidase Activity

Figure 31 shows the plots of β-napthylamine concentration released as a function of time at various pH's. The pH-activity curves for the LAP were then obtained by plotting the concentration of the resultant product (β-napthylamine)as a function of pH as seen in Figure 32. The individual data are shown in Appendix XVI. Each experiment was carried out by measuring the extent of β-napthylamine produced during 240 min incubation period. The effect of pH on the LAP activity was varied from 4.0-9.0. It is evident from Figures 31 and 32 that maximum LAP activity was observed at an alkaline pH of 8.0. At pH 9.0, the LAP activity appeared to decrease slightly indicating a tendency of the enzyme to be less active at the more alkaline pH values. Since both CS J and CS G were

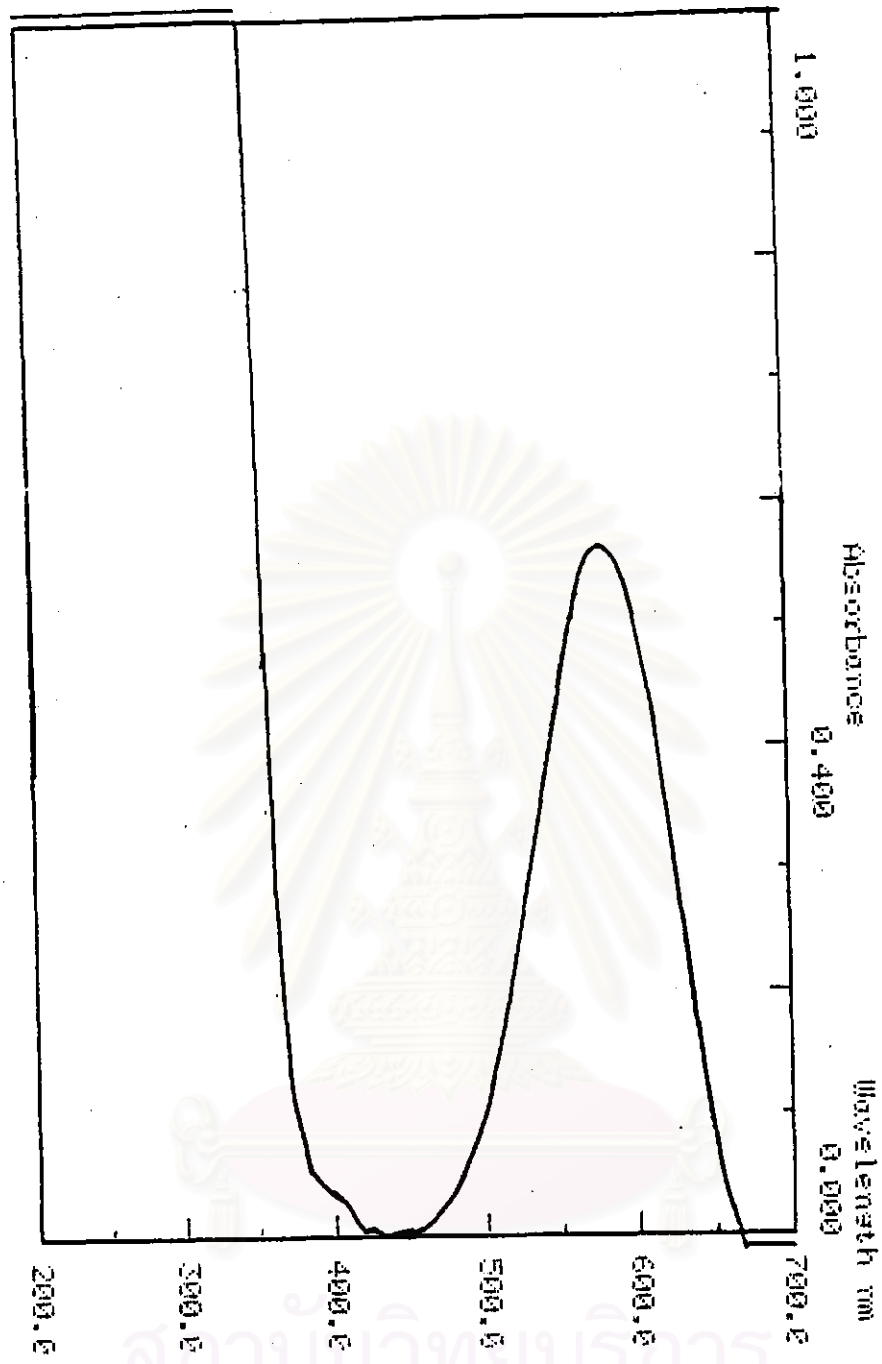


Figure 30 Absorption spectra of the β-napthylamine at concentration of (5.0×10^{-4} mg/ml) at pH 6.0

Figure 31 Plots of B-naphthylamine concentration released as a function of time at various pH's

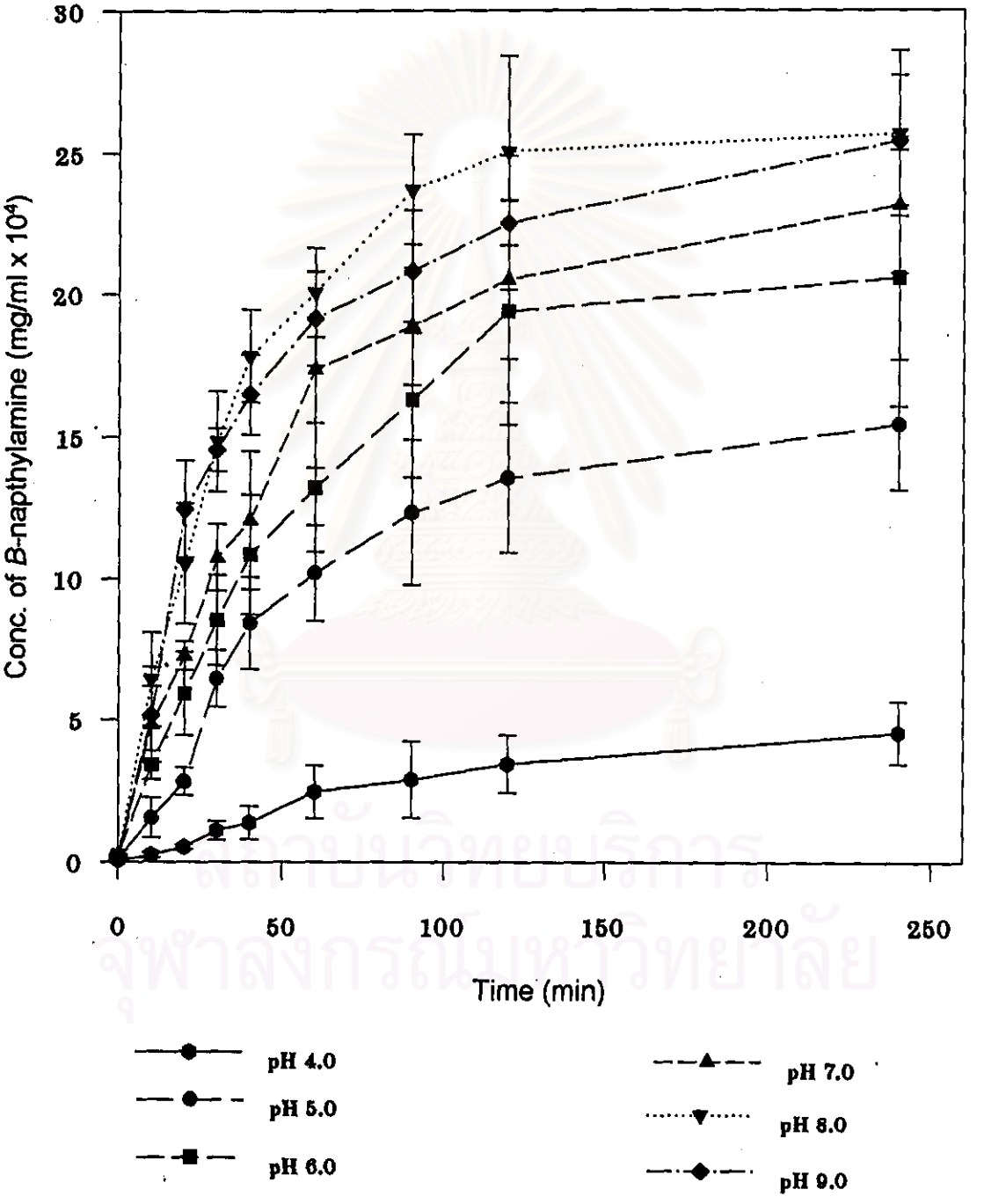
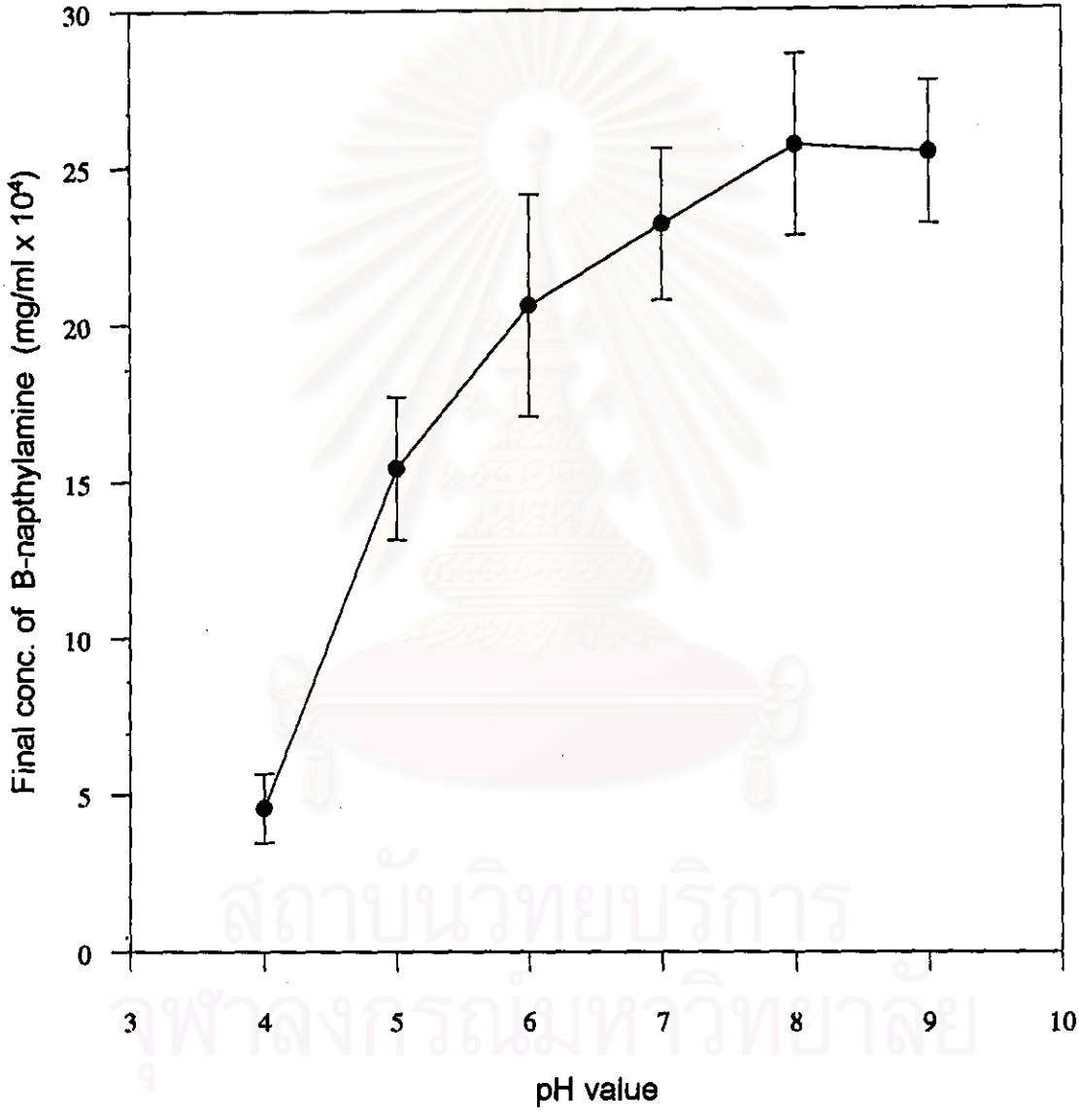


Figure 32 The pH-activites curves for the leucine aminopeptidase : Plots of the finalconcentration of B-napthylamine versus pH value at 240 min.

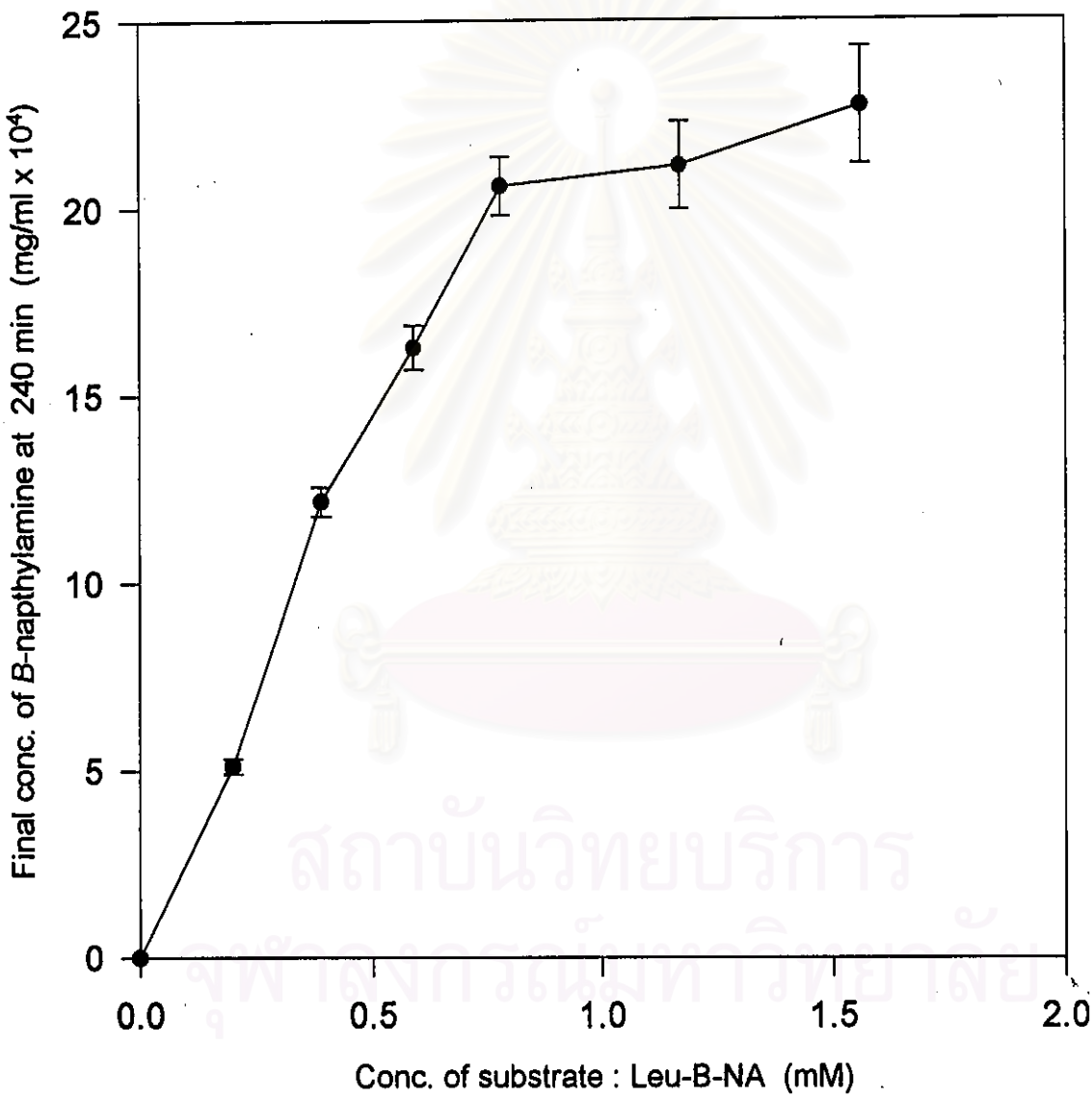


unable to dissolve at pH 8.0 and the LAP activity was still found to be high in the lower pH range (between 5.0 and 8.0), pH 6.0 was selected to be the optimum pH condition for the two chitosans to study their possible inhibitory effect on LAP. As a result, the degradation experiments in this study were performed at pH 6.0 similar to trypsin since chitosans can be completely dissolved at this pH.

Effect of Substrate Concentration on Leucine Aminopeptidase Activity

At the optimum pH of 6.0, the substrate concentrations in range of 0.20 to 1.56 mM (0.20, 0.39, 0.59, 0.78 1.17 and 1.56 mM) were investigated determine the optimum substrate concentration at this condition. The degradation experiments were started by adding LAP to the mixtures with different concentrations of substrate. The preparations were incubated at 37^0C for 240 min and after quenching the reaction with 0.5 ml 2 N HCl, the samples were analysed as described in step 4 to 7 of the analytical method. From the studies outlined above, 0.78 mM L-Leu-β-NA in phosphate buffer at pH 6.0 was selected as the optimum substrate concentration. The curves in Figure 33 represent the plots between the final concentration for β-napthylamine produced at 240 min versus substrate concentration. The concentration of β-napthylamine was found to be directly proportional to the substrate concentration in the range of 0 to 0.78 mM. At concentration higher than 0.78 mM, however, the curve started to level off indicating the saturation of LAP by the excess substrate. As a result, the value of 0.78 mM was considered to be optimum and

Figure 33: Formation of B-napthylamine as a function of substrate concentration following substrate incubation with LAP at 240 min.



always used in the subsequent experiments. The individual data of the substrate concentration effect are provided in Appendix XVII.

Determination of Leucine Aminopeptidase Activity

Figure 34 illustrates the representative calibration curve of β-naphthylamine by making a plot of absorbance at 580 nm versus the concentration of β-naphthylamine . The curve was linear and passed through the origin. A representative curve is express by the equation $Y = 0.077\ X$ with the regression coefficient was 0.9997 where Y = absorbance at 580 nm and X is the concentration of β-naphthylamine in mg/ml x 10^{-4} . Figure 35 and 36 show the formation of β-naphthylamine at various times following incubation of L-Leu-β-NA and LAP enzyme with and without CS J and CS G, respectively. The average values of AUC and k are showed in Table 23 and 24 whereas the individual data are provided in Appendix XVIII.

As seen from these figures, LAP activity was not inhibited by both CS J and CS G. ANOVA results at 5% significance level on AUC also indicated that both chitosans at all concentrations did not differ siginificantly from the control buffer group between the AUC and k values among various concentration in each chitosan ($p > 0.05$, Appendices XIXa and XIXb), i.e. they possess no inhibitory effect on the LAP activity, resulting in practically no changes in the β-naphthylamine versus time profiles when compared with the control (Figures 35 and 36) .On the other hand, the same figures show that bestatin (0.145 mM) strongly inhibited the LAP activity. The effect was also highly significant from the control as

Figure 34 Representative calibration curve of *B*-Napthylamine in 0.15 M phosphate buffer (pH 6.0)

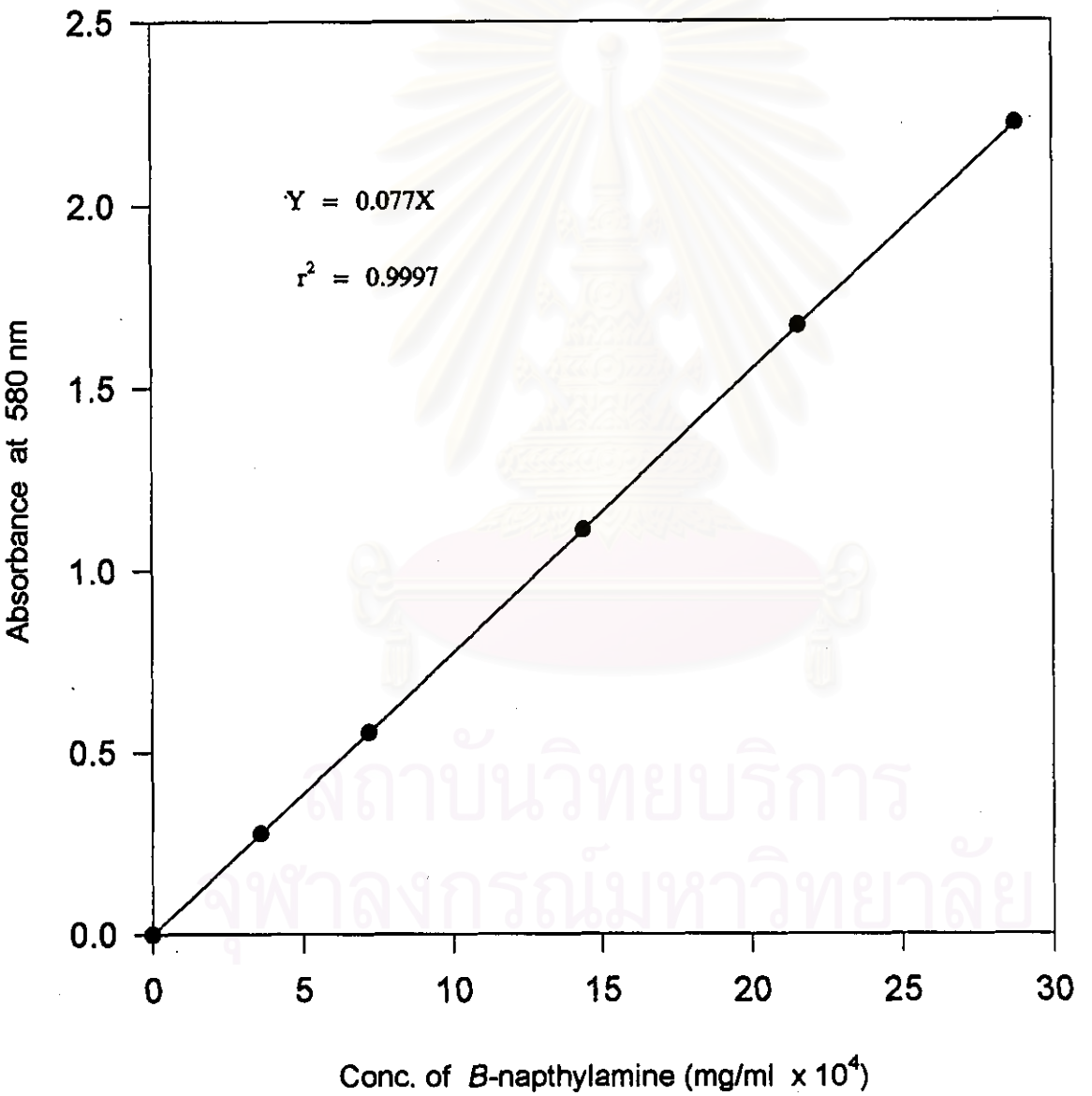


Table 23 Comparison of inhibitory effect of CS J at various concentrations (pH 6.0) on leucine amino peptidase activity

B-naphthylamine	Time (min)									$AUC_{0\text{-}240min}$	k
(mg/ml x 10^4)	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% 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)

Table 24 Comparison of inhibitory effect of CS G at variuos concentration (pH 6.0) on leucine amino peptidase activity

B-naphthylamine	Time (min)									$AUC_{0\text{-}240min}$	k
(mg/ml x 10^4)	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)

Figure 35 Formation of *B*-napthylamine following incubation of L-Leu-*B*-NA with LAP and CS J (pH 6.0) at various concentrations.

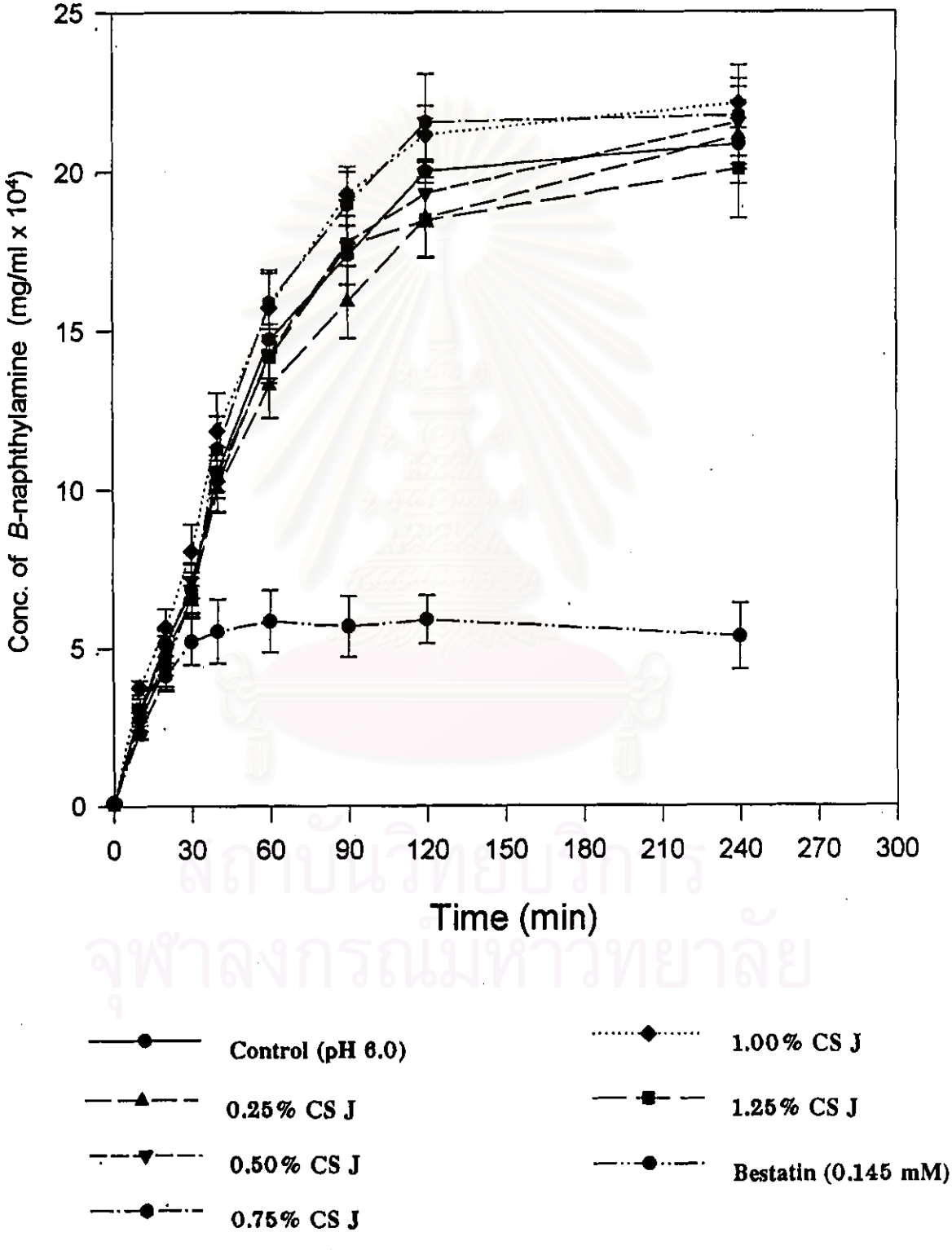
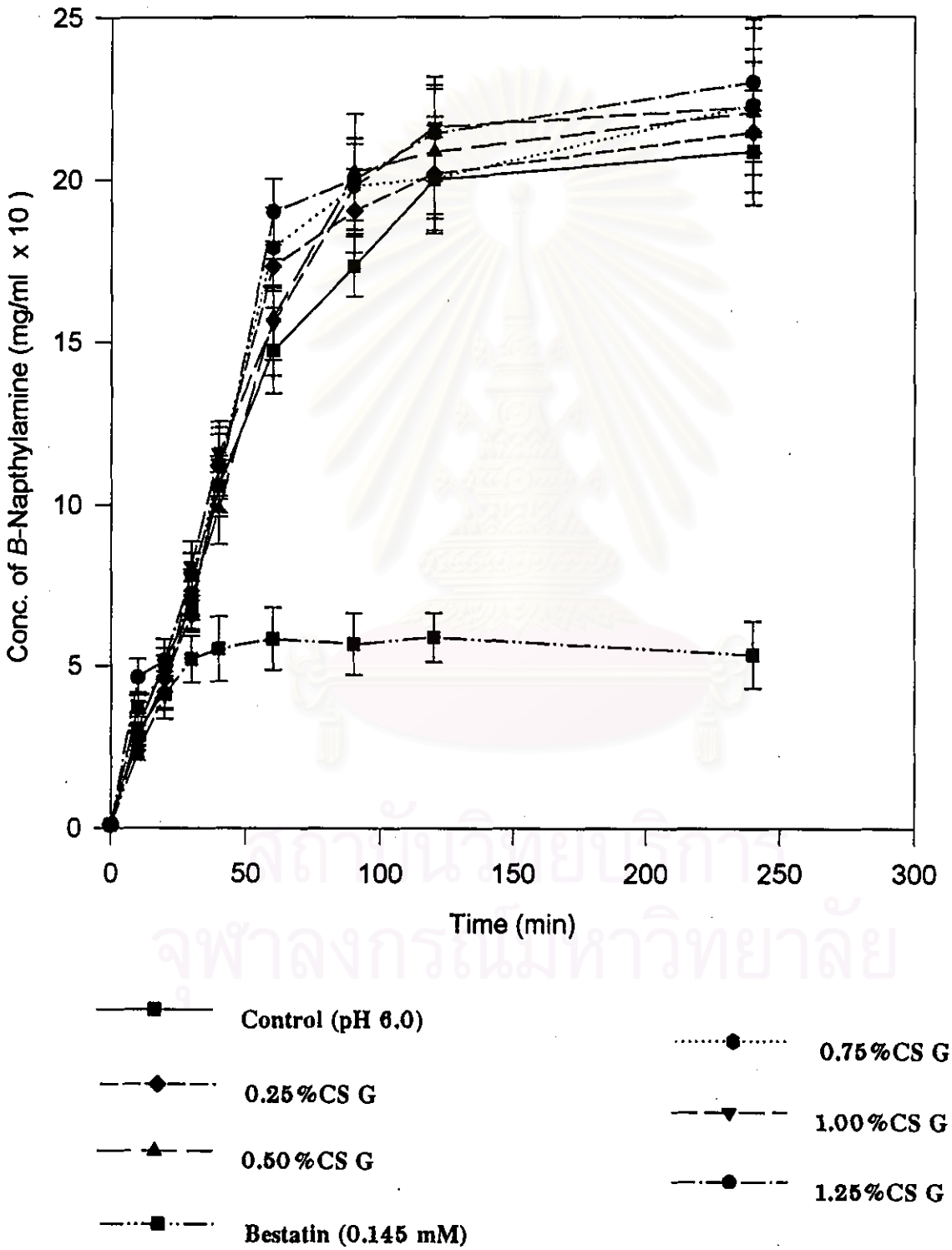


Figure 36 Formation of *B*-napthylamine following incubation of L-Leu-*B*-NA with LAP and CS G (pH 6.0) at various concentrations.



juded from the AUC values ($p < 0.05$, Student's t-test, Appendix XIX_c). The values of k or the initial rate of β-naphthylamine formation also show the same results (Table 23 and 24), i.e. only bestatin was able to dramatically reduce the rate of L-Leu-β-NA degradation(50% reduction) whereas both chitosans did not produce any noticeable reduction in the k values of k when compared to the control buffer group. (However, ANOVA were not performed on the values of k due to variation in selecting the appropriate time points for calculation of this parameter). 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 activities of the enzyme LAP as judged from the values of AUC and k