CHAPTER IV

RESULTS AND DISCUSSION

The results of the experiments are divided into two major sections but they are also interrelated. The data from the first part led to the development of the next investigations. Each part also has its own results and discussion and all of these are then summarized.

Part I. Studies to determine the reversible effect of the enhancers on the nasal membrane integrity following their removal from the rat nasal cavity.

1.1) Comparison of the efficacy of different enhancers on the nasal membrane permeability

High pressure liquid chromatographic analysis

The chromatograms obtained from the HPLC conditions described in Chapter III are shown in Figure 7. Figure 7A, shows a representative chromatogram of L-Phenylalanine which is an internal standard and Figure 7B shows a representative chromatogram of [D-Arg^2]-Kyotorphin. The retention time of [D-Arg^2]-Kyotorphin and L-Phenylalanine are about 9.2 and 7.0 min, respectively. These compounds are well separated from each other with completely resolved baseline. In addition, their peaks did not overlap with any of the solvent peaks and the addition of chitosans (CS J and CS G), cyclodextrins (HP-β-CD and DM-β-CD) or lauroylcarnitine chloride (LCC) did not interfere with their retention times (Figure 8).

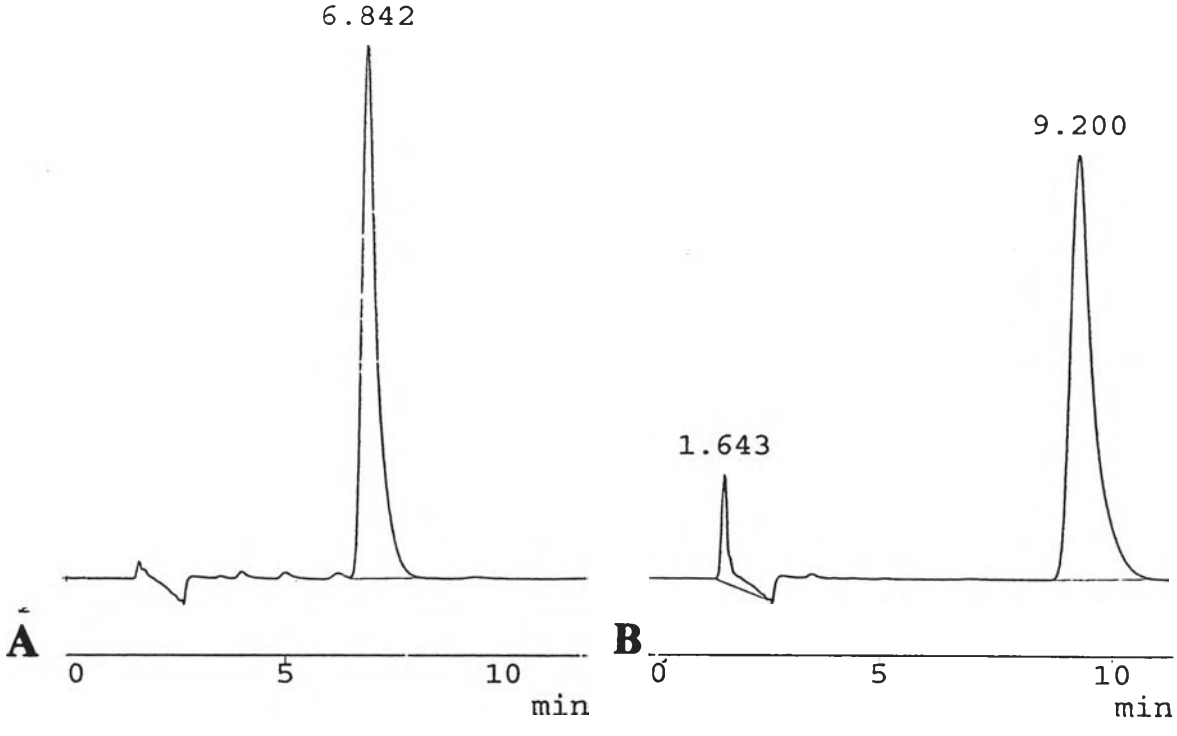


Figure 7 High pressure liquid chromatogram of (A): L-Phenylalanine (7.0 min) and (B): [D-Arg^2]-Kyotorphin (9.2min)

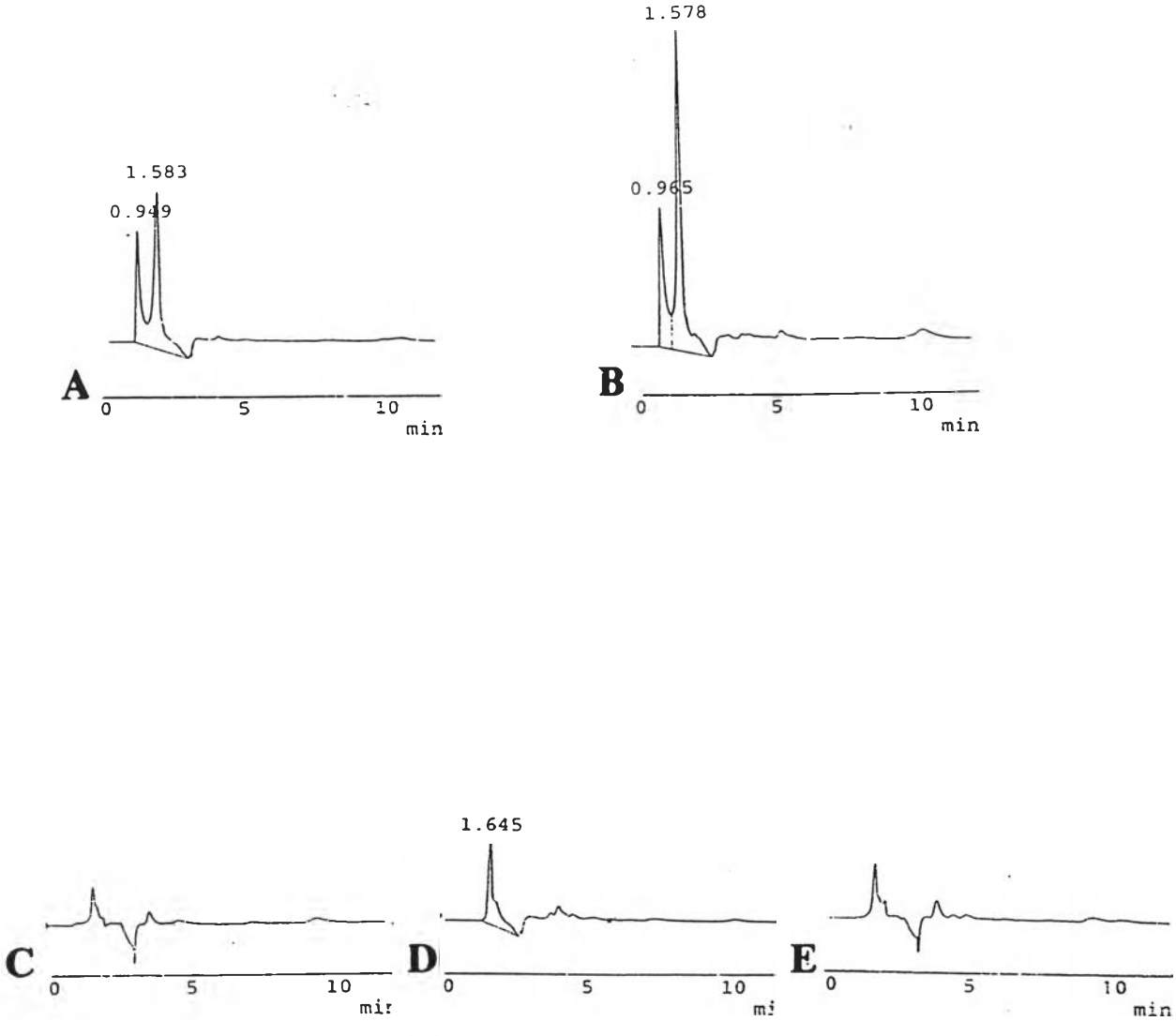


Figure 8 High pressure liquid chromatogram of (A): CS J (B): CS G (C): HP-β-CD (D): DM-β-CD and (E): LCC in isotonic saline

Standard Curve

Standard [D-Arg^2]-Kyotorphin solutions were prepared at the final concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mM in isotonic saline solution pH 6.0. After adding the internal standard, the solution was injected to the HPLC. Figure 9 shows the representative chromatograms of standard [D-Arg^2]-Kyotorphin and L-Phenylalanine solutions dissolved in isotonic saline solution pH 6.0.

Figure 10 shows the representative calibration curve of standard [D-Arg^2]-Kyotorphin in isotonic saline solution pH 6.0. The plot was linear. The regression coefficient (r^2) was 0.9999. All other standard curves gave similarly good linearity with the values in the ranges of 0.999-0.9999 in most cases.

Testing of tubing and cannula for possible adsorption of [D-Arg^2]-Kyotorphin

To confirm that loss of [D-Arg^2]-Kyotorphin from the perfusion solution was not due to adsorption of the dipeptide onto the tubing and esophageal cannula during the nasal perfusion, 0.5 mM solution of [D-Arg^2]-Kyotorphin in isotonic solution pH 6.0 was recirculated through the perfusion system without the rat. Analyses of [D-Arg^2]-Kyotorphin in the perfusion solution at 0 and 120 min revealed that there was no change in the chromatograms of the dipeptide during this period, indicating its good physicochemical stability in the solution without any apparent adsorption onto the tubing and cannula during perfusion.

The testing of interference from the nasal mucosa during perfusion

Figure 11 shows the chromatogram of the nasal perfusate of the isotonic saline solution after recirculating through the rat nasal cavity for 60 min. From this Figure, it is clear that other contents of the rat nasal perfusate (e.g. mucus protein) did not interfere with the chromatograms of the dipeptide and the internal standard.

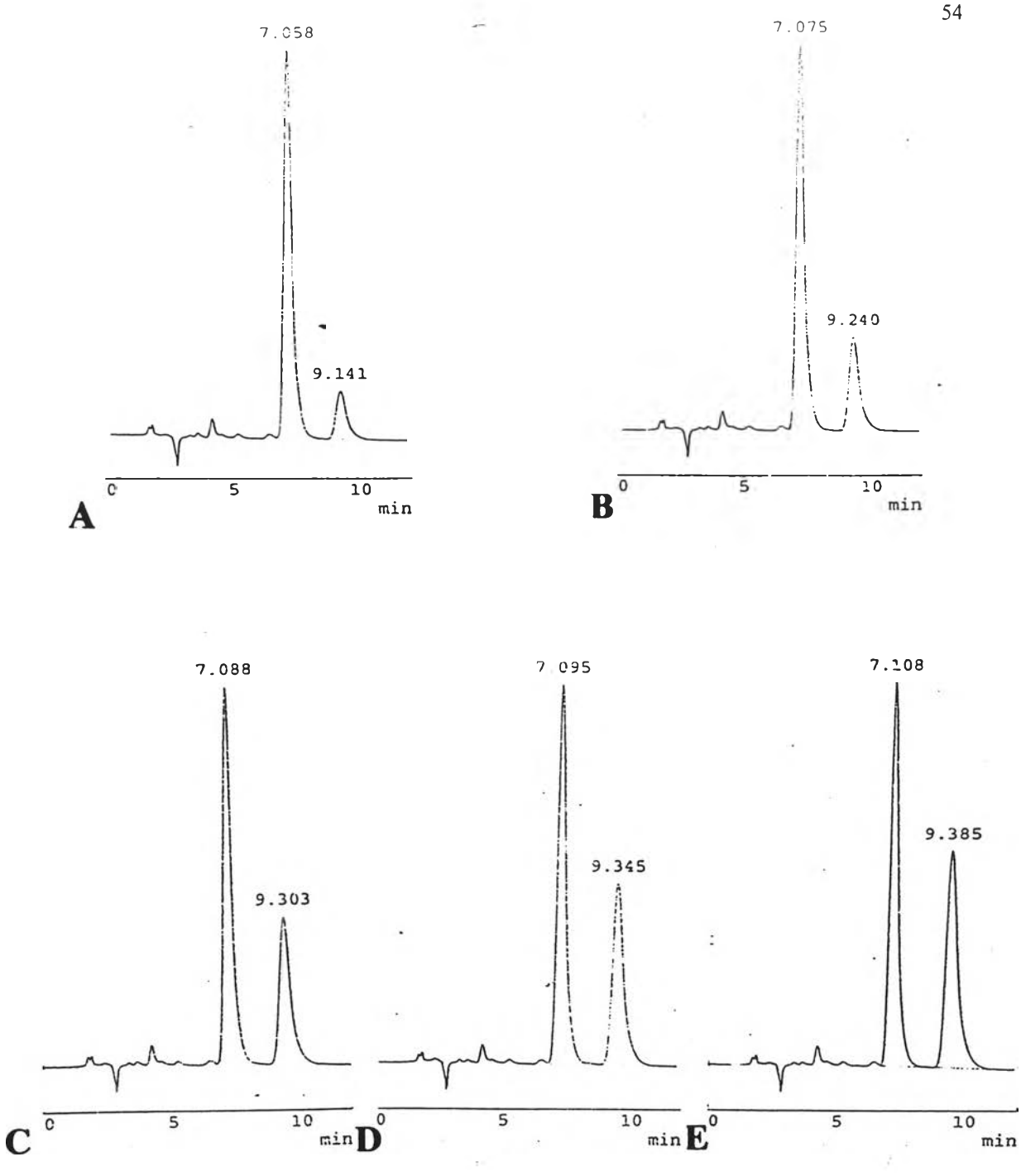
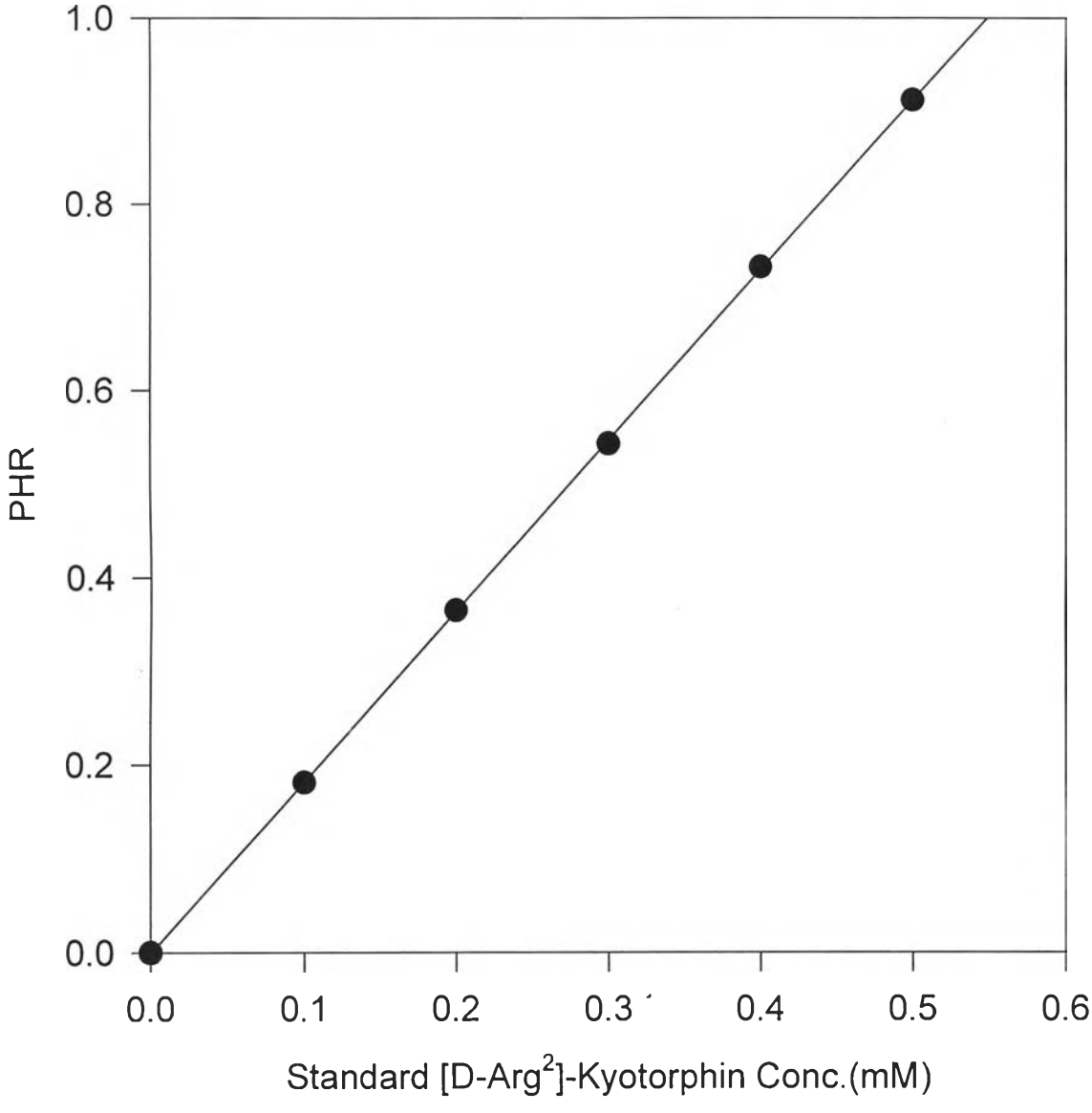


Figure 9 High pressure liquid chromatogram of the calibration curve of [D-Arg^2]-Kyotorphin (9.2min) at the concentration of 0.1 (A), 0.2 (B), 0.3 (C), 0.4(D) and 0.5 mM (E) with 5 mM L-Phenylalanine (7.0 min) as internal standard, in isotonic saline pH 6.0



Y = -0.001+1.826x $r^2 = 0.9999$

PHR = peak height ratio of standard [D-Arg^2]-Kyotorphin to L-Phenylalanine

X = standard [D-Arg^2]-Kyotorphin conc. (mM)

Figure 10 Representative calibration curve of [D-Arg^2]-Kyotorphin in isotonic saline pH 6.0

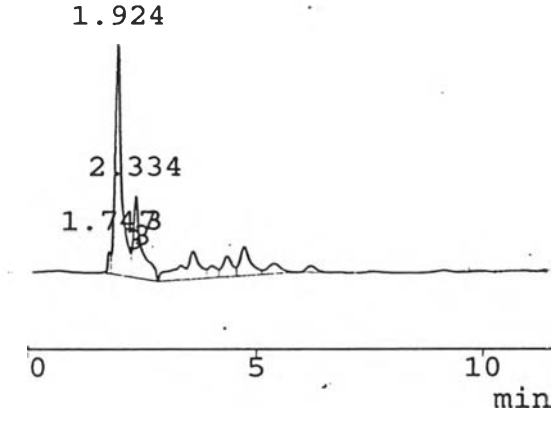


Figure 11 High pressure liquid chromatogram of nasal perfusate containing only saline after recirculating through the rat nasal cavity for 60 min.

All the endogenous peaks eluted early during the first five minutes. In addition, Figure 12 shows the chromatograms of the perfusate at 0 and 60 min which contained only [D-Arg^2]-Kyotorphin and L-Phenylalanine in isotonic saline solution pH 6.0 (no enhancer). Results in this figure confirmed the previous report (Tengamnuay and Mitra, 1990a) that [D-Arg^2]-Kyotorphin was enzymatically stable in the nasal perfusate without hydrolysis into amino acids L-Tyr and D-Arg. If enzymatic hydrolysis were to occur, a sharp peak of L-Tyr should have been observed at about 3.5 min . (D-Arg, on the other hand, did not absorb UV light at 274 nm and therefore did not give peak under this HPLC condition). Consequently, loss of the dipeptide from the perfusate should be caused by its absorption across the rat nasal mucosa and not by local metabolism. Thus, the enhancers which were more efficacious, should be able to produce pronounced decrease in the dipeptide content of the nasal perfusate. The pH value was fixed at 6.0 in all perfusion experiments regardless of the enhancers because LDH enzyme which was utilized in the toxicity evaluation of nasal membrane in the next part of experiments demonstrates high activity at this pH. Martinek (1972) reported that LDH has an optimum activity at pH 6.8 while Pujara et al. (1995) observed the loss of LDH activity at pH below 4.0. Since the perfusion could not be run at pH 6.8 because chitosan will precipitate from the solution at pH greater than 6.0. Thus, pH 6.0 was selected as the most favorable common pH condition for all the enhancers under study.

The concentrations of CS J and CS G were selected at 0.1 and 0.5%w/v for each chitosan according to the results obtained from previous experiments (Sahamethapat, 1996). Although 0.1% was already effective in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin, the concentration of 0.5% was also studied in order to see more clearly any specific effect of chitosans on the permeability and the integrity of the rat nasal epithelium. Comparison was then made with 5% HP-β-CD as

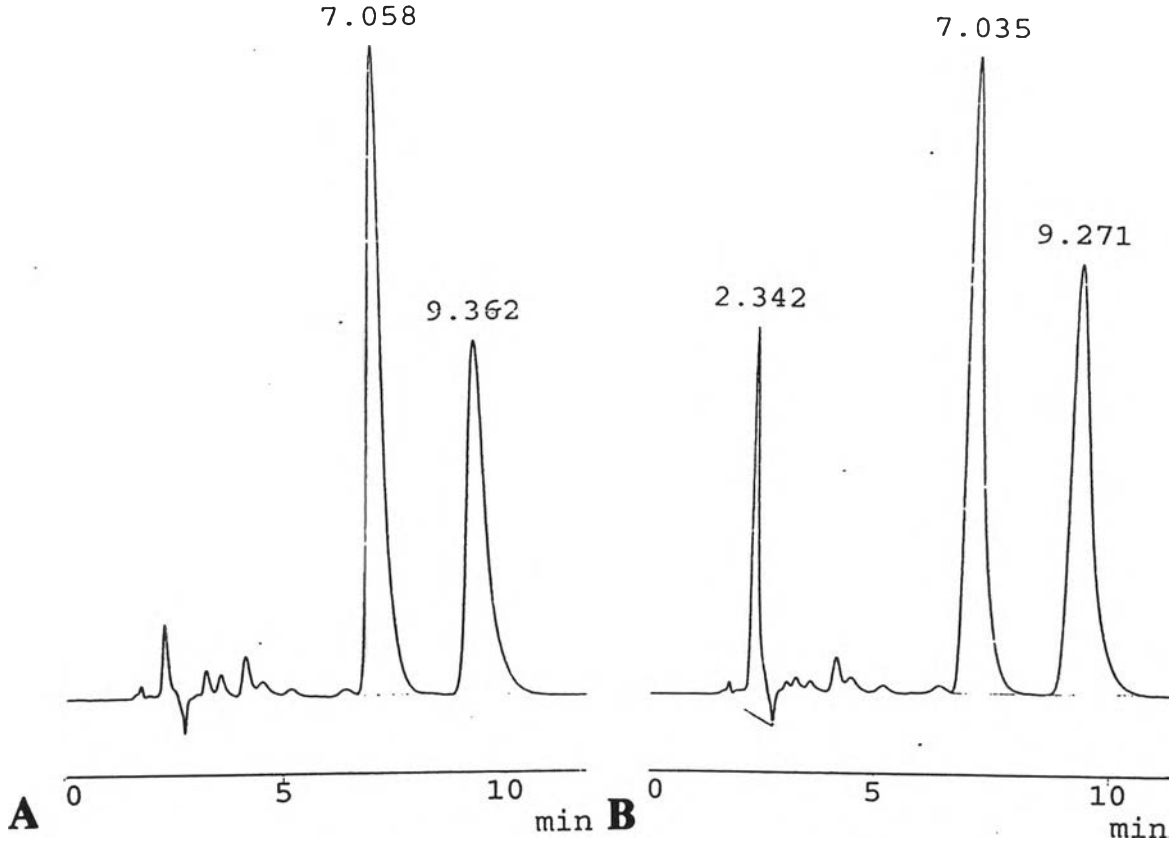


Figure 12 High pressure liquid chromatogram of [D-Arg^2]-Kyotorphin (9.2 min) and L-Phenylalanine (7.0 min) in isotonic saline pH 6.0 at 0 (A) and 60 min (B) of the perfusion

usual since it is one of the least irritating enhancers reported so far with respect to nasal morphology (Chandler et al.,1991b), mucosal component release (Shao et al., 1992; Marttin et al.,1995), and cytotoxicity (Merkus, Schipper et al., 1991) et al., 1991). Another cyclodextrin-type enhancer (DM-β-CD) was also studied for comparison since it has been shown to be a very effective nasal absorption enhancer of insulin and other steroid hormones (Schipper et al., 1990; Merkus, Schipper et al., 1991). The concentration of DM-β-CD used in this study was set at 1.25% which was lower than that usually employed in the *in vivo* nasal absorption studies (2-5%). However, preliminary results have shown that the *in situ* nasal perfusion using lower concentrations of DM-β-CD can better maintain the viability of the rat throughout both perfusion periods.

Table 4 shows percent [D-Arg^2]-Kyotorphin remaining in the nasal perfusate at different times during the first 60-min nasal perfusion in the presence of various enhancers. The first set was a control group. The control solution contained only 0.5 mM [D-Arg^2]-Kyotorphin in an isotonic saline solution (no enhancer). From data in Table 4, there is very little absorption of [D-Arg^2]-Kyotorphin through the nasal mucosa from the control group after perfusion for 60 min (only about 1.9±6.7% absorbed). Perfusion with 5% HP-β-CD also shows very little dipeptide absorption with the same result as the control group (only 0.4 ± 2.3%). In contrast, 0.1% LCC shows 70.33 ± 8.4 percent drug remaining after perfusion for 60 min which is equivalent to about 30% absorption. Those of 0.5% CS J, 1.25% DM-β-CD and 0.5% CS G were 78.2 ± 12.5, 80.3 ± 7.7 and 83.2 ± 1.6%, respectively, which are equivalent to respective nasal absorption of 21.8, 19.7 and 16.8%.

enhancers	% remaining of [D-Arg^2]-Kyotorphin in the nasal perfusate					Apparent first order rate constant, k_{obs} (min)x10^3	n
	0 min	15 min	30 min	45 min	60 min		
Isotonic saline	100±0.0	97.1±3.9	97.7±3.9	97.7±6.0	98.1±6.7	-	8
0.5% CS G	100±0.0	97.5±1.3	94.3±3.6	87.0±2.6	83.2±1.6	3.39±0.22	4
0.5% CS J	100±0.0	98.8±5.7	89.7±7.1	81.5±17.4	78.2±12.5	4.80±3.40	4
0.1% LCC	100±0.0	90.4±8.3	81.4±7.3	79.0±7.8	70.3±8.4	5.66±1.58	3
1.25% DM-β-CD	100±0.0	94.7±6.0	90.4±4.6	86.0±6.5	80.3±7.7	3.61±1.30	4
5.0% HP-β-CD	100±0.0	97.7±3.5	98.3±7.3	100.4±3.6	99.6±2.3	-	7

Data = mean ± SD (n = 3-8 rats/group)

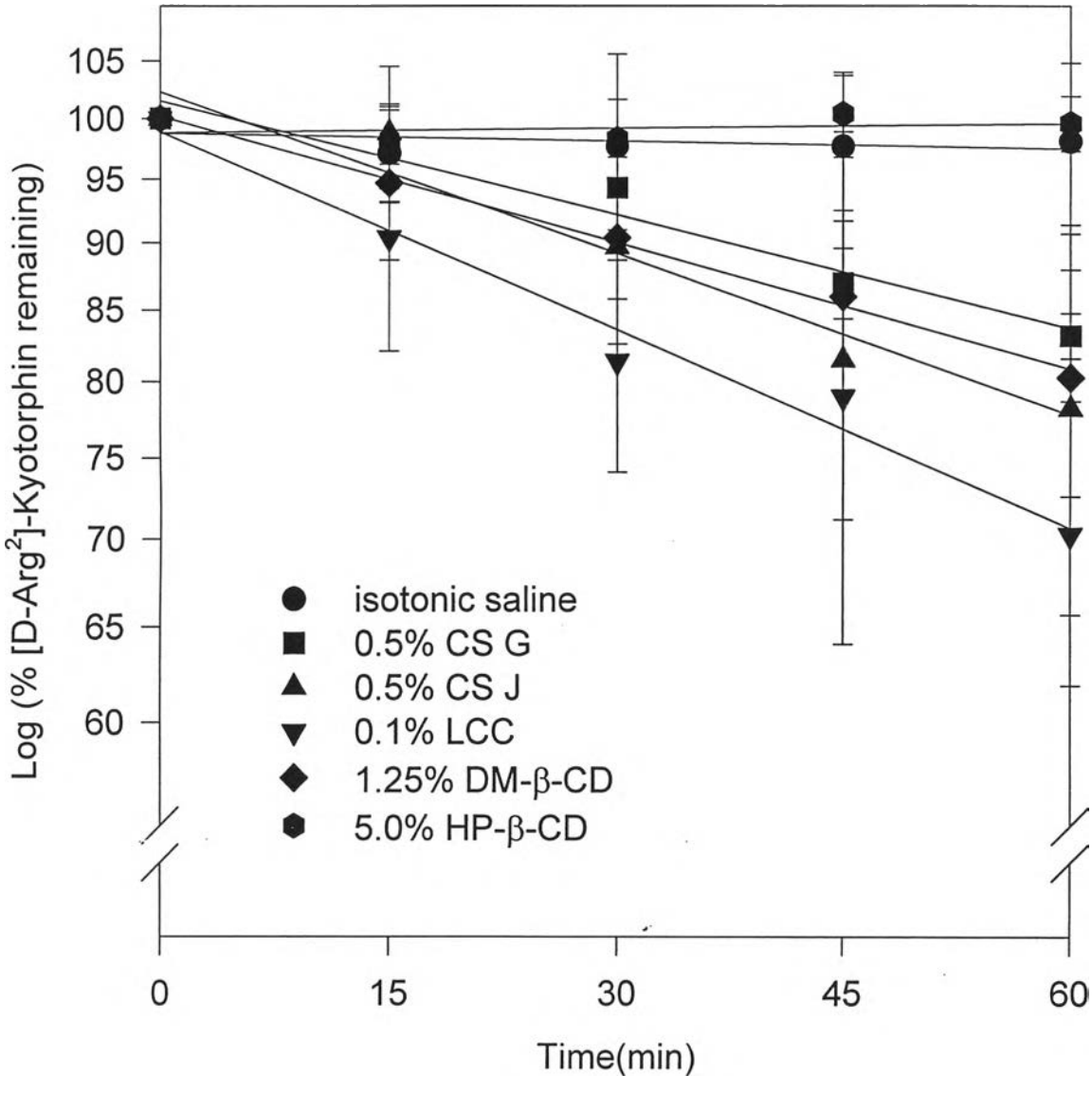
Table 4 The percent [D-Arg^2]-Kyotorphin remaining in the nasal perfusate during the first hour of nasal perfusion

Analysis of variance (ANOVA) was then applied to the data obtained from the studies at 5% significance level. From ANOVA table (Appendix IIa), it is obvious that there was a significant difference in the % remaining of drug in the perfusate at T_{60} of the first hour among the five enhancers and control group ($p < 0.05$). A multiple rank test (Duncan's test) was further applied in order to rank these differences at the same significance level. The ranking of these results, in an increasing order, are as follows:

	LCC	CS J	DM-β-CD	CS G	Control	HP-β-CD
Enhancer conc.	0.1%	0.5%	1.25%	0.5%	0%	5.0%
%Remaining at T_{60}	70.33	78.2	80.3	83.2	98.1	99.7

The line underneath the letters signifies that there was no significant difference between the enhancers on the same line ($p > 0.05$). Duncan's test result indicates that all the enhancers except HP-β-CD were significantly effective over the control group in enhancing the nasal absorption of this dipeptide. Moreover, 0.1% LCC appears to be more effective than 0.5% CS G whereas 0.5% CS J and 1.25% DM-β-CD gave an absorption enhancing effect intermediate between 0.1% LCC and 0.5% CS G. On the other hand, 5.0% HP-β-CD was not effective with the value of % remaining at T_{60} similar to that of control ($p>0.05$).

Figure 13 represents the semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusate at pH 6.0 versus time during the first period in the presence of each enhancer.



Value = mean ± SD (n=3-8 rats/group)

Figure 13 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusate versus time during the first period in the presence of various enhancers..

Derivation of the equation describing these plots was first characterized by Huang et al.(1985) and based on the first order transport kinetics, i.e. passive diffusion of drug across the absorptive membrane.

If loss of the drug from the perfusate appears to follow the first order kinetics, the Fick's first law of diffusion may be applied:

$$dm/dt = -DA \bullet dC/dx \quad \text{.....(1)}$$

where
- m = amount of solute present at time t
- dm/dt = rate of change of amount of solute in the perfusate
- D = diffusion coefficient of drug, cm^2 / time
- A = surface area of the nasal mucosa, cm^2
- dC/dx = concentration gradient across the nasal membrane barrier, conc/cm

The conventional unit for the amount and time in equation(1) are gm and sec, respectively. However, it was more preferable to express the time in term of a larger unit such as minute since the length of time involved in each experiment was 60 min. In addition, since all the solutions were prepared on a millimolar basis, the respective amount of the drug and concentration were thus expressed as mmole and mmole / liter (mM).

Since $dm/dt = V \bullet dC/dt$ .....(2)

Where dC /dt = rate of change of drug concentration in

the perfusate, mM/min

and V = volume of the perfusion medium, cm^3

It is possible, therefore, to rearrange equation (1) in terms of dC/dt using the following approximation:

$$dC/dx = \Delta C/\Delta x = Cd\text{-}Cr/h \quad \ldots\ldots\ldots\ldots\ldots\ldots(3)$$

Where h = thickness of the nasal membrane and associated unstirred aqueous layer, cm

Cd = concentration in the donor side, mM

= concentration of drug in the perfusion medium

Cr = concentration in the receptor side, mM

= concentration of drug in the blood

By substituting equation (2) and (3) in equation (1), equation (4) can be obtained

$$dC/dt = -(DA/hV)(Cd\text{-}Cr) \quad \ldots\ldots\ldots\ldots\ldots\ldots(4)$$

If sink conditions are assumed (Cd >> Cr), then equation (5) is obtained

$$dC/dt = -k_{obs}Cd \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(5)$$

Where $k_{obs} = DA/hV = k_{in}/V \quad \ldots\ldots\ldots\ldots\ldots\ldots(6)$

k_{in} = intrinsic rate constant for nasal absorption (cm^3/min) which is a parameter unique to the nasal mucosa

= DA/h

k_{obs} = apparent *in situ* first order absorption rate constant, min^{-1}

Equation (5) is similar to the equation which describes the first order elimination of the drug from a single compartment model following an intravenous bolus injection.

If equation (5) is integrated between the limits of C_0 and C, equation (7) is achieved.

$$InC = InC_0 - k_{obs} \bullet t \quad \text{.....(7)}$$

or $$Log\ C = LogC_0 - k_{obs} \bullet t/2.303 \quad \text{.....(8)}$$

By making a plot of log C as a function of time, a straight line would be obtained with a slope and an intercept of $-k_{obs}/2.303$ and $logC_0$, respectively. Table 4 also shows the mean and SD of k_{obs} which was calculated from the slope of the individual plot of the first perfusion period. The perfusion data of the individual rat as well as its k_{obs} value are provided in Appendix I. It can be seen from these data and Figure 13 that the perfusion profiles fairly followed the first order kinetics. All the semilogarithmic plots show good linearity with the regression coefficient in the range of 0.9-0.99 in most cases, Appendix I. The value of k_{obs} reflects the rate of nasal drug absorption. This parameter together with the percent [D-Arg^2]-Kyotorphin remaining at 60 min ($\%T_{60}$) after the first perfusion period were used for statistical comparison in order to determine the optimal enhancing activity for each enhancer. Since HP-β-CD was not effective, it was excluded from further comparison and only CS J, CS G, DM-β-CD and LCC were compared for their relative absorption enhancing activity.

One-way analysis of variance (ANOVA) was also applied to the values of k_{obs} and % T_{60} at the same significance level so as to compare the absorption enhancing

effect of the four enhancers i.e. CS G, CS J, LCC and DM-β-CD. It was found that there were no significant differences in the values of k_{obs} and % T_{60} ($p>0.05$, Appendix IIc).

Thus, the results from these experiments indicate that the four enhancers, 0.5% CS G, 0.5% CS J, 0.1% LCC and 1.25% DM-β-CD, have about the same ability in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin. However, the enhancing effect of 0.1 % LCC appears to be more pronounced than 0.5%CS G, especially when the Duncan's test was applied on %T_{60} of all the enhancer-treated groups and control.

Illum et al. (1994) proposed that the cationic nature of chitosan could have a transient effect on the gating function of the tight junctions leading to its enhancing effect on membrane permeability. 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 eletronegative sites (such as tight junctions) on a membrane which require coordination with cations (such as calcium) for dimensional stability. Removal of these " pivot " ions could result in a loosening or opening of the tight junction and increased absorption of drug via paracellular pathway. In addition, other unknown mechanisms of absorption enhancement may exist for chitosan such as its possible direct effects on the transcellular permeability.

Artursson et al.(1994) also suggested that chitosan may have a different mechanism of action in enhancing the transport of dipeptide across epithelial membranes to that displayed by "classical" materials in the form of surfactants and bile salts. The interaction between the apical membrane of the epithelial cell and

chitosan appears to be specific and saturable, as opposed to the non-specific and non-saturable effects seen for surfactant. LCC, one of the surfactants derived from acylcarnitines was reported by Kagatani et al.(1996) to have a critical micellar concentration (CMC) of about 0.029%, and that its enhancing effect reached maximum at 0.1% which is about 3 times the CMC at pH 4.0.

Kagatani et al.(1996) suggested that the contribution to the transport via inhibition of proteolytic enzyme by LCC seems low. Maximum enzyme inhibition by aprotinin (trypsin inhibitor) resulted in only 1.4 times enhancement in the rat nasal sCT absorption at pH 4.0 (Morimoto, Miyazaki and Kakemi, 1995) which is difficult to explain 9.5 times enhancement in the rat nasal sCT absorption in the same condition by LCC. Thus, micelle formation was considered to be an important factor in the mechanism of absorption enhancement by LCC on the nasal absorption of peptide drugs.

It is interesting to note that perfusion with 0.1% LCC was found to stimulate the release of some red blood cells from the nasal mucosa into the perfusate by the end of first hour. Thus, increasing concentration of LCC greater than 0.1% may cause more release of red blood cells which are indicative of severe membrane damaging effects. Although the enhancing effect of 0.1% LCC appears to be comparable or slightly superior to the two chitosans and DM-β-CD in this study, its potential toxicity must be carefully evaluated before any clinical testing can be conducted.

For HP-β-CD and DM-β-CD which are derivatives of cyclodextrin, the results show differences in their ability to increase the membrane permeability of dipeptide drug. HP-β-CD and DM-β-CD were found to have good solubilizing power

(exceeding 50% w/v) (Yoshida et al., 1988). Several mechanisms of action by which cyclodextrins can improve nasal peptide absorption have been suggested (Irie et al., 1992). For instance, cyclodextrins are able to interact with membrane lipids and proteins in the nasal epithelium, which may reduce the barrier function of the epithelium. Cyclodextrins can also inhibit proteolytic enzyme activities in the nasal mucosa and, finally, they may act directly upon the peptide or protein molecule thereby inhibiting aggregation of the molecules.

In this study, 1.25% DM-β-CD was significantly effective over the control in increasing the nasal membrane permeability, while 5% HP-β-CD was not. 5% DM-β-CD was reported to exert a direct effect on the nasal mucosa as evidenced by the release of several mucosal components which was much greater than that caused by 5%HP-β-CD (Shao, Krishnamoorthy and Mitra, 1992). In addition, the previous experiment demonstrated that the *in situ* nasal perfusion using 5% DM-β-CD could not maintain the viability of the rat throughout the two perfusion periods because of the bleeding of nasal mucosa. This membrane-damaging mechanism may be important in promoting the nasal drug absorption in a manner similar to 0.1% LCC. Therefore, the concentration of DM-β-CD was limited to only 1.25% in all the perfusion experiments to avoid excessive damage to the rat nasal mucosa and its vitality.

Since the nasal permeation enhancement mechanisms of these enhancers may be different, they should be further investigated in terms of the efficacy and toxicity as well as the extent of membrane reversibility.

1.2) Comparison of the membrane-damaging effect of different enhancers based on the extent of LDH release.

Table 5 shows the content (U/ml) of lactate dehydrogenase (LDH) that was released from the rat nasal mucosa during the first hour of *in situ* perfusion in the presence of various enhancers.

These data reveal that perfusion with only the isotonic saline pH 6.0 (control group) resulted in very low levels of LDH release. At the end of the first hour, the average LDH content was 38.33 ± 9.93 U/ml. The value of the control group agreed well with the results of Shao and Mitra (1992) who reported that nasal perfusion with only isotonic phosphate buffer (pH 7.4) for 90 min gave LDH release of 33.0 ± 18.0 U/ml.

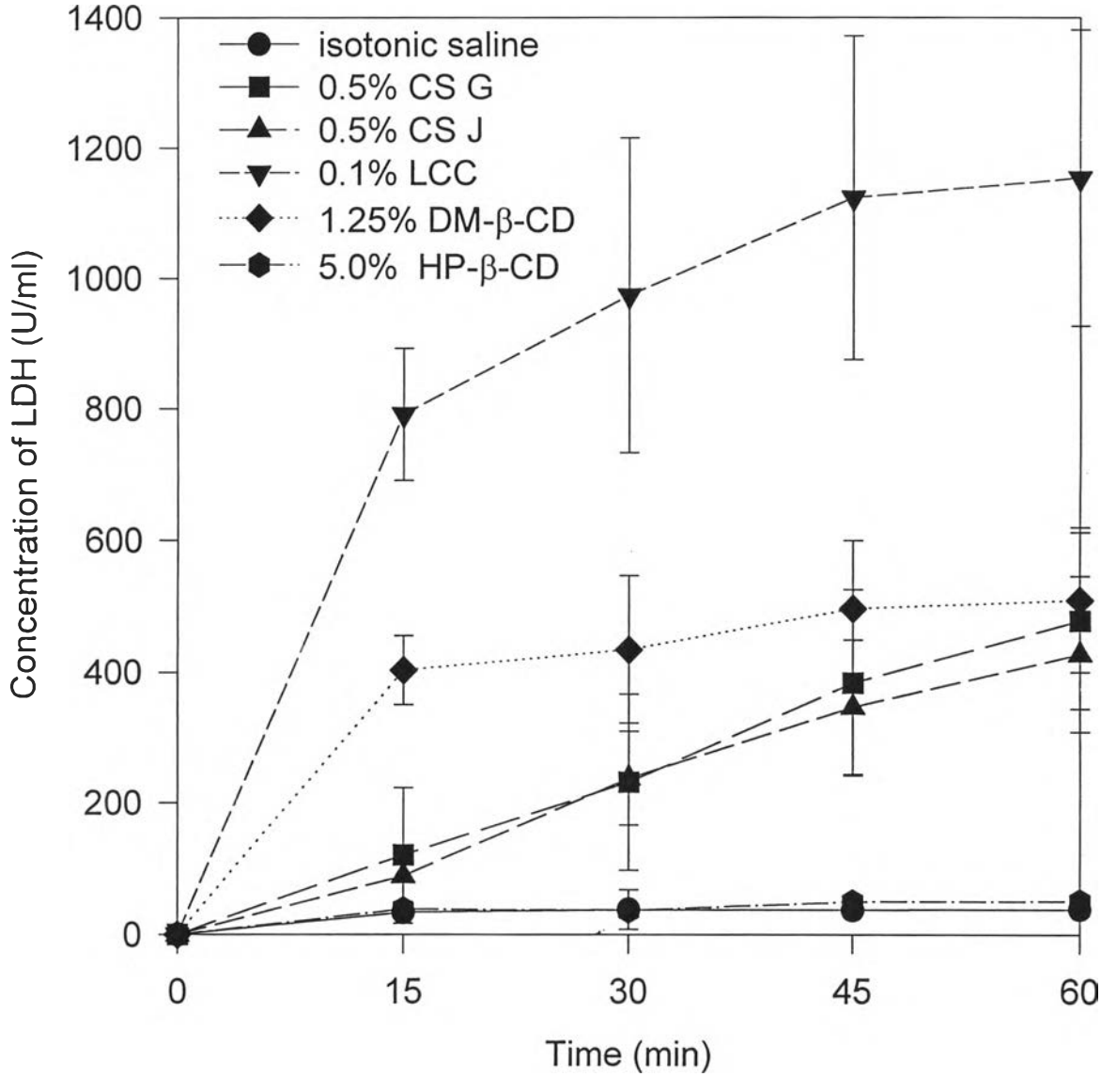
Perfusion with 5% HP-β-CD yielded the same results as that of control, i.e., there was very small amount of LDH release at the end of the first perfusion period, with the average content at 60 min of 50.15 ± 8.93 U/ml. On the other hand, perfusion with other enhancers (0.5% CSJ, 0.5% CSG, 0.1% LCC and 1.25% DM-β-CD) resulted in substantial release of LDH in the nasal perfusates. The LDH contents at the end of first hour were 427.87 ± 118.74, 478.91 ± 133.96, 1154.40 ± 226.93 and 510.43 ± 109.53 U/ml for CS J, CS G, LCC and DM-β-CD, respectively .

The release profiles of LDH during the first hour are shown in Figure 14. When ANOVA was applied at 5% significance level to the values of LDH at the end of the first hour, significant difference was detected among the six groups under study ($p << 0.05$), Appendix VIb. Multiple comparison using Duncan's test was further employed in order to rank the effect. The results, in an increasing order, are as

enhancers	Concentration (U/ml) of LDH in the nasal perfusate				n
	15 min	30 min	45 min	60 min	
Control group	33.33±16.33	37.67±30.04	37.23±9.43	38.33±9.93	4
0.5% CS G	120±103.42	231.95±135.15	384.12±142.04	478.91±133.96	4
0.5% CS J	88.34±46.31	238.11±72.24	347.04±102.77	427.87±118.74	4
0.1% LCC	791.67±101.16	974.26±241.03	1,123.74±248.00	1,154.40±226.93	4
1.25% DM-β-CD	403.33±52.35	434.89±112.27	497.08±102.83	510.43±109.53	4
5.0% HP-β-CD	38.33±6.39	36.38±11.26	49.31±9.49	50.15±8.93	4

Data = mean ± SD (n = 4 rats/group)

Table 5 Concentration (U/ml) of lactate dehydrogenase (LDH) that released from the rat nasal mucosa during the first hour of *in situ* perfusion in the presence of various enhancers.



Value = mean ± SD (n = 4 rats/group)

Figure 14 The release profiles of LDH concentration in rat nasal perfusates versus time during the first period in the presence of various enhancers.

follows:

	Control	HP-β-CD	CS J	CS G	DM-β-CD	LCC
Enhancer conc.	0%	5.0%	0.5%	0.5%	1.25%	0.1%
LDH (U/ml)	38.33	50.15	427.87	478.91	510.43	1,154.40

The lines underneath the enhancers indicate that there was no significant difference in the LDH content at the end of the first hour among the groups on the same line ($p > 0.05$). Therefore, Duncan's test results reveal that HP-β-CD caused minimal leakage of the enzyme and its effect was similar to saline control. This was also in agreement with Shao et al. (1992) and Marttin et al. (1995) who reported that 5% HP-β-CD was one of the least membrane damaging enhancers, giving minimal release of mucosal components. On the other hand, the other four enhancers caused significantly greater extent of LDH release than the control and 5% HP-β-CD ($p < 0.05$). Furthermore, LCC appears to induce significantly greatest extent of LDH release (Slight bleeding was observed in the nasal perfusates of the LCC-treated group but all the rats retained their viability throughout both perfusion periods).

It is interesting to note that the ranking of LDH release at T_{60} bear some similarity that of %[D-Arg^2]-Kyotorphin remaining at T_{60}. 5% HP-β-CD which is not an effective enhancer also caused very small release of LDH whereas 0.1% LCC, which gave the highest [D-Arg^2]-Kyotorphin absorption, also induced the greatest extent of LDH release. It thus appears that there may be some correlation between the absorption enhancing property and membrane-damaging effect among these enhancers.

Figure 15 is a plot between the average percent [D-Arg^2]-kyotorphin remaining at the end of the first perfusion and the average content of LDH (U/ml)

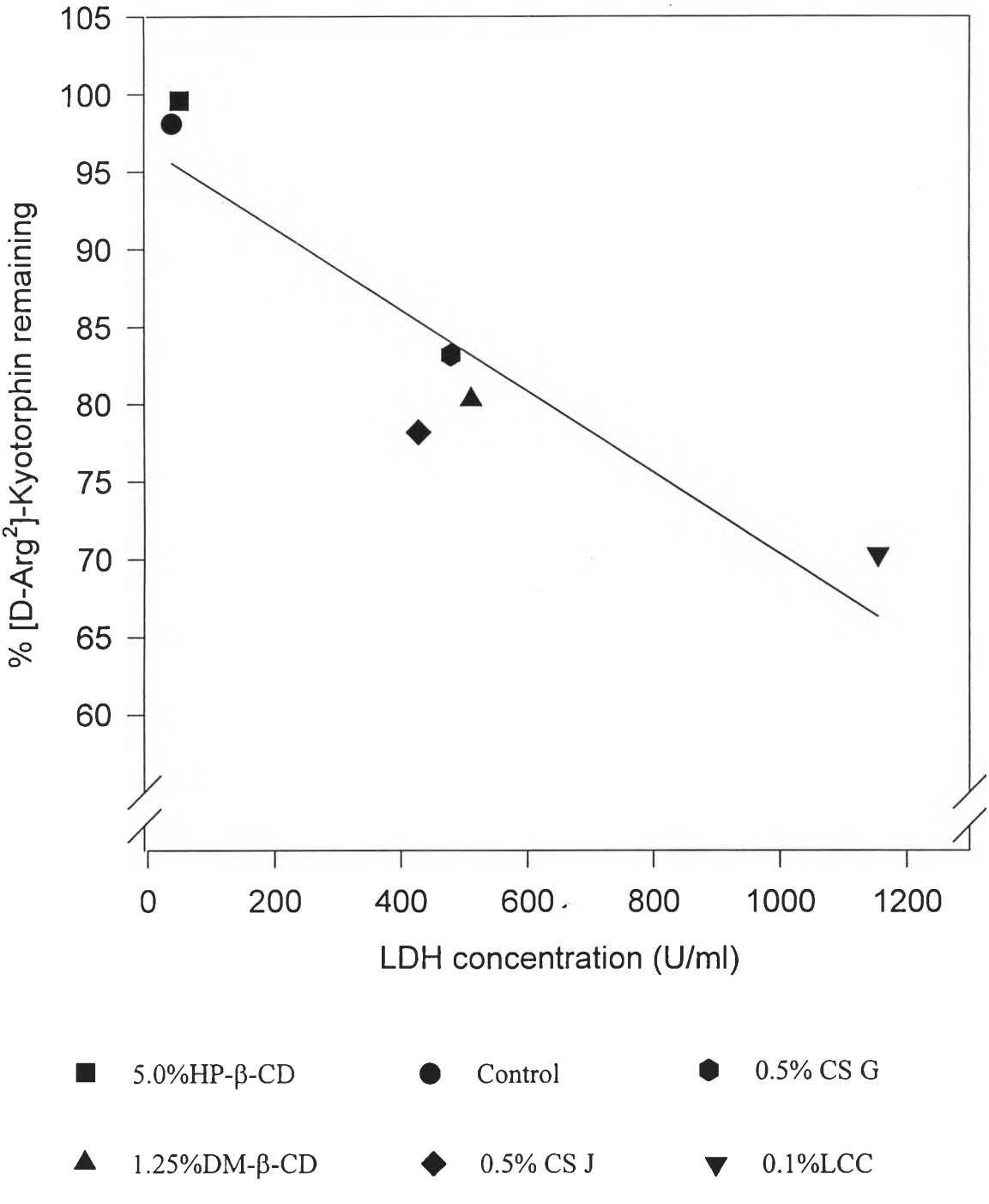


Figure 15 The average percent [D-Arg^2]-Kyotorphin remaining and the average content of LDH (U/ml) release into nasal perfusate at the end of the first perfusion period.

released into the nasal perfusate at the same time point. From this figure it can be seen that significant correlation between the two parameters was observed with the correlation coefficient of 0.9215 ($p < 0.05$), Appendix XI. 0.1% LCC, which induces the highest LDH release, also gives the highest nasal absorption of [D-Arg^2]-kyotorphin. On the other hand, the saline and 5%HP-β-CD-treated groups show very poor absorption of the dipeptide in agreement with the very low level of LDH release at 60 min. 1.25% DM-β-CD, 0.5% CS J and 0.5% CS G exhibit the average values of both percent dipeptide and the LDH content at 60 min in the intermediate region of the correlation line. Since LDH is an intracellular enzyme, correlation with the dipeptide absorption suggests that all the enhancers under study may somehow be able to directly interact with the nasal membrane which may lead to the nasal absorption of [D-Arg^2]-Kyotorphin by a transcellular pathway. The greater the degree of enhancer-membrane interaction, the greater the extent of [D-Arg^2]-Kyotorphin absorption via this route. The exact mechanisms by which these enhancers can directly interact with the nasal membrane are not fully known. However, LCC was reported to interact with the membrane by micelle formation (Kagatani et al., 1996) whereas cyclodextrins were found to be able to extract phospholipids and cholesterol from the nasal membrane, probably by forming inclusion complexes (Shao et al.,1992; Marttin et al.,1995). Although chitosans have previously been suggested to enhance the nasal absorption via paracellular pathway (Illum et al., 1994), evidence of direct membrane interaction has been reported by Sahamethapat (1996) who observed that both CS J and CS G at 0.1% were able to cause some small release of membrane phospholipids after *in situ* nasal perfusion. Therefore, correlation results appear to suggest that the enhancers under study, especially 1.25% DM-β-CD, 0.1% LCC, 0.5% CS J and 0.5% CS G may be able to enhance the nasal absorption of [D-Arg^2]-Kyotorphin to some extent by the transcellular pathway. The enhancing activity of each enhancer via this route can be further differentiated based on its effect on the

LDH release. However, it should be reminded that transcellular route is just one of the two major absorption pathways. No information is available regarding the different abilities of these enhancers in promoting the nasal absorption of peptides via the paracellular pathway.

1.3) Comparison of reversible effect of different enhancers on the nasal membrane integrity and mucosal permeability

A desirable feature of any absorption enhancer is that the compound in question must act only temporarily on the absorptive epithelium. Recovery of the nasal mucosa should occur in due time following removal of the absorption enhancers from the application site. In this part of the study the reversible effect of different enhancers on the nasal mucosa was investigated in terms of both mucosal permeability and membrane integrity using the *in situ* nasal perfusion. As in the previous part, absorption of [D-Arg^2]-Kyotorphin was used as an indicator of mucosal permeability whereas the release of LDH in the nasal perfusate implicated the loss of nasal membrane integrity. At the end of the first perfusion period, the nasal cavity of each rat was flushed with isotonic saline to remove any residual enhancer of the previous perfusion. Then, the perfusion was restarted using only [D-Arg^2]-Kyotorphin in isotonic saline as the perfusing solution.

Figures 16 to 20 represent the release profiles of LDH in both perfusion periods for each enhancer and the control group. The data are also shown in Table 6. From these figures it can be seen that the release of LDH during the second perfusion period, after the enhancer had been removed from the nasal cavity, markedly decreased from the first perfusion period. This reduction in LDH release was

	n	1st hour				2nd hour				%ΔC_{60}
		(min)15	30	45	60	(min)15	30	45	60	
Control group	4	33.33 ±16.33	37.67 ±30.04	37.23 ±9.4.3	38.33 ±9.93	33.33 ±17.64	46.84 ±38.50	58.40 ±34.97	70.20 ±37.04	74.07 ±55.76
5.0% HP-β-CD	4	38.33 ±6.39	36.38 ±11.26	49.31 ±9.49	50.15 ±8.93	16.67 ±8.61	22.27 ±10.24	48.05 ±35.60	49.73 ±19.93	8.07 ±68.35
1.25% DM-β-CD	4	403.33 ±52.35	434.89 ±112.27	497.08 ±102.83	510.43 ±109.53	38.33 ±16.67	57.29 ±16.08	86.57 ±37.26	76.36 ±37.40	-84.72 ±7.65
0.1%LCC	4	791.67 ±101.16	974.26 ±241.03	1,123.74 ±248.00	1,154.40 ±226.93	48.33 ±10.00	68.09 ±19.79	75.05 ±22.10	102.23 ±22.10	-91.08 ±1.64
0.5% CS J	4	88.34 ±46.31	238.11 ±72.24	347.04 ±102.77	427.87 ±118.74	75.00 ±23.33	114.55 ±22.52	129.78 ±24.28	153.63 ±34.22	-62.99 ±7.45
0.5% CS G	4	120.00 ±103.42	231.95 ±135.15	384.12 ±142.04	478.91 ±133.96	78.33 ±38.25	121.27 ±66.15	133.28 ±58.37	170.52 ±78.56	-64.79 ±13.62

Data : mean ± SD (n = 4 rats/group)

%ΔC_{60} = Percentage change in conc. LHD at the end of 2nd hour relative to that of 1st hour

$= \Delta C_{60}/C_1 x100\%$

$= [(C_2-C_1)/C_1]x100\%$

Table 6 Comparison of the LDH release profiles between the first perfusion period (with enhancer) and the second perfusion period (without enhancer).

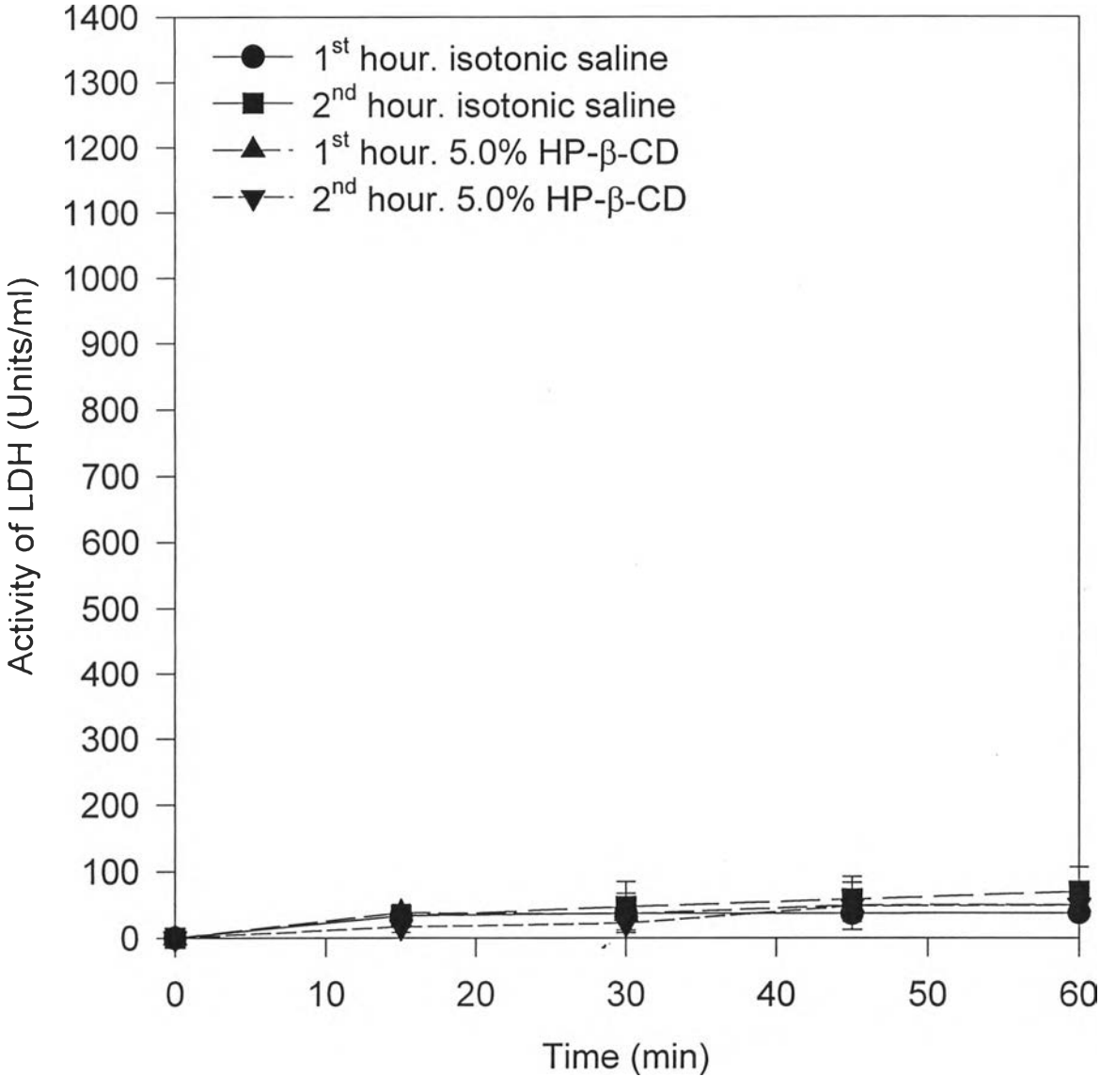


Figure 16 The release profiles of LDH in both perfusion periods for 5.0% HP-β-CD and control group.

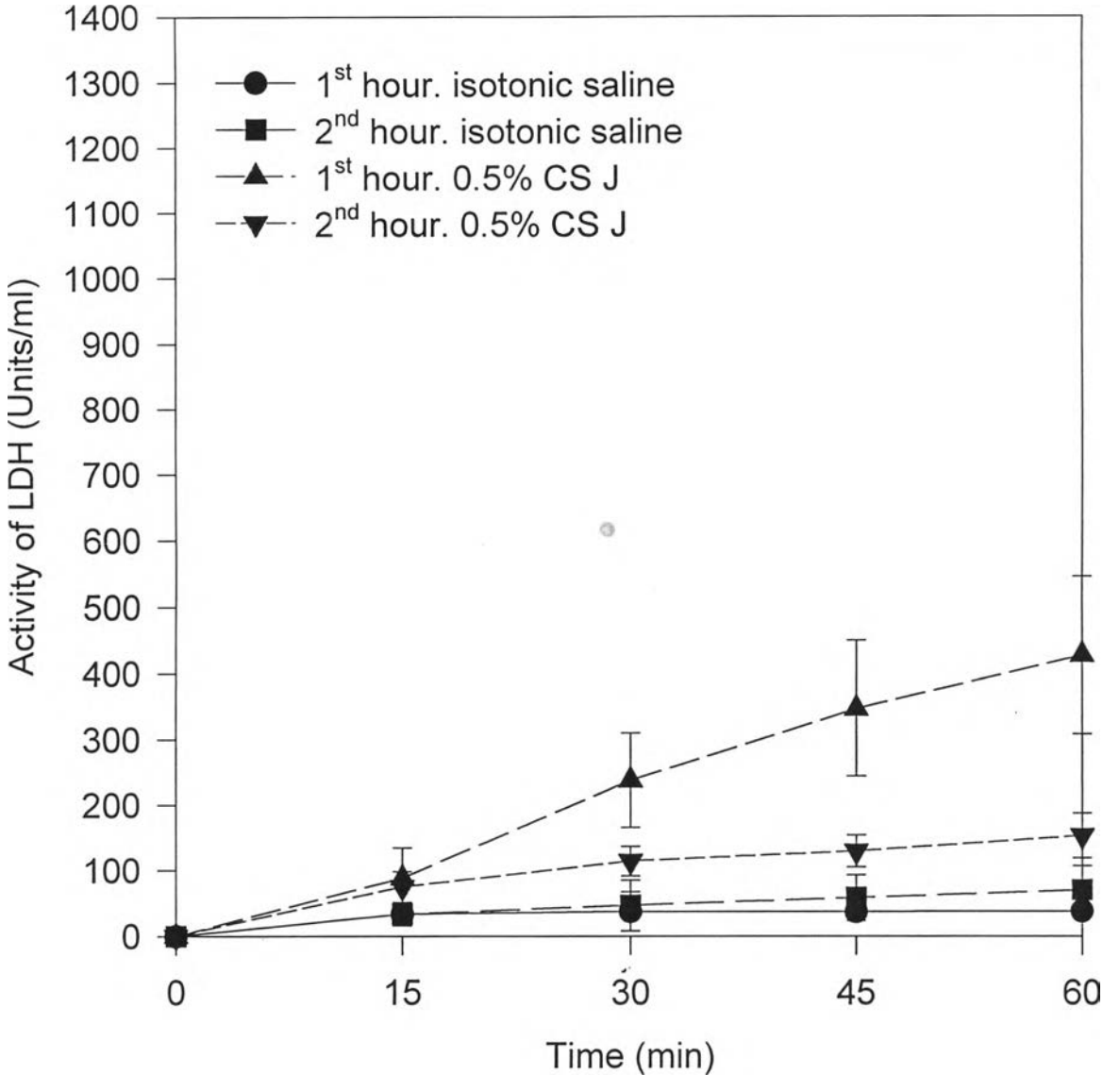


Figure 17 The release profiles of LDH in both perfusion periods for 0.5% CS J and control group.

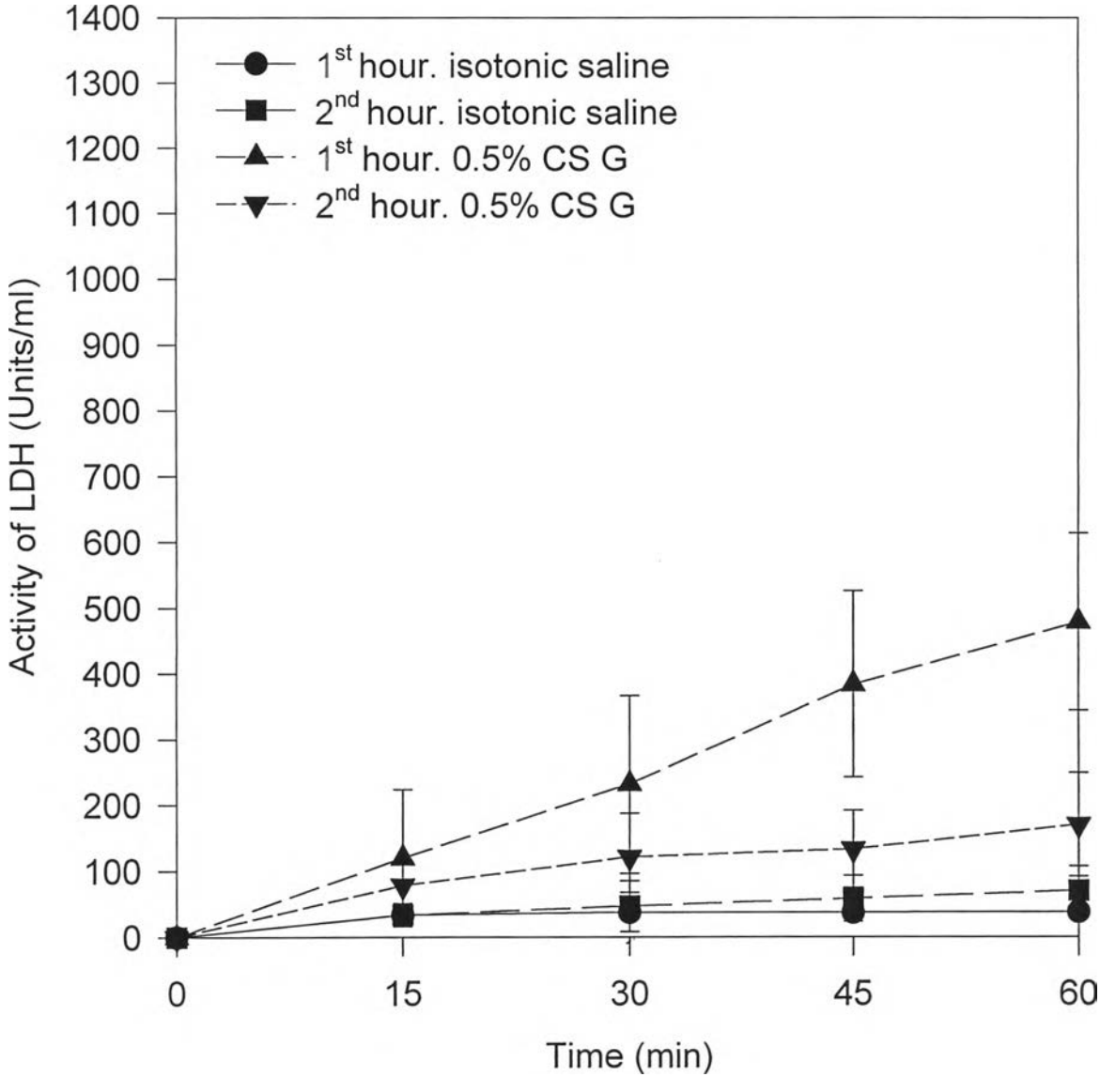


Figure 18 The release profiles of LDH in both perfusion periods for 0.5% CS G and control group.

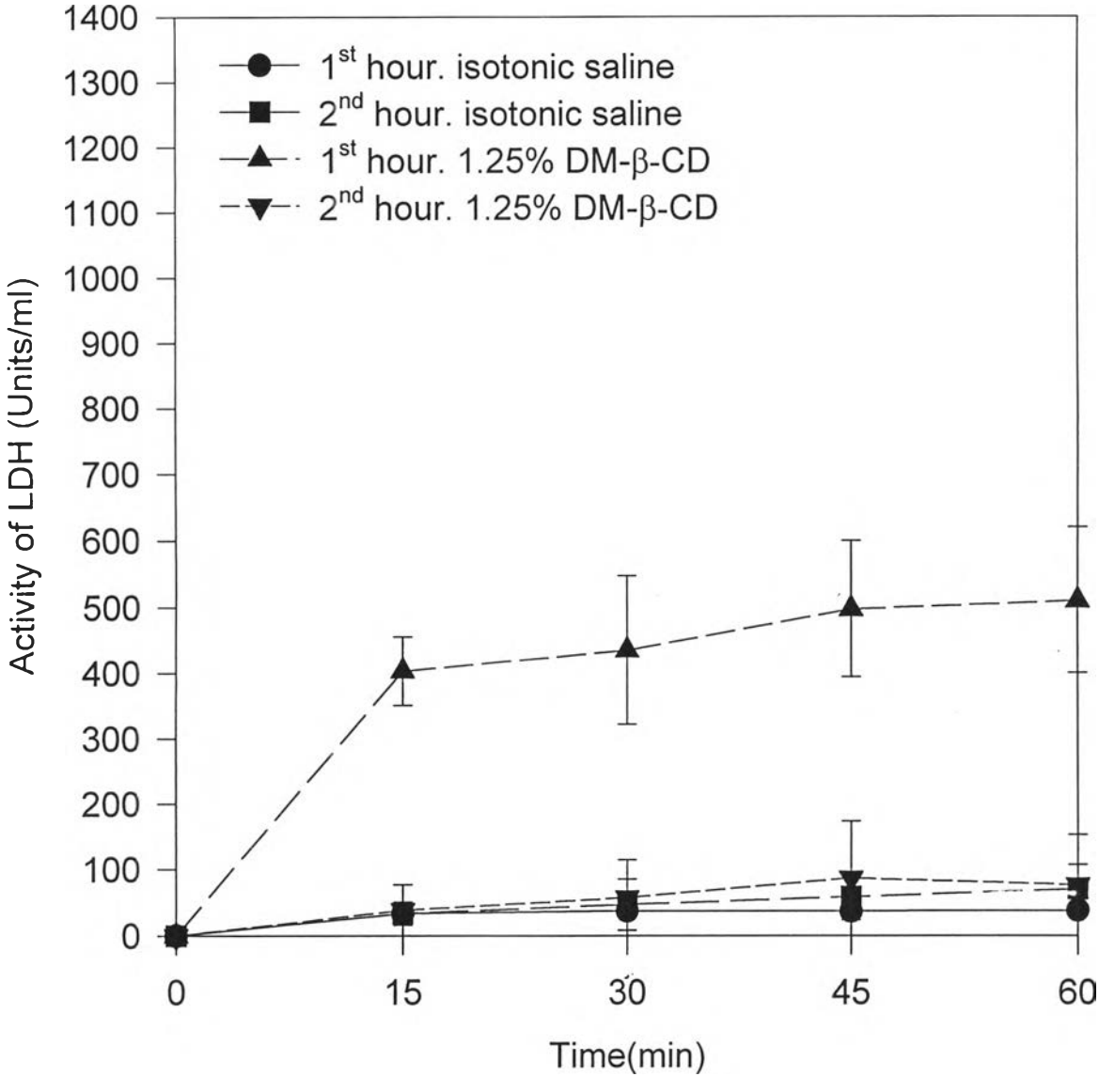


Figure 19 The release profiles of LDH in both perfusion periods for 1.25% DM-β-CD and control group

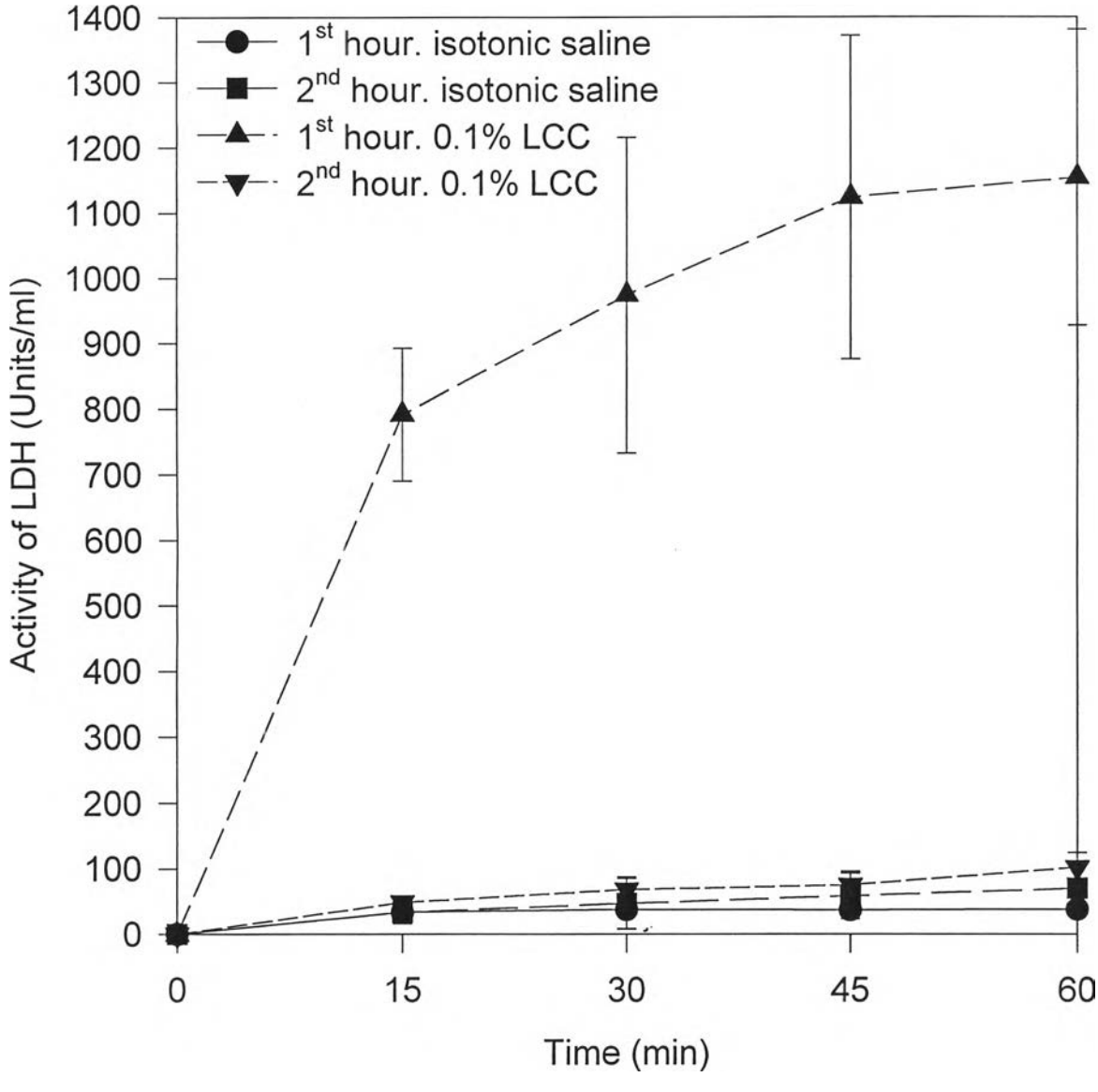


Figure 20 The release profiles of LDH in both perfusion periods for 0.1% LCC and control group

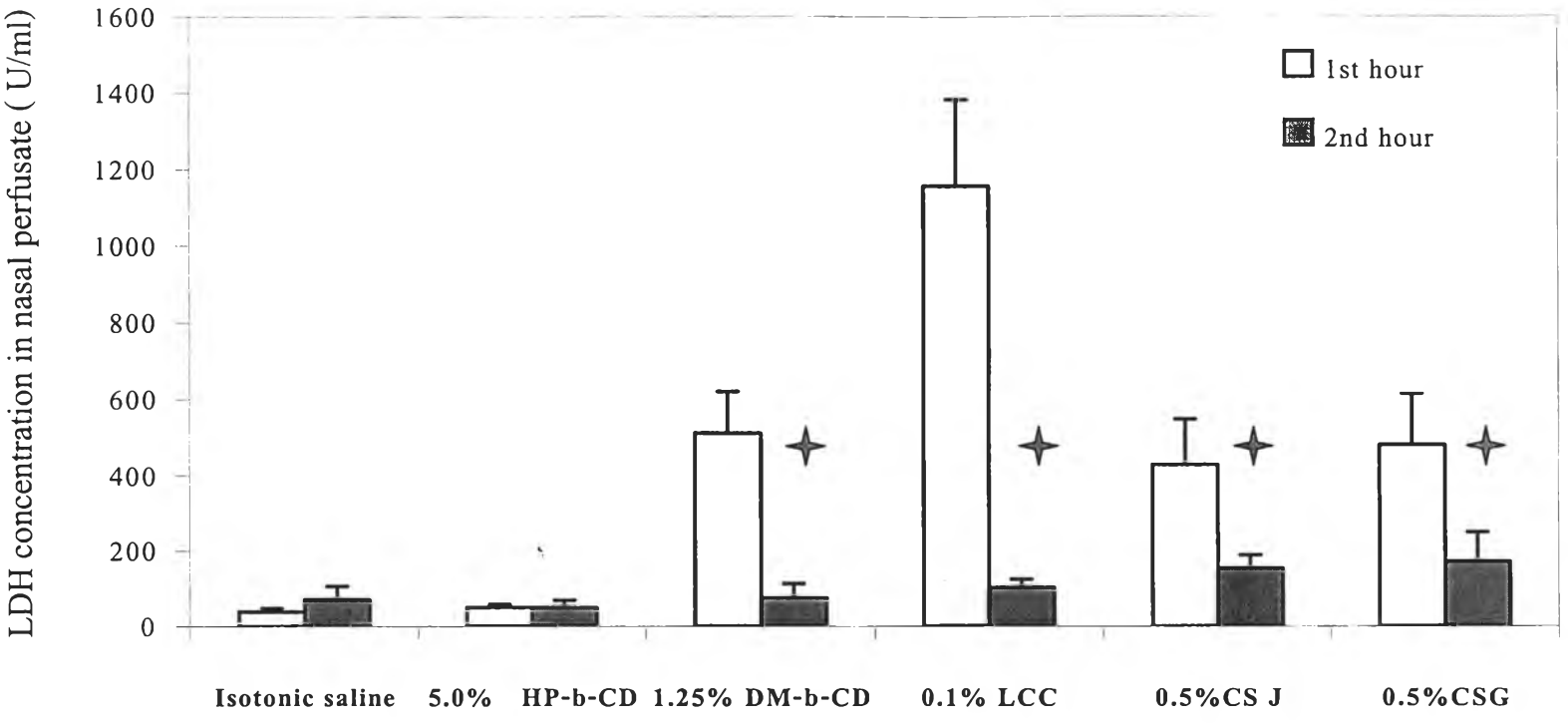
observed in most of the enhancer-treated groups, except for the control and 5% HP-β-CD, which gave similar LDH release profiles regardless of the perfusion period.

Comparisons were then made between the LDH content at the end of the first and second perfusions (T_{60}) using paired Student's t-test at 5% significance level for each treatment group, Appendix VII. As expected, there were no significant differences in the LDH content at T_{60} for the control (1st hr = 38.33 ± 9.93 vs 2nd hr = 70.20 ± 37.04 U/ml) and HP-β-CD-treated groups (1st hr = 50.15 ± 8.93 vs 2nd hr = 49.73 ± 19.93 U/ml) ($p > 0.05$, paired Student's t-test). However, there were significant differences in the concentrations of LDH at T_{60} between the first and second periods for all the other enhancers, i.e. 0.5% CS J, 0.5% CS G, 1.25% DM-β-CD and 0.1% LCC ($p < 0.05$, paired Student's t-test).

After removing CS J from the rat nasal cavity, the extent of LDH release at T_{60} dropped nearly three folds from 427.87 ± 118.74 to 153.63 ± 34.22 U/ml. In the case of CS G, the LDH content decreased from 478.91 ± 133.96 to 170.52 ± 78.56 U/ml. The values for LCC and DM-β-CD also reduced sharply from 1,154.40 ± 226.93 to 102.23 ± 22.10 and from 510.43 ± 109.53 to 76.36 ± 37.40 U/ml, respectively. Therefore, it is apparent that 0.5% CS J, 0.5% CS G, 1.25% DM-β-CD and 0.1% LCC demonstrated some degree of reversible effect on the nasal membrane integrity. On the other hand, 5% HP-β-CD and the control groups failed to produce such an effect. However, the lack of membrane reversibility observed with the control and the HP- β-CD-treated groups is clearly a result of their intrinsically weak interaction with the nasal membrane. Results from the first part of study has previously shown that the two groups failed to produce any noticeable absorption of [D-Arg^2]-Kyotorphin during the first perfusion period. They also induced similar

minimal release of LDH into the nasal perfusates. Consequently, the second perfusion in which no enhancer was present would hardly have any effects on the nasal mucosa and it was found that the LDH contents were not different from the first perfusion ($p > 0.05$). Summary of the extent of LDH release at the end of both perfusion periods is Histogram represented in Figure 21 for all the enhancer-treated and the control group.

The significant decrease in LDH content after removal of CS J, CS G, LCC and DM-β-CD indicates that changes in the membrane integrity as modified by these enhancers is reversible and that the mucosa is able to return to its normal original state. Rapid decrease in LDH release should not be caused by depletion of the LDH content of the nasal epithelium since greater amount of LDH could be released from the nasal mucosa upon exposure to higher concentrations or different types of enhancers. For example, Shao et al. (1992) reported that 5% DM-β-CD was able to induce a much higher release of LDH. Accumulation of LDH was observed to increase linearly with time, reaching 6,120 U/ml after 90 min-perfusion. 0.1% LCC, the most membrane-perturbing enhancer used in this study, was shown to cause maximum LDH accumulation of only 1,230 U/ml. Thus, the decrease in LDH should rather be caused by rapid normalization of the nasal membrane. Rapid recovery of the mucosal integrity has been previously reported by Erickson (1988) who observed that mucosal injury to the rat small intestinal mucosa by bile salt, chenodeoxycholate, was reversed within 1 hr following its removal. The author suggested that this rapid repair involves (a) villus shortening which reduces the surface area of injury and (b) epithelial cell migration to cover the injured area (Moore, Carlson and Madara, 1989a and 1989b). However, the complex mechanisms by which the nasal mucosa can recover itself remain to be investigated.



✦ Significant from 1st hr ($p < 0.05$, paired student's t-test)

Figure 21 Histogram comparing the LDH content release into the nasal perfusate at the end of both perfusion periods

Since the absorption of [D-Arg^2]-Kyotorphin was correlated with the LDH release, leakage of this enzyme from the cytosol into the nasal perfusates could be a result of an increase in the membrane permeability and/or disruption of the epithelial cells caused by the absorption enhancers. In the preliminary study, it was found that perfusion of DM-β-CD at concentration equal to or higher than 2.5% caused bleeding of the rat nasal cavity, indicating severe disruption of the nasal epithelium and underlying endothelium. This was in agreement with the strong hemolytic activity of DM-β-CD (Yoshida et al., 1988). At 5% concentration, DM-β-CD caused rapid bleeding of the rat nasal epithelium within 10 min after perfusion. The rats often died prematurely and the perfusion experiments could not be conducted successfully at this concentration. Perfusion of 0.1% LCC also induced slight bleeding but the extent was much smaller and the rat viability was maintained throughout both perfusion periods. However, bleeding resulted in a sharp rise in LDH level which surpassed all the other enhancers (Figure 14). On the other hand, perfusion with a lower concentration of DM-β-CD (1.25%), 5% HP-β-CD and the two chitosans (0.5%) did not cause any bleeding and the LDH release was accordingly lower than that by 0.1% LCC ($p < 0.05$, Duncan's test on LDH at the end of the first perfusion (T_{60})). Thus, it is apparent that the level of membrane interaction exerted by these enhancers at the concentrations studied was milder than 0.1% LCC. Furthermore, Duncan's test result also revealed that the effects of 1.25% DM-β-CD, 0.5% CS J and 0.5% CS G on LDH release at T_{60} were not different from each other ($p > 0.05$) but were significantly greater than the control and HP-β-CD-treated groups ($p < 0.05$). However, when ANOVA and subsequent Duncan's test were applied on the LDH contents at 15 min perfusion (T_{15}) (AppendixVIa), 1.25% DM-β-CD was found to give greater LDH release than the two chitosans, with the following ranking result:

Enhancers	control	HP-β-CD	CS J	CS G	DM-β-CD	LCC
Conc.	0%	5.0%	0.5%	0.5%	1.25%	0.1%
LDH at T_{15} (U/ml)	33.33	38.33	88.33	120	403.33	791.67

Thus, even at a lower conc. of 1.25%, DM-β-CD may still be able to cause greater membrane irritation than the two chitosans, at least during the initial period of perfusion. Also, it is interesting to note that other types of enhancers such as bile salts and synthetic surfactants can cause a much greater LDH release and thus are potentially more membrane irritating than all the enhancers used under this study. For example, Shao et al.(1992) reported that nasal perfusion of sodium deoxycholate resulted in LDH release at 90 min of 12,600 U/ml which is about 10 times the value observed for 0.1% LCC at 60 min (1,154 U/ml). Therefore, the extent of membrane interaction and epithelial damages could vary widely among different enhancers, depending on their chemical structure and concentration (Marttin et al., 1995).

Regardless of the severity of membrane interaction, however, the nasal mucosa appears to recover itself quite rapidly, as determined from the percent recovery of the nasal membrane integrity or %ΔC. %ΔC is defined as percent reduction in the extent of LDH release at T_{60} of the second perfusion relative to that of the first perfusion. It is calculated from the formula:

$$\%\Delta C = [(C_2-C_1)/C_1] \times 100\ \%$$

where C_1 and C_2 are the contents of LDH at the end (T_{60}) of the first and second perfusion, respectively.

In the case of LCC reversal of membrane integrity was nearly complete within 1 hr (close to 100%), indicating a very rapid recovery after enhancer removal with average %ΔC of -91.08 ± 1.64 %. (The minus sign implies the reduction in LDH content). 1.25% DM-β-CD also gave rapid recovery with average %ΔC of -84.72 ± 7.65 % within an hour after its removal. The two chitosans, CS J and CS G at 0.5% each, showed slightly lower membrane recovery at one hour, with average %ΔC of -62.99 ± 7.45 % and -64.79 ± 13.62 %, respectively. On the contrary, the positive values for %ΔC observed with the control and 5% HP-β-CD groups indicated respective increases in LDH of 74.07 ± 55.76% and 8.07 ± 68.35%. The data agree well with the paired Student's t-test result that these two groups showed no sign of membrane reversibility due to their poor membrane interaction, Appendix VII.

The four enhancers which demonstrated significant reversible effect on the LDH release, i.e. 0.1% LCC, 1.25% DM-β-CD, 0.5% CS J and 0.5% CS G, were then compared together with regard to extent of membrane recovery by applying one-way ANOVA on the values of %ΔC at 5% significance level, Appendix VIII. ANOVA results indicated a significant difference in %ΔC values. Duncan's new multiple range test was further applied at 5% level with the following ranking result:

	0.1% LCC	1.25% DM-β-CD	0.5% CS G	0.5% CS J
%ΔC	-91.08	-84.72	-64.79	-62.99

The line joining each enhancer indicates that there was no significant difference in %ΔC among the enhancers on the same line. Therefore, the Duncan's test result suggests that the four enhancers can be classified into two groups of different membrane reversibility. The first group consists of LCC and DM-β-CD which showed significantly more rapid membrane recovery than the second group

which consists of the two chitosans ($p < 0.05$). The percentage recovery was similar between the two enhancers within the same group ($p > 0.05$).

Interestingly, results from previous parts have shown that 0.1% LCC and 1.25% DM-β-CD interacted with the rat nasal membrane more strongly than 0.5% CS J and CS G as judged from the extent of LDH release during the first perfusion period. Hence, it had previously been expected that recovery of membrane integrity for LCC and DM-β-CD following their removal should have occurred more slowly than the two chitosans which exhibited milder membrane interactions and, in turn, should have had faster recovery. However, as can be seen the from the above Duncan's test result, LCC and DM-β-CD demonstrated rapid membrane recovery, even better than CS J and CS G.

The reasons as to this observation are not clearly known. Nevertheless, it may involve the mucoadhesive properties of chitosans. Artursson et al. (1994) suggested that chitosans possess fairly good mucoadhesion via an electrostatic attraction between the positively charged chitosan molecule and the negatively charged sialic acid residues of the mucus. After flushing the nasal cavity with isotonic saline, it is possible that a residual amount of chitosan may remain in the nasal mucosa which could lead to some extent of membrane interaction. On the contrary, LCC and DM-β-CD are not mucoadhesive polymers. Their removal from the nasal cavity by flushing with saline appeared to be complete, thereby allowing the membrane to recover rapidly without concomitant interferences from the enhancer. This rapid recovery could even be observed during the first perfusion period. Figures 19 and 20 show that LDH level increased sharply during the first 15 min when rats were nasally perfused with respective solutions of 1.25% DM-β-CD and 0.1% LCC. After 15 min, however, the rise in LDH concentration appears to level off. This may indicate that

some degree of nasal membrane recovery was already occurring during the first perfusion period in the presence of the enhancer, leading to saturation of the LDH level. After their removal, the extent of LDH release was reduced to the same level as saline for both enhancers (Figures 19 and 20). On the other hand, Figures 17 and 18 indicate that removal of 0.5% CS J and CS G from the nasal cavity showed a slower membrane recovery rate. Comparison of LDH content at the end of the second perfusion (T_{60}) revealed that there was a significant difference among the five enhancers and the control group (one-way ANOVA, $p < 0.05$), Appendix VIc. Subsequent Duncan's test gave the following ranking result:

	HP-β-CD	Control	DM-β-CD	LCC	CS J	CS G
Enhancer conc.	5%	0%	1.25%	0.1%	0.5%	05%
LDH at T_{60}	49.73	70.20	76.36	102.23	153.63	170.52

After removal of DM-β-CD and LCC, the release of LDH in the nasal perfusates dropped to the level not significantly different from the control (isotonic saline) and HP-β-CD-treated groups ($p > 0.05$). The similarity in LDH contents suggests that removal of DM-β-CD and LCC resulted in a near complete membrane recovery. On the other hand, the values for CS J and CS G are significantly greater than the control and the other enhancers ($p < 0.05$), indicating the residual effects of chitosans which still persisted long after their removal from the nasal cavity. As previously suggested, this could be due to their mucoadhesive nature. Another possibility is that, despite their relatively milder interactions with the nasal membrane, the effects of chitosans could be more sustained than those of 0.1% LCC and 1.25% DM-β-CD. This may account for a low but gradual increase in LDH during the first perfusion and a slower recovery after their removal in the second period. Nevertheless, more studies are needed to support this hypothesis. Also, whether such

sustained effects are associated with their mucoadhesive properties are not presently known. In addition, rapid reversal of membrane integrity may be observed for LCC and DM-β-CD at only low concentrations as utilized here. Shao et al.(1992) reported that nasal perfusion with 5% DM-β-CD resulted in a linear increase in LDH level throughout the 90 min-perfusion, with a very high LDH level at the end of perfusion (6,120 U/ml). Preliminary perfusion studies with higher concentrations of LCC and DM-β-CD also resulted in substantial bleeding of the nasal mucosa. It is therefore possible that the extent of membrane interaction exerted by higher concentrations of LCC and DM-β-CD could be too severe to allow for rapid recovery of the nasal membrane.

The above discussion was based on the reversible effects of the enhancers on the nasal membrane integrity, as determined from the extent of LDH release. Recovery of the nasal mucosa was also evaluated in terms of its permeability to peptide transport using [D-Arg^2]-Kyotorphin as a model dipeptide. Figures 22 to 26 depict the semilogarithmic plots of % [D-Arg^2]-Kyotorphin remaining in the nasal perfusates versus time in the presence of different enhancers (first hour) and after their removal from the rat nasal cavity (second hour). The data of the control group (perfusion of the dipeptide without enhancer) are also provided in every plot for direct comparison. The inidividual data together with the average values are shown in Table 7. As can be seen from these figures, reversal of nasal permeability to [D-Arg^2]-Kyotorphin was observed for all enhancers except 5% HP-β-CD. Paired Student's t-test was applied to compare the values of the dipeptide remaining at the end of the first and second perfusions (%T_{60}), Appendix III. In agreement with the LDH results, significant reduction in [D-Arg^2]-Kyotorphin absorption (or increase in %T_{60})was found for 0.1% LCC, 1.25% DM-β-CD, 0.5% CS J and 0.5% CS G ($p < 0.05$, paired

	n	1st hour (min)					2nd hour (min)					%ΔC
		0	15	30	45	60	0	15	30	45	60	
Control group	8	100.0±0.0	97.1±3.9	97.7±3.9	97.7±6.0	98.1±6.7	100.0±0.0	97.5±3.4	97.0±3.2	97.8±5.2	96.0±4.4	-1.69±8.69
0.5% CS G	4	100.0±0.0	97.5±1.3	94.3±3.6	87.0±2.6	83.2±1.6	100.0±0.0	96.2±3.2	94.1±3.1	95.5±2.2	91.4±2.4	9.98±3.54
0.5% CS J	4	100.0±0.0	98.8±5.7	89.7±7.1	81.5±17.4	78.2±12.5	100.0±0.0	99.3±3.0	96.3±4.1	95.1±3.0	95.0±6.4	23.18±14.42
0.1% LCC	3	100.0±0.0	90.4±8.3	81.4±7.3	79.0±7.8	70.33±8.4	100.0±0.0	93.9±4.1	94.6±8.5	89.0±7.7	83.8±8.3	19.53±8.37
1.25%DM-β-CD	4	100.0±0.0	94.7±6.0	90.4±4.6	86.0±6.5	80.3±7.7	100.0±0.0	98.0±4.5	95.7±4.7	92.8±6.5	92.1±6.5	14.99±5.48
5.0% HP-β-CD	7	100.0±0.0	97.7±3.5	98.3±7.3	100.4±3.6	99.6±2.3	100.0±0.0	98.9±3.0	96.5±3.2	100.9±6.0	101.0±5.9	1.44±7.49

Data : mean ± SD (n = 3-8 rats/group)

$\%\Delta C = C_2\text{-}C_1/C_1 \times 100\%$

C_1 = [D-Arg^2]-Kyotorphin conc. at T_{60} of the first perfusion period

C_2 = [D-Arg^2]-Kyotorphin conc. at T_{60} of the second perfusion period

Table 7 %[D-Arg^2]-Kyotorphin remaining in the nasal perfusates versus time in the presence of different enhancers(first hour) and after their removal from the rat nasal cavity (second hour).

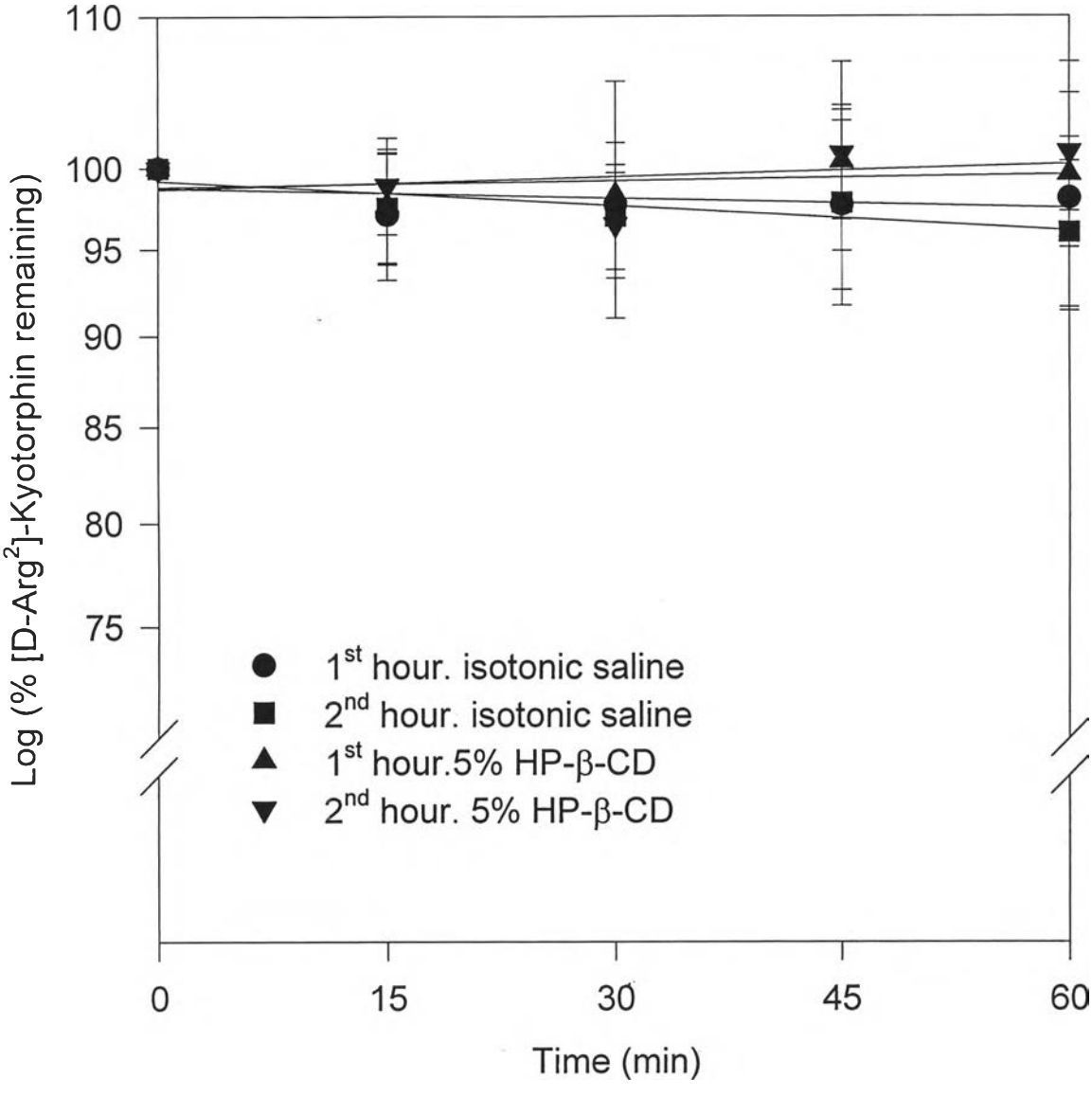


Figure 22 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time in presence of 5.0% HP-β-CD (first hour) and after its removal from the rat nasal cavity (second hour) and control group.

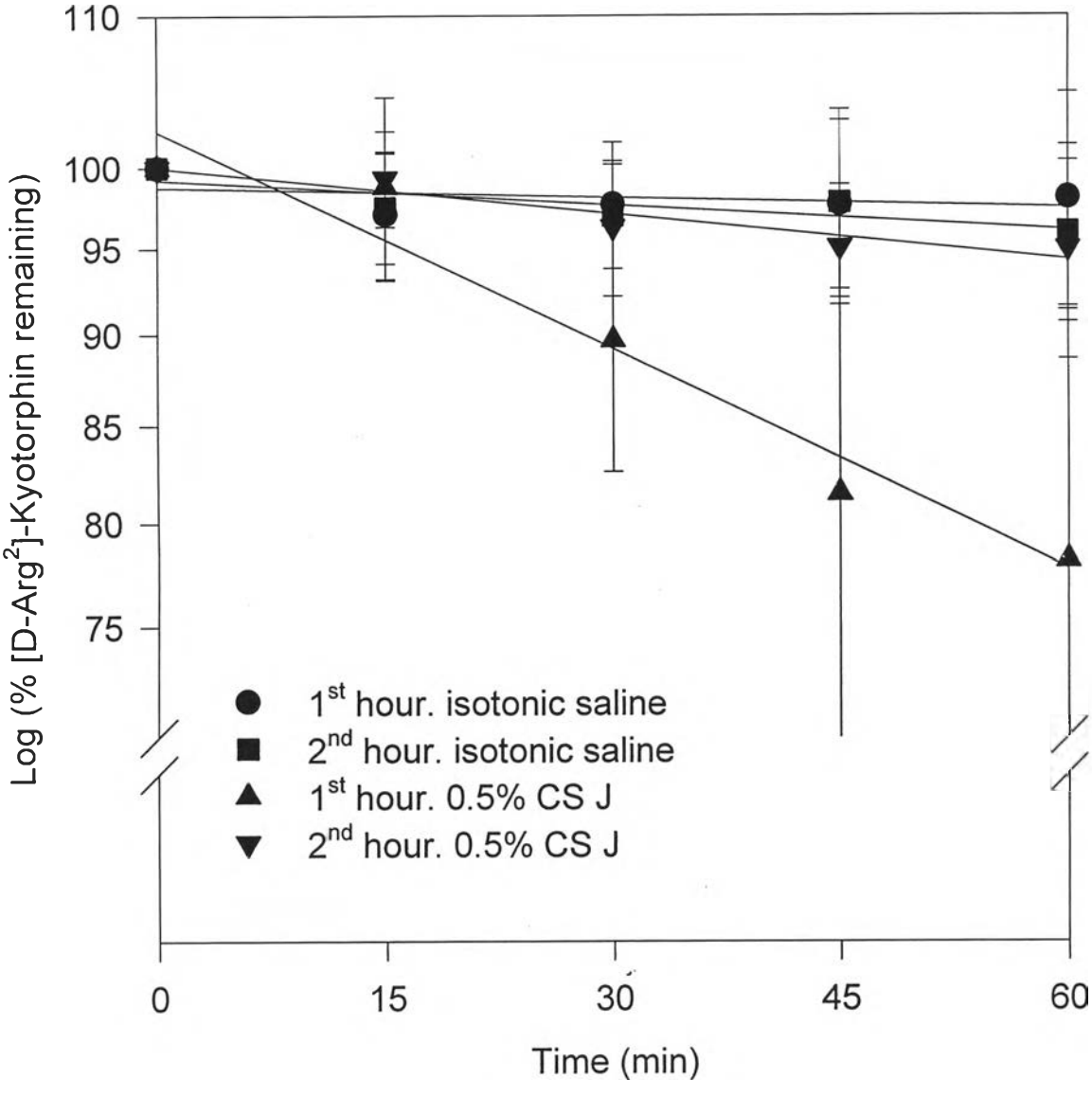


Figure 23 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time in presence of 0.5 % CS J (first hour) and after its removal from the rat nasal cavity (second hour) and control group.

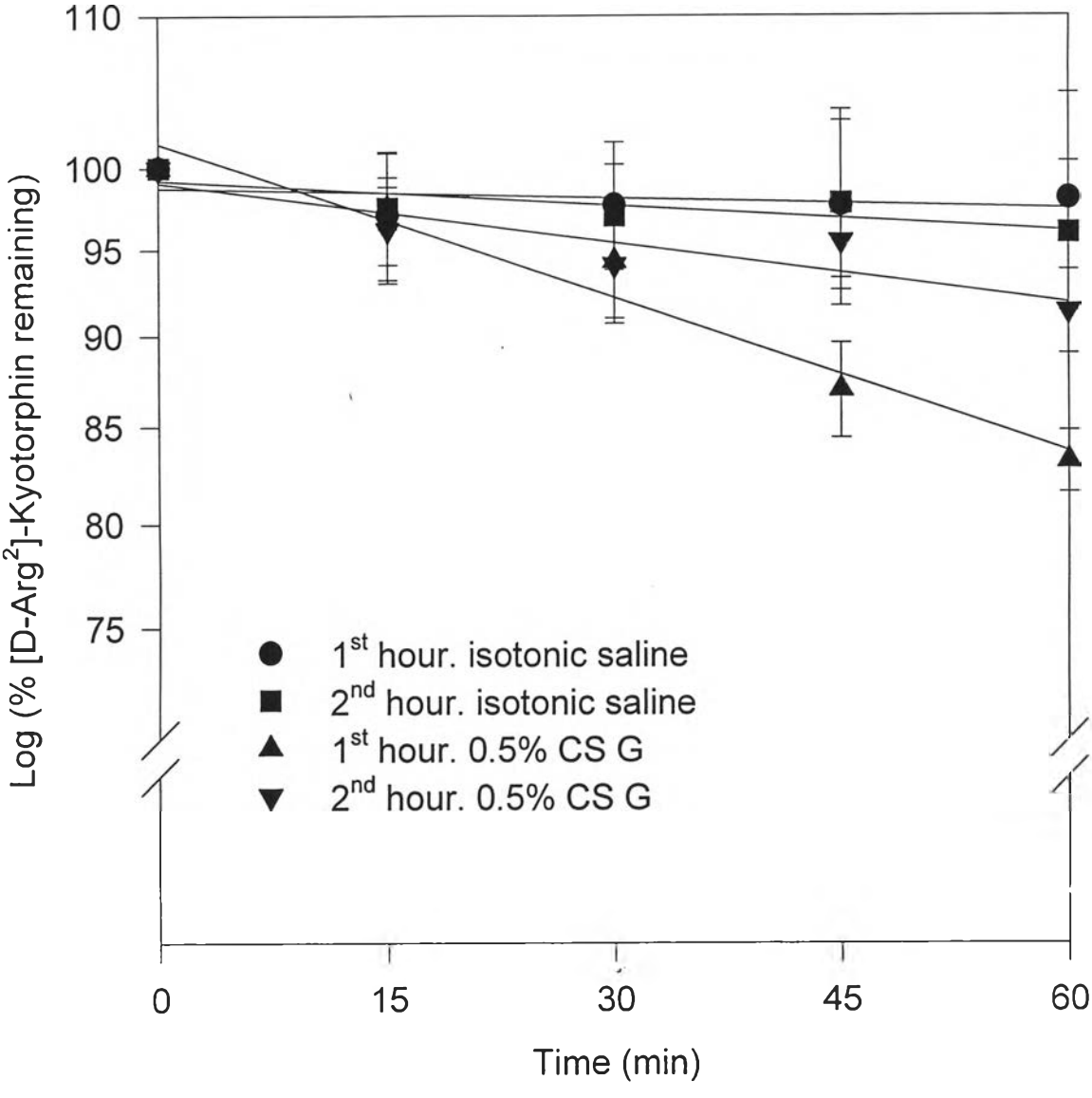


Figure 24 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time in presence of 0.5 % CS G (first hour) and after its removal from the rat nasal cavity (second hour) and control group.

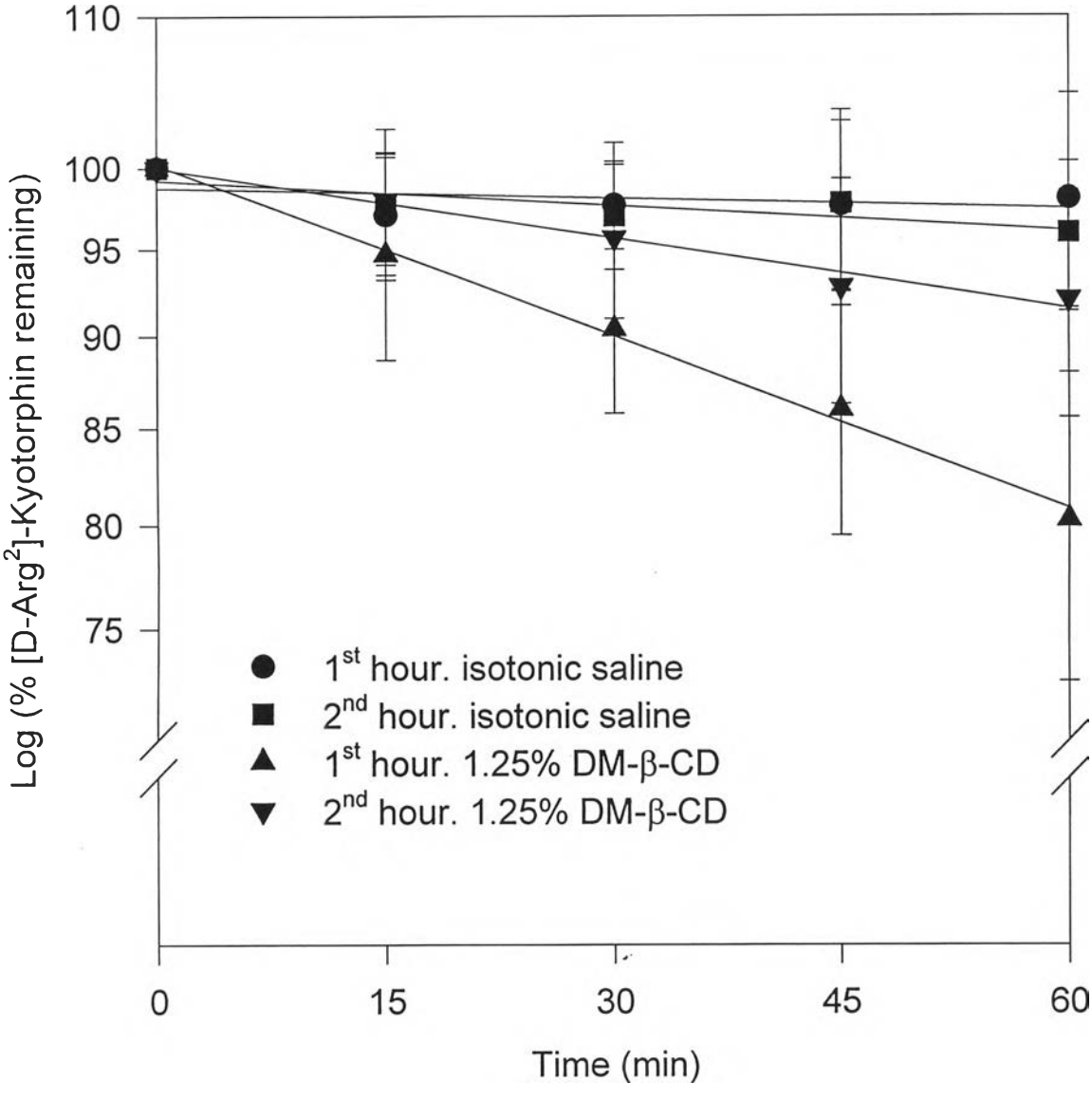


Figure 25 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time in presence of 1.25 % DM-β-CD (first hour) and after its removal from the rat nasal cavity (second hour) and control group.

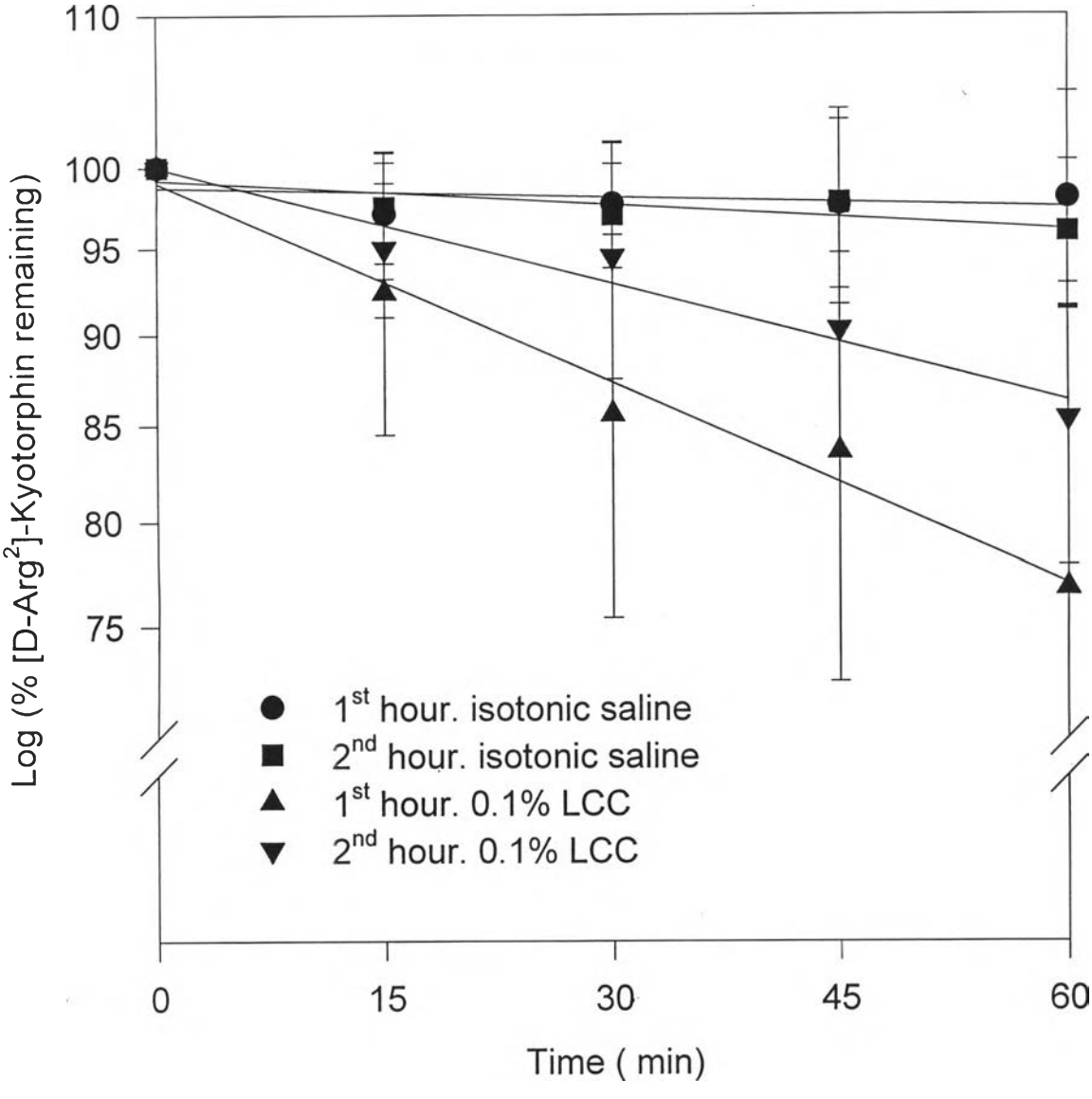


Figure 26 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time in presence of 0.1% LCC (first hour) and after its removal from the rat nasal cavity (second hour) and control group.

Student's t-test). In contrast, no significant difference in $\%T_{60}$ was observed between the first and second perfusions for the control and HP-β- CD-treated groups ($p > 0.05$, paired Student's t-test). Thus, the data confirmed the LDH results that these two groups exerted very weak effects on the nasal mucosa in terms of both membrane integrity and permeability to peptide absorption, leading to an apparent lack of mucosal reversibility as opposed to the other four enhancers. Histogram comparing the percent [D-Arg^2]-Kyotorphin remaining in the nasal perfusate at the end of the first and second perfusions is illustrated in Figure 27.

The percent change in % [D-Arg^2]-Kyotorphin remaining at the end of the second perfusion relative to that of the first perfusion was calculated from the following formula:

$$\%\Delta C = [(C_2-C_1)/C_1] \times 100\ \%$$

where C_1 and C_2 are % [D-Arg^2]-Kyotorphin remaining at the end ($\%T_{60}$) of the first and second perfusion, respectively.

The data for $\%\Delta C$ are also given in Table 7 for each of the enhancers. The average values are -1.69, 1.43, 9.98, 23.18, 19.53 and 14.99 % for the control (saline), 5% HP-β-CD, 0.5% CS G, 0.5% CS J, 0.1% LCC and 1.25% DM-β-CD, respectively. (The negative sign indicates a decrease in $\%T_{60}$ relative to the first period and vice versa, the positive sign implies an increase in $\%T_{60}$ (or reduction in peptide absorption)). One-way ANOVA was then applied on the $\%\Delta C$ values to compare the reversible effects of 0.5% CS G, 0.5% CS J, 0.1% LCC and 1.25% DM-β-CD on [D-Arg^2]-Kyotorphin nasal absorption. (The control and HP-β-CD-treated groups were

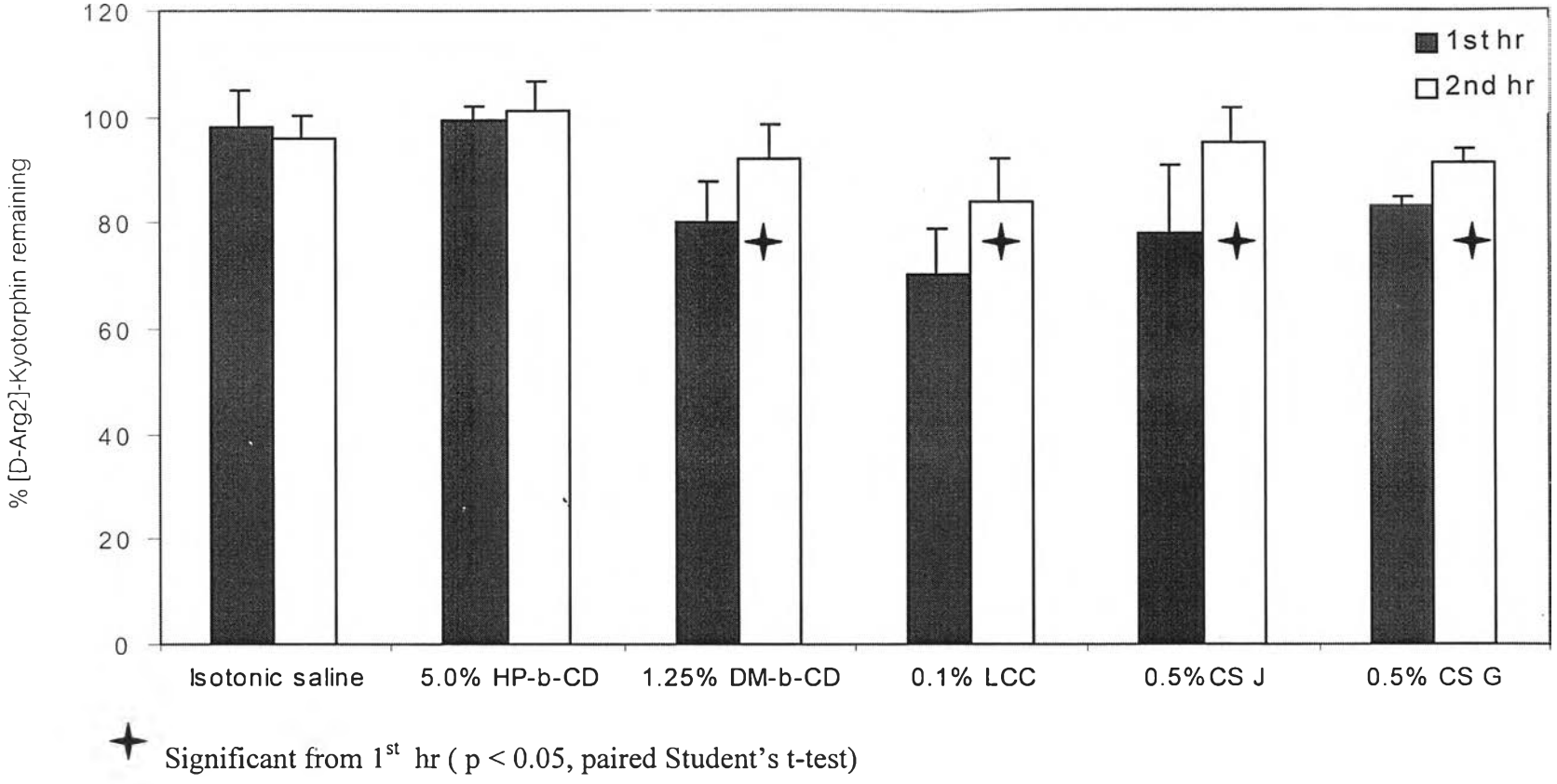


Figure 27 Histogram comparing the percent [D-Arg^2]-Kyotorphin remaining in the nasal perfusate at the end of the first and second perfusion.

excluded from comparison since they had failed to enhance the nasal absorption of this dipeptide and did not possess any reversibility). ANOVA result shows that there was no significant difference among the four enhancers with respect to %ΔC ($p > 0.05$), Appendix IV. However, it is interesting to note that 0.5% CS J gave the highest %ΔC value of 23.18 % whereas CS G, at the same concentration, exhibited the slowest recovery, with the %ΔC value of only 9.98 %. On the other hand, 0.1% LCC and 1.25% DM-β-CD showed intermediate values of 19.53 and 14.99 %, respectively. The ranking result, although not significant statistically, was somewhat in conflict with the LDH result. According to the LDH data, 0.1% LCC and 1.25% DM-β-CD showed significantly faster recovery than the two chitosans, with the LDH contents dropped down to the control level after their withdrawal. However, their reversal in [D-Arg^2]-Kyotorphin absorption appears to occur at a slightly slower rate than 0.5% CS J but faster than 0.5% CS G. CS J and CS G thus seem to differ markedly with respect to the reversibility of [D-Arg^2]-Kyotorphin absorption. No correlation was also observed between %ΔC (LDH release) and %ΔC (dipeptide remaining) ($p > 0.05$; test of zero correlation), Appendix XII. Figure 28 shows the correlation between the LDH and [D-Arg^2]-Kyotorphin recovery.

Lack of correlation and the discrepancies between the LDH and [D-Arg^2]-Kyotorphin recovery results may be explained in terms of the pathways of dipeptide absorption and LDH release. Since LDH is an intracellular enzyme, its release into the nasal perfusate is a result of direct membrane perturbation caused by the enhancer via a transcellular pathway. Absorption of [D-Arg^2]-Kyotorphin, on the other hand, can occur through both the paracellular and transcellular routes. Differential abilities of these enhancers in promoting the nasal absorption of this dipeptide by the transcellular pathway have been characterized based on the LDH release profiles.

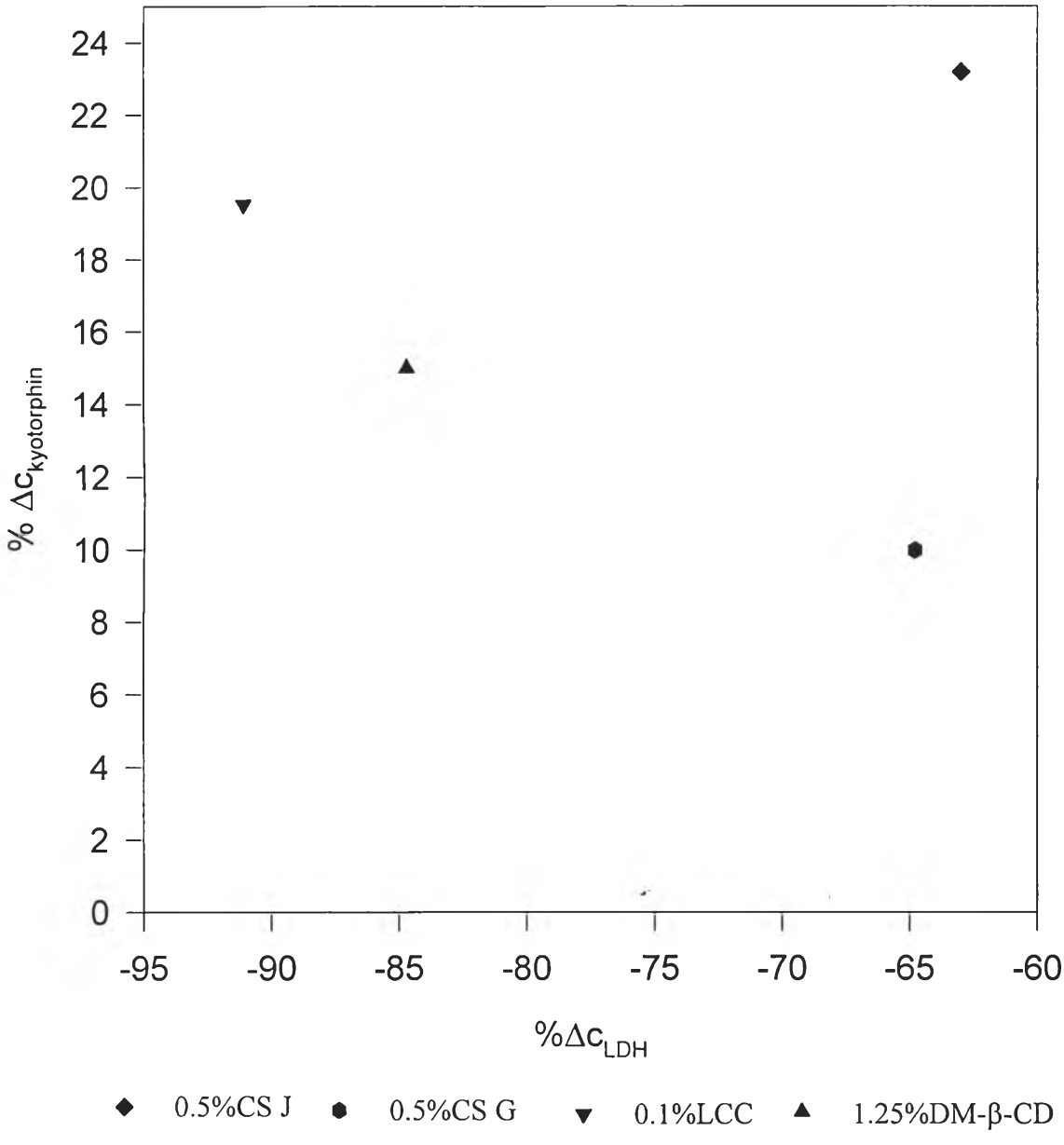


Figure 28 The correlation between % ΔC (LDH release) and % ΔC ([D-Arg^2]-Kyotorphin remaining)

However, the mechanisms and the extent to which various enhancers can act via the paracellular route may differ considerably and currently are not known. Therefore, the differential contribution of this pathway to the overall absorption may lead to different reversible effects of the enhancers on the mucosal permeability, yielding a ranking result that may not be in the same direction as that of membrane integrity.

To see the effect of chitosans more clearly, perfusion experiments were repeated using 0.1% and 0.5% of CS J and CS G as the perfusing solutions. As shown in Table 8, the release profiles of LDH as well as the percent [D-Arg^2]-Kyotorphin remaining in the perfusates were similar between 0.1% and 0.5% concentration for both CS J and CS G regardless of the perfusion period. The average LDH values at the end of the first perfusion with CS J are 442.56 $\pm$ 173.05 and 427.87 $\pm$ 118.74 U/ml for 0.1 and 0.5% conc., respectively. The average LDH values at 60 min after removal of CS J decreased to 185.23 $\pm$ 98.30 and 153.63 $\pm$ 34.22 U/ml for 0.1 and 0.5% conc., respectively. No significant differences in the LDH values were observed between the 0.1 and 0.5 % CS J in both periods ($p > 0.05$, unpaired Student's t-test), Appendix IXd and IXe, CS G also showed similar results to that of 0.5% CS J, i.e. the LDH values at the end of both periods are not significantly different between the two concentrations (0.1% vs 0.5%, first period = 479.24 $\pm$ 99.08 vs 478.91 $\pm$ 133.96 and 0.1% vs 0.5%, second period = 192.81 $\pm$ 43.59 vs 170.52 $\pm$ 78.56 U/ml; $p > 0.05$, unpaired Student's t-test), Appendix Xd and Xe. The percentage recovery of membrane integrity ($\%\Delta C_{LDH}$) was also calculated for 0.1% CS J and 0.1% CS G. The values are -54.87 $\pm$ 31.05 % and -57.40 $\pm$ 17.20 %, respectively, which are not different from 0.5% CS J (-62.99 $\pm$ 7.45%) and 0.5% CS G (-64.79 $\pm$ 13.62%) using the same t-test ($p > 0.05$), Appendix IXf and Xf. Also, there were no significant differences with respect to % [D-Arg^2]-Kyotorphin remaining between 0.1 and 0.5%

	n	1st hour (min)					2nd hour (min)				%Δc
		0	15	30	45	60	0	15	30	45	60	
%dipeptide remaining												% ΔC_{Kyo}
0.1 % CS J	4	100.0±0.0	97.1±4.1	87.0±2.7	81.7±5.8	77.5±4.8	100.0±0.0	100.9±4.2	99.2±2.1	99.0±2.9	97.4±4.1	26.04±9.27
0.5% CS J	4	100.0±0.0	98.8±5.7	89.7±7.1	81.5±17.4	78.2±12.5	100.0±0.0	99.3±3.0	96.3±4.1	95.1±3.0	95.0±6.4	23.18±14.42
LDH content												% ΔC_{LDH}
0.1 % CS J	3	-	28.89 ±13.88	233.80 ±133.92	291.00 ±205.47	442.56 ±173.05	-	93.33 ±43.72	143.74 ±78.55	172.78 ±105.34	185.23 ±98.30	-54.87 ±31.05
0.5 % CS J	4	-	88.34 ±46.31	238.11 ±72.24	347.04 ±102.77	427.87 ±118.74	-	75.00 ±23.33	114.55 ±22.52	129.78 ±24.28	153.63 ±34.22	-62.99 ±7.45
%dipeptide remaining												% ΔC_{Kyo}
0.1 % CS G	3	100.0±0.0	99.0±8.3	89.4±7.0	86.7±8.6	86.0±9.7	100.0±0.0	100.3±6.1	94.0±5.1	94.2±4.3	95.5±6.9	11.48±7.46
0.5% CS G	4	100.0±0.0	97.5±1.3	94.3±3.6	87.0±2.6	83.2±1.6	100.0±0.0	96.2±3.2	94.1±3.1	95.5±2.2	91.4±2.4	9.98±3.54
LDH content												% ΔC_{LDH}
0.1 % CS G	3	-	48.89 ±13.88	198.57 ±17.00	372.96 ±71.54	479.24 ±99.08	-	137.78 ±69.39	193.35 ±101.15	174.32 ±48.65	192.81 ±43.59	-57.40 ±17.20
0.5 % CS G	4	-	120.00 ±103.42	231.95 ±135.15	384.120 ±142.04	478.91 ±133.96	-	78.33 ±38.25	121.27 ±66.15	133.28 ±58.37	170.52 ±78.56	-64.79 ±13.62

% ΔC_{Kyo} = Percentage change in conc. of %[D-Arg^2]-Kyotorphin at T_{60} of 2nd hr relative to that of 1st hr

% ΔC_{LDH}= Percentage change in conc. of LDH at T_{60} of 2nd hr relative to that of 1st hr

Table 8 Comparison of the release profiles of LDH and the percent [D-Arg^2]-Kyotorphin remaining in the perfusates of 0.1%, 0.5% of CS J and CS G

concentration of both CS J and CS G. The values are not different from each other regardless of the perfusion period ($p > 0.05$, unpaired Student's t-test. The data are provided in Table 8, together with the percentage change in the level of dipeptide remaining at the end of each perfusion period (%ΔC). For example, the % [D-Arg^2]-Kyotorphin remaining at the end of the first perfusion with 0.1% and 0.5% CS J are 77.5 $\pm$ 4.8 % and 78.2 $\pm$ 12.5 %, respectively. After their removal from the nasal cavity, the respective % dipeptide remaining increased to 97.4 $\pm$ 4.1 % and 95.0 $\pm$ 6.4 % which corresponded to %ΔC of 26.04 $\pm$ 9.27 % and 23.18 $\pm$ 14.42 %.), Appendix IX and X (a, b and c)

Therefore, the data from LDH release and [D-Arg^2]-Kyotorphin absorption indicate that the effects of the two chitosans on the rat nasal membrane integrity and mucosal permeability appear to be concentration-independent or saturable, at least in the concentration range of 0.1 to 0.5%. The results obtained here are somewhat different from those previously reported by Sinswat (1997) who found that the absorption enhancing effect of the two chitosans was concentration dependent from 0.25 to 1.0%. The enhancing effect started to level off only at concentration higher than 1.0%. The difference in the observed results could be due to differences in the experimental technique and the type of model peptide that were used in each study. In Sinswat's study, the *in vivo* nasal absorption technique was employed instead of the *in situ* nasal perfusion and salmon calcitonin, a polypeptide with 32 amino acid residues, was used as a model drug to investigate the absorption enhancing effects of chitosans as opposed to a smaller dipeptide [D-Arg^2]-Kyotorphin utilized in this study. It is possible that the chitosan concentrations at which the enhancer effects start to level off or become saturated could vary from one study to another, depending on the experimental methodology being employed.

Although the two chitosans gave significantly higher release of LDH than the control and 5% HP-β-CD-treated groups, their LDH levels were significantly smaller than that induced by 0.1% LCC and 1.25% DM-β-CD, suggesting a lesser extent of membrane irritation. The difference in LDH release was clearly noticeable during the initial period of the first perfusion ($p < 0.05$, Duncan's test on LDH level at 15 min perfusion). They also showed fairly good reversibility in terms of both the nasal membrane integrity and permeability to peptide absorption. On the other hand, HP-β-CD is not an effective enhancer despite its excellent safety profiles as reported here and elsewhere (Shao, Krishnamoorthy and Mitra, 1992). In many cases, it failed to enhance nasal absorption of several peptides even at a high concentration of 5 % (Verhoef et al., 1994).

Therefore, the results accumulated so far have indicated that CS J and CS G may have a promising potential for use as a safe and effective nasal absorption enhancer. They possess several advantages over the two cyclodextrins and LCC in terms of absorption enhancing efficacy and safety. At 0.1 and 0.5%, both chitosans are more effective than 5% HP-β-CD and less membrane irritating than 0.1% LCC and 1.25% DM-β-CD. A recent study in human volunteers, Aspden et al.(1997), has found that chitosans did not produce any deleterious effects on the nasal epithelium and its normal mucociliary function following daily nasal administration of chitosan solutions for one week. Hence, an *in vivo* experiment was carried out as described in the next section to confirm the absorption enhancing effect and tolerability of the chitosan in healthy volunteers using salmon calcitonin as a model peptide. The results were then compared with the control saline and DM-β-CD, a reference enhancer.

Part II Study to confirm the *in vivo* efficacy chitosans in improving the nasal absorption of sCT in healthy volunteers

Although it has been found that 0.1 and 0.5% of CS G did not differ in their nasal absorption enhancing effect and safety on the rat nasal mucosa. From previous studies, Sahamethapat (1996) reported that 0.5% of CS G at pH 6.0 had the strongest enhancing effect on [D-Arg^2]-Kyotorphin absorption in rat. Thus, 0.5% CS G at pH 6.0 was selected in order to determine its nasal absorption enhancing efficacy of sCT in human volunteers and the results were compared with 1.33% DM-β-CD and the control group at the same pH.

Since the normal pH of the adult nasal secretions ranges approximately from 5.5 to 6.5 (Chien , Su and Chang, 1989) and sCT is known to be stable only in acidic conditions (Lee et al.,1992). Therefore, the pH 6.0 selected for this experiment appeared to be suitable for this study.

In addition, the plasma sCT levels following intranasal administration of sCT was previously shown to have a dose-dependent increase over the dosage range of 200 to 400 IU and plasma calcium concentrations dropped significantly 60 min after administration (Kurose et al.,1987). A dose-dependent increase in plasma sCT was also demonstrated in a study evaluating lower doses (50 to 200 IU) administered as a nasal spray in 40 postmenopausal women (Thamsborg et al.,1990). No reduction in serum calcium was demonstrated at these doses and it was found that the minimum effective dose to reduce plasma calcium should be between 200-300 IU (Thamsborg et al.,1990).

Thus, the dosage of nasal sCT chosen for this study was 300 IU Miacalcic® (equivalent to 300 IU sCT). The preparation of the nasal solutions and the method of nasal administration have previously been described in Chapter III.

Measurement of plasma calcium level was used to assess the efficacy of chitosans in enhancing the sCT absorption. Increased sCT absorption was indicated by a gradual fall in plasma calcium level and thus a smaller AUC value.

Table 9 shows the percent plasma calcium level relative to the initial point at various times and its AUC values during the 9-hr period following nasal administration of sCT in the presence of 0.5% CS G, 1.33% DM-β-CD and the control group. The data of the individual subjects are provided in Appendix XIV. Plots of changes in percent plasma calcium as a function of time are illustrated in figure 29. Statistical comparison using randomize block ANOVA at 5% significant level revealed that the $AUC_{0\text{-}9\ hr}$ of the plasma calcium levels was significantly different among the three groups ($p<0.05$). ANOVA results are provided in table 10.

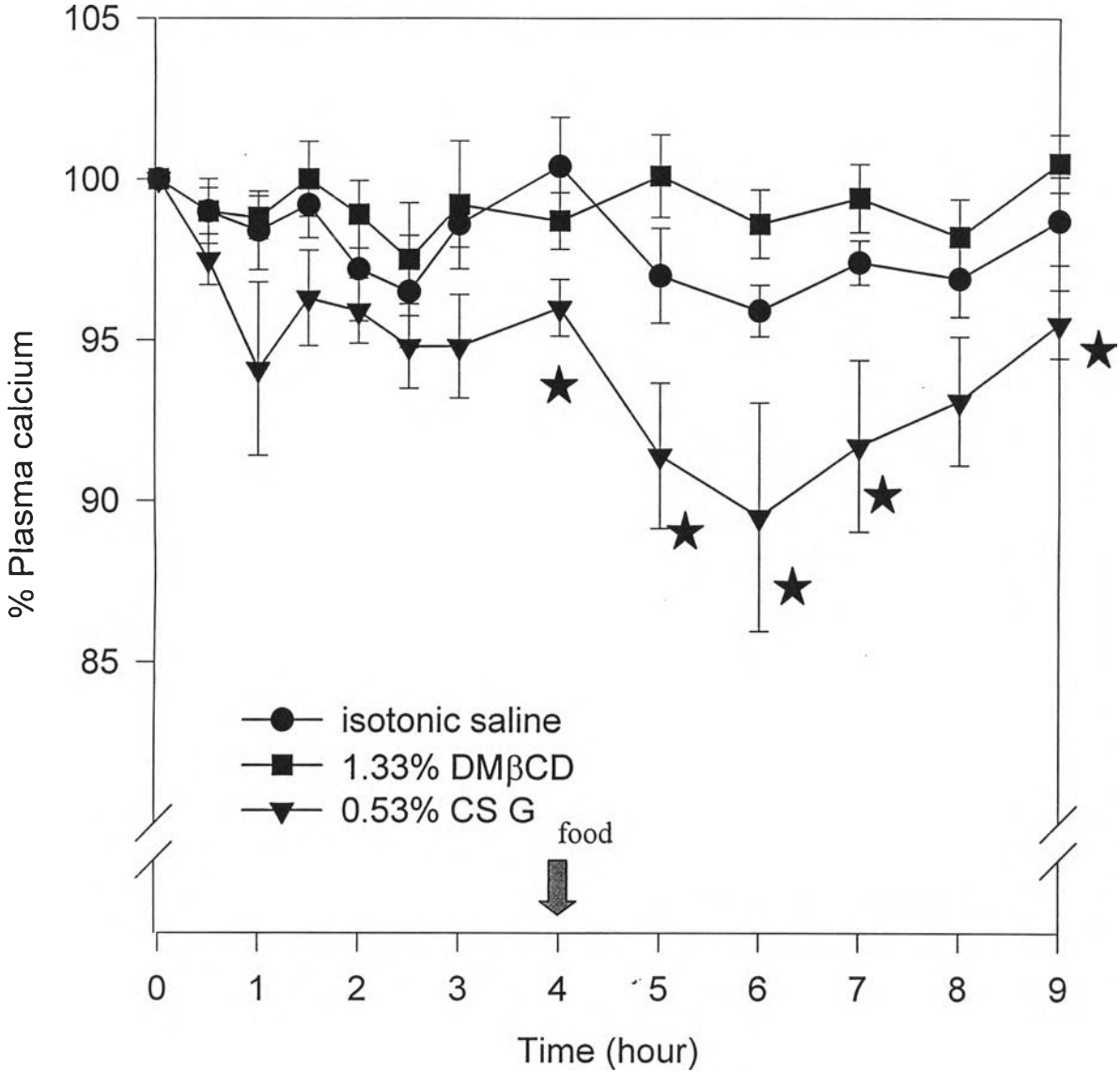
Table 10 The randomized block ANOVA result of the $AUC_{0\text{-}9hr}$ of the plasma calcium levels

Source	df	SS	MS	F	Prob.
Subjects	7	4986	712.2857	1.272913	0.3306
Enhancers	2	9640	4820	8.613735	0.0040
Error	14	7834	559.5715		
Total	23	22460			

Table 9 The percent plasma calcium level relative to the initial point and its AUC values during the 9-hr period following nasal administration of sCT in the presence of various enhancers.

Data : mean ± SEM (n = 8 subjects/group)

Time (hr)	Control	1.33% DM-β-CD	0.53% CS G
0	100±0.00	100±0.00	100±0.00
0.5	99.0±1.01	99.0±0.72	97.5±0.79
1	98.4±1.22	98.8±0.67	94.1±2.7
1.5	99.2±1.03	100.0±1.17	96.3±1.48
2	97.2±1.61	98.9±1.05	95.9±1.01
2.5	96.5±1.75	97.5±1.76	94.8±1.31
3	98.6±0.73	99.2±1.99	94.8±1.61
4	100.4±1.53	98.7±0.88	96.0±0.88
5	97.0±1.47	100.1±1.29	91.4±2.26
6	95.9±0.80	98.6±1.07	89.5±3.55
7	97.4±0.69	99.4±1.06	91.7±2.67
8	96.9±1.18	98.2±1.17	93.1±2.00
9	98.7±1.37	100.5±0.90	95.5±1.06
$AUC_{0\text{-}9hr.}$	881.15±7.40	891.67±5.32	844.88±12.08



★ Significantly different from isotonic saline and 1.33% DM-β-CD at $\alpha = 0.05$ using Duncan's new multiple range test

Figure 29 Percent plasma calcium level relative to the initial value after nasal administration of sCT with and without enhancers.

Duncan' s test was further applied in order to rank this difference. The ranking result, in an increasing order, was

	0.5% CS G	<	control	<	1.33% DM-β-CD
mean $AUC_{0\text{-}9hr}$ (%.hr)	844.89		881.15		891.67

The line underneath the control and 1.33% DM-β-CD-treated groups implies that there was no significant difference in $AUC_{0\text{-}9hr}$ between the two groups ($p>0.05$). However, the $AUC_{0\text{-}9hr}$ of 0.5% CS G was significantly smaller than control and 1.33% DM-β-CD ($p<0.05$), indicating that the extent of sCT absorption was greater than those two groups. Figure 30 demonstrates a histogram comparing the AUC of the percent plasma calcium relative to the initial level ($AUC_{0\text{-}9}$) between the control, 0.5% CS G and 1.33% DM-β-CD.

As can be seen from Figure 29 the values of percent plasma calcium for the control and DM-β-CD-treated groups decreased only slightly throughout 9 hr-period. On the other hand, a significant hypocalcemic response was observed after nasal administration of sCT in 0.5% CS G. The minimum serum calcium level was observed at 6 hr after nasal administration. This was slightly different from the results of Thamsborg et al.(1993) who reported that there was a significant decrease in serum ionized calcium with a nadir at 4 hr after nasal spray administration of calcitonin 200 IU as a single dose in healthy volunteers. This may be caused by the difference in the nasal formulations and the methods of administration Thamsborg et al. (1993).used commercial nasal spray at pH 4.0 whereas in this study the commercial spray was prediluted with isotonic saline pH 6.0 (with or without

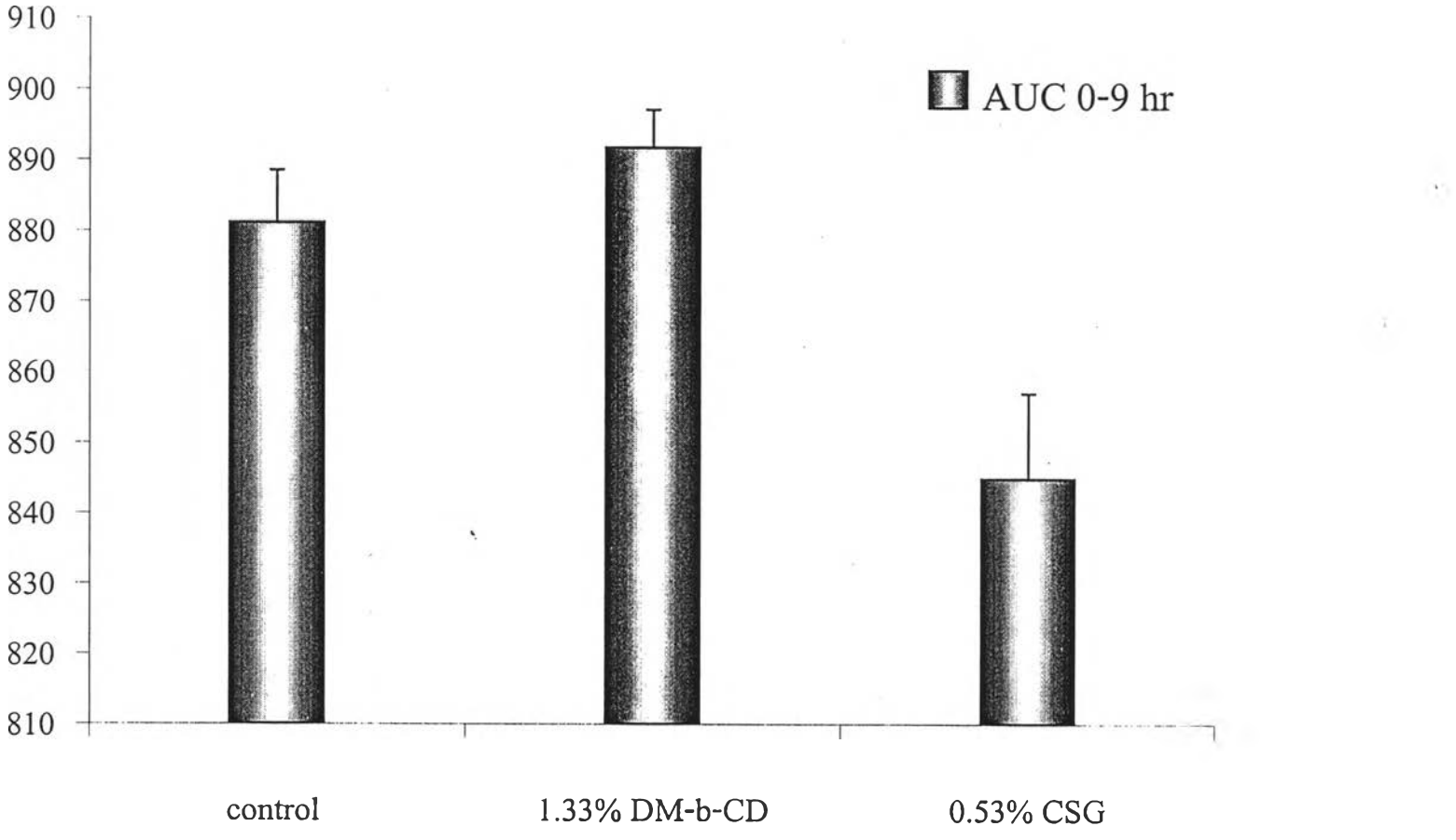


Figure 30 Histogram comparing the AUC of the percent plasma calcium relative to the initial level (AUC_{0-9}) between the control, 0.53% CS G and 1.33% DM-β-CD.

enhancer) and administered by a micropipette. In this study, sCT in saline (control) showed first minimum in the calcium level of about 96.5% at 2.5 hour post-dose. Then, the level increased to the initial value at 4 hr before decreasing again, reaching second minimum of 95.9% at 6 hr post-administration. When CS G was included in the nasal formulation, the first fall in plasma calcium level could be observed as early as at 1 hr post-dose (94.1%). However, this was not significantly different from the control and DM-β-CD ($p>0.05$, ANOVA). After 4 hr post-administration, the plasma calcium for CS G became significantly lower than that of control and DM-β-CD at all points except at 8 hr ($p<0.05$, Duncan's test). The lowest drop in plasma calcium occurred at 6 hr with the mean value of 89.5%. At 9 hr, the calcium level of CS G was still lower than the other two groups ($p<0.05$, Duncan's test). Therefore, statistical comparison of plasma calcium at different time points, especially at 4, 5, 6, 7, 9 hr, agreed with the results of AUC values in that 0.5% CS G is an effective nasal absorption enhancer of sCT. The hypocalcemic effect is also more prolonged. The mucoadhesive property of CS G may be responsible for a sustained hypocalcemic effect of sCT observed here. On the other hand, the enhancing effect of 1.33% DM-β-CD was very poor and did not differ from the control at all time points ($p>0.05$, Duncan's test).

Besides, Kurose et al.(1987) found that a decrease in the plasma calcium concentration level was not significant if the sCT level was not high enough (lower than 90 pg/ml). Thus, the control and DM-β-CD may enhance the nasal absorption of sCT to a level lower than 90 pg/ml. Moreover, it was found that in human, the nasal drops were deposited posteriorly and cleared very rapidly (Harris et al.,1986). Since sCT was applied to the nasal mucosa by micropipette (direct nasal instillation) in this study, the absorption of sCT could be lower than that after nasal spray. Nevertheless, inclusion of 0.5% CS G was still able to significantly increase the nasal absorption of

sCT as evidenced from the hypocalcemic effect despite the not-so-effective method of nasal administration used here.

It is expected that improvement in the method of sCT delivery, such as using a metered-dose nasal spray, would lead to an even better absorption since application of the nasal drops resulted in slight discomfort of the nose some subjects who have complained of swallowing some portion of the preparation as a result of rapid mucociliary clearance. It is therefore possible that the enhancing effects of chitosans on sCT absorption could be further improved if the nasal spray dosage form was used and the pH of the solution was adjusted to 4.0, an optimal pH value for sCT.

It is also interesting to note that the response of plasma calcium level in this result was rather variable. This is in agreement with those of Buclin et al.(1987) who found that the response to the nasal form was variable, and the response to the suppository was more constant.

In previous study, Verhoef et al.(1994) suggested that the influence of DM-β-CD on the polypeptide nasal absorption may differ largely between animal species. For example, studies in rats showed that DM-β-CD largely improved the absorption of insulin, resulting in bioavailability of about 109% (Merkus, Schipper et al., 1991a). In contrast to the results in rats, 5% DM-β-CD in solution did not have a significant effect on nasal insulin absorption in healthy volunteers (Merkus,Verhoef et al.,1991b).

Therefore, the combined results from part I and II indicate the advantages of chitosans over the two cyclodextrins (HP-β-CD and DM-β-CD) in terms of absorption enhancing efficacy and safety. Their enhancing effects also appear to be

reversible. Apparently, 0.5% CS G exhibited a stronger absorption enhancing effect for sCT in human volunteers than 1.33% DM-β-CD. The effect of DM-β-CD, on the other hand, was found to be almost ineffective in enhancing the nasal sCT absorption of this peptide in humans.