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Enhancing effect of chitosan on nasal absorption of salmon calcitonin in rats: comparison with hydroxypropyl- and dimethyl-β-cyclodextrins

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Abstract

Two types of chitosan, i.e. the free amine (CS J) and the glutamate salt (CS G), were evaluated for their enhancing effect on in vivo nasal absorption of salmon calcitonin (sCT) in rats. The results were subsequently compared with β-cyclodextrins, one of the most commonly studied enhancers. Solutions containing sCT and chitosan (0–1.25% w/v) in isotonic phosphate buffers (IPB; pH 3.0–6.0) were nasally administered at the dose of 10 IU/kg. The plasma calcium lowering effect in each sCT-treated rat was determined by calculating the total percent decrease in plasma calcium (%*D*). CS J showed an increase in %*D* as the solution pH was decreased in accordance with the increased ionization and hydration of the free amine chitosan at the more acidic pH. However, CS G showed an increase in %*D* with increasing pH, with maximum hypocalcemic effect observed at pH 6.0. At their optimal pH (4.0 for CS J and 6.0 for CS G), the absorption enhancing effect of both chitosans was concentration dependent from 0.25 to 1.0% and leveled off at 1.25%. Using specific RIA, the absolute bioavailability of sCT after comparison with i.v. administration was determined to be 2.45, 1.91, and 1.22% for 1% CS J, 5% dimethyl-β-cyclodextrin (DM-β-CD) and control group (intranasal (in) sCT alone), respectively. Although the absolute nasal bioavailability seemed to be low when compared to the i.v. administration, the inclusion of 1% CS J resulted in two-fold increase in the AUC_{0-180} of plasma sCT relative to that of the control group. Addition of 5% DM-β-CD also led to 1.56-fold increase in absorption over the control group. All the enhancers showed significant absorption enhancement ($P < 0.05$) with the highest effect observed with CS J. In conclusion, cationic polymer chitosan may have promising potential as a safe and effective nasal absorption enhancer of sCT.

Keywords: Salmon calcitonin; Nasal absorption enhancers; Free amine chitosan; Chitosan glutamate; Hydroxypropyl-β-cyclodextrin; Dimethyl-β-cyclodextrin; Nasal bioavailability

1. Introduction

Salmon calcitonin (sCT) is an endogenous polypeptide hormone consisting of 32 amino acids which plays a vital role in both calcium homeostasis and bone remodeling. Nasal administration of sCT has been used therapeutically for the treatment of hypercalcemia, Paget's disease, and osteoporosis (Stevenson and Evans, 1981; Jacobs, 1985). However, the relative bioavailability of sCT was reported to be poor, only 1.6% that of intramuscular preparation (Kagatani et al., 1996). To enhance the absorption efficacy of sCT, various enhancers have been investigated including bile salts, lauroylcarnitine chloride, and

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sodium tauro-24,25-dihydrofusidate (Kagatani et al., 1991, 1996; Lee et al., 1994). However, the use of these enhancers was often associated with some types of nasal membrane damages. For example, bile salts can induce significant release of several membrane components following nasal exposure in rats (Shao and Mitra, 1992). Other compounds reported to enhance the nasal absorption of peptides include laureth-9, lysophosphatidylcholine, and dimethyl-β-cyclodextrin (DM-β-CD; Gill et al., 1994; Shao et al., 1992; Merkus et al., 1991). However, all of these enhancers have been reported their membrane-irritating effect according to histology, ciliotoxicity, and membrane component release studies (Merkus et al., 1993; Marttin et al., 1995).

Chitosan is a cationic polysaccharide obtained from deacetylation of chitin, a structural polymer abundant in cretaceous animals like crabs and shrimps. Due to its biocompatibilty, biodegradability, and low toxicity, chitosan represents an attractive biopolymer for a variety of pharmaceutical applications (Illum, 1998; Paul and Sharma, 2000; Takahashi et al., 1990; Meshali and Gabr, 1993). Chitosans are potent absorption enhancers for poorly absorbed hydrophilic drugs. They improve, for example, the uptake of polypeptides such as atenolol, insulin, and buserelin across nasal and intestinal epithelia (Schipper et al., 1997; Illum et al., 1994; Luessen et al., 1996). The mechanism of action was suggested to be a combination of mucoadhesion (Henriksen et al., 1996; Witschi and Mrsny, 1999) an effect on the gating properties of the tight junction (Artursson et al., 1994; Illum et al., 1994).

In our previous work, we compared the absorption enhancing activity and safety of different types of chitosan with that of DM-β-CD and hydroxypropyl-β-cyclodextrin (HP-β-CD) using in situ rat nasal perfusion techniques (Tengamnuay et al., 2000). We found that both the free amine and soluble salt forms of chitosans were effective nasal absorption enhancers of L-Tyr-D-Arg, a model opioid dipeptide. The more soluble salt form appeared to be less dependent on pH whereas the enhancing activity of the free amine chitosan increased as the pH was lowered from 6 to 4. We also found that both chitosans had a relatively mild effect on the rat nasal membrane. The extent of total protein, phosphorus, and lactate dehydrogenase (LDH) release after nasal perfusion with chitosans (0.1 and 0.5%) was low and much less than that induced by 1.25 and 5% DM-β-CD, an effective enhancer reported to have marked membrane-irritating effects (Muangkum and Tengamnuay, 1999; Krishnamoorthy et al., 1995; Marttin et al., 1995; Yoshida et al., 1988). Despite the promising potential of chitosan as a safe and effective nasal absorption enhancer, studies to confirm its in vivo efficacy are limited. Also, its in vivo enhancing activity relative to other commonly studied enhancers has not been extensively investigated.

Thus, the primary purpose of this study was to evaluate the in vivo efficacy of chitosan as a nasal absorption enhancer of sCT in rat. Changes in plasma calcium (hypocalcemic effect) and sCT levels were used as indicators of sCT absorption. The effects of chitosan type, pH, and concentration were investigated and results were subsequently compared with that of DM-β-CD and HP-β-CD.

2. Materials and methods

2.1. Chemicals

Chitosan free amine (CS J) with viscosity average molecular weight (MW_v) of 1860 kDa and 80% deacetylation was purchased from Kyowa Technos Co. (Japan). Chitosan glutamate (CS G) (Seacure G 210+, MW_v 800 kDa and >70% deacetylated) was donated by Pronova Biopolymer (Norway). The actual molecular weight of the chitosan content in CS G approximately 480 kDa taken into account of the 35–45% presence of glutamic acid in the CS G. Salmon calcitonin, synthetic (sCT) was purchased from Sigma Chemicals Co. (St. Louis, MO). Salmon calcitonin radioimmunoassay kit was purchased from Peninsula Lab. Inc. (Belmont, USA). Calcium assay kit (*o*-cresolphthalein complexone) was from Clinag Co. (Thailand). HP-β-CD and DM-β-CD were from Aldrich Chemicals (USA) and Sigma Chemicals Co., respectively. All other reagents were of analytical grade and used as received.

2.2. In vivo absorption studies in rats

Male Sprague–Dawley rats (250–300 g) were surgically treated according to the method of Hirai et al. (1981) with slight modifications (Tengamnuay and

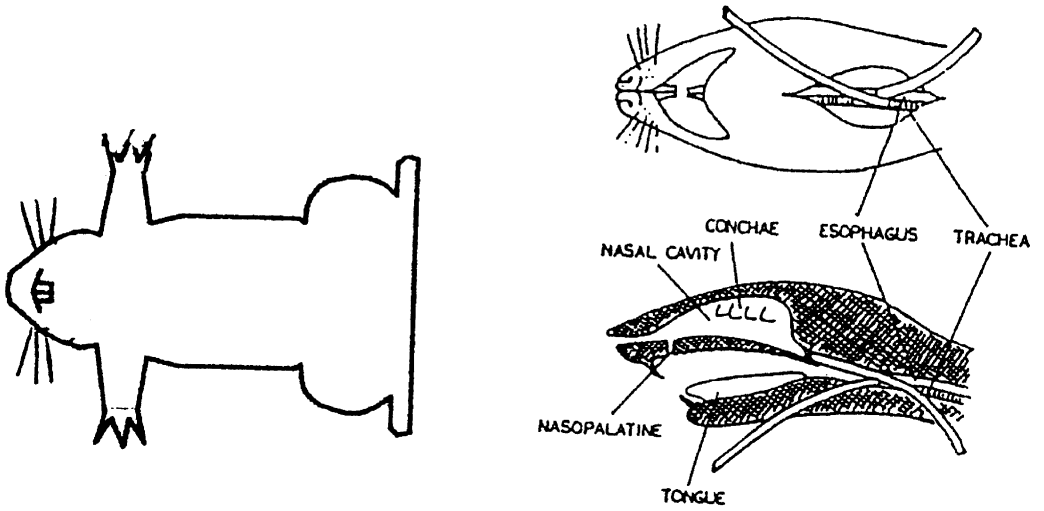

Fig. 1. Respective diagrams of the surgical procedure of in vivo nasal absorption experiment.

Mitra, 1990). After anesthetization, an incision was made at the neck of the rat to expose the trachea. A polyethylene tube was inserted about 1.5 cm deep into the trachea toward the lungs to maintain respiration during the experiment. The esophagus was also cannulated with another similar polyethylene tube, which was closed at the end with an adhesive agent and inserted toward the posterior nasal cavity. The nasopalatine was sealed with an adhesive agent to prevent drainage of the drug solution from the nasal cavity into the mouth. Respective diagrams of the surgical procedure of in vivo nasal absorption experiment are illustrated in Fig. 1.

2.2.1. Preparation of sample solutions

Stock sCT solutions (3.5 μM) were separately prepared by dissolving in 0.15 M isotonic phosphate buffers (IPB) of different pH (3.0, 4.0, 5.0, and 6.0). Stock solutions of the two chitosans (CS J and CS G) at 2.0% w/v were prepared by separately dissolving in 1% v/v acetic acid and allowing them to swell overnight. The pH was adjusted to the same as sCT solutions by adding either 1N HCl or 1N NaOH. The osmotic pressure of the chitosan stock solutions was also adjusted to be isotonic by gradual addition of NaCl using an osmometer (Osmomat 030-D, Gonotec, Berlin, Germany). The final solution containing 1.0% w/v chitosan and 1.75 μM sCT was subsequently obtained by mixing equal volumes of the stock sCT and chitosan solutions of the same pH value. In addition, nasal solutions containing 1.75 μM sCT with varying concentrations of the two chitosans (0.25, 0.5, 0.75, and 1.25%) were also prepared using the same procedures.

2.2.2. Drug administration

Groups of five animals were used in the plasma calcium study and a separate group of animals ($n = 3$) was used plasma sCT study. The animals were manually restrained in a supine position while the solution was instilled into the nostril (Fig. 1). Test solutions were always administered through the rat's right nostril at a dose of 10 IU/kg and appropriate administered volume (40–50 μl) via a microsyringe which was attached to a blunt needle. Intravenous administration was also carried out by injecting a bolus dose of 0.15 IU/kg of sCT in IPB pH 4.0 through the jugular vein. Blood samples were periodically collected from the same vein for 240 min (plasma calcium study) or for 180 min (plasma sCT study). After each blood withdrawal, the same volume of sterile normal saline was put back into the circulation to maintain total blood volume. Plasma samples were separated by centrifugation at 3000 rpm for 15 min and kept frozen at −20 °C for subsequent analysis of both plasma calcium and immunoreactive sCT.

The respective baseline groups were also carried out by nasal administration of only the IPB buffers. The enhancing activities of chitosans were then compared with that of DM-β-CD and HP-β-CD. The reference enhancer solutions contained 1.75 μM sCT with 5.0% w/v of either DM-β-CD or HP-β-CD in IPB pH 7.4.

2.2.3. Analytical method

2.2.3.1. Measurement of plasma calcium concentration. Plasma calcium levels following sCT administration were determined colorimetrically using a commercial assay kit. The principles are based on the ability of *o*-cresolphthalein complexone to form purple-colored complex with calcium in an alkaline medium, which can be measured at 570 nm.

2.2.3.2. Measurement of plasma sCT concentration. Plasma immunoreactive sCT was quantitated by radioimmunoassay using a commercial kit. This technique is essentially a double antibody assay and has detectable concentrations ranging from 10 to 1280 pg/ml. All samples were assayed in duplicate using the standard preparations and procedures provided with the kit. The radioactivity was detected using a Gammatec™ II gamma counter (Nucleus Inc., Oak Ridge, CA).

2.2.4. Determination of hypocalcemic effect and nasal bioavailability

The percent reduction in plasma calcium during 0–240 min (%*D*) relative to the appropriate baseline group (only IPB without sCT and enhancer) was calculated for each rat according to the following equation:

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

in which $\overline{\mathrm{AUC}}_{\mathrm{baseline}}$ is the average area under plasma calcium curve from 0 to 240 min after nasal administration of 0.15 M IPB (the baseline group) at the corresponding pH and $\mathrm{AUC}_{\mathrm{sCT\,treated}}$ is the individual area under the plasma calcium curve from 0 to 240 min after nasal administration of sCT (with or without enhancer) to the individual rat. The values of AUC, either from plasma calcium or sCT data, were calculated using the trapezoidal rule.

2.3. Statistical evaluation

Analysis of the data from rat absorption studies was made by Student's *t*-test, one-way ANOVA, and multiple comparison of the means (Duncan's test) where appropriate. Difference between group means was considered significant at $P < 0.05$. For human data, randomized block ANOVA was applied and followed by Duncan's test at the same significant level. The computation was performed using a statistical software package (SAS Inc.).

3. Results and discussion

3.1. Effect of pH of chitosan on in vivo nasal absorption enhancing efficacy

The enhancing activity of both chitosans appear to depend on pH. The enhancing effect of CS J was observed to increase as the pH is decreased (Fig. 2a). This could be due to the ability of the free amine form which require an acidic condition for ionization and hydration. Chitosan is a basic polymer with an intrinsic pK_a value of about 6.5 (Schipper et al., 1996). As the pH is lowered below its pK_a, the fraction of the ionized groups in the chitosan molecules increases. However, too acidic pH such as pH 3.0 may be associated with nasal irritation (Ohwaki et al., 1985, 1987). As observed earlier, nasal administration of buffer pH 3.0 also interfered with the baseline calcium level in rats. Thus, pH 4.0 was chosen over pH 3.0 to be the optimum pH for CS J and was always used in subsequent studies.

On the other hand, the result obtained for CS G seemed to be opposite to CS J (Fig. 2b). The enhancing effect of 1% CS G on sCT nasal absorption was maximal at pH 6.0. The results obtained here also agreed with our previous report (Tengamnuay et al., 2000). We attributed this observation to the greater solubility of the glutamate salt. It is possible that CS G might be able to assume the highly ionized, elongated shape which helped to maintain its enhancing activity at less acidic bulk pH values. Nevertheless, the reasons for the reverse order of the pH effect observed with CS G are not clearly known.

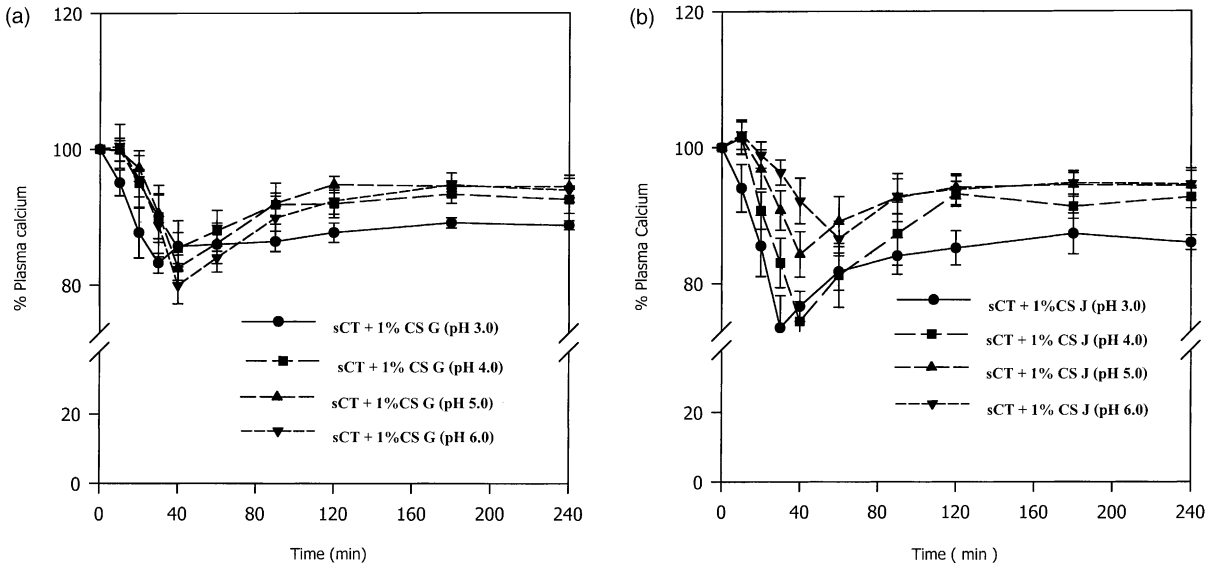

Fig. 2. Effect of pH of chitosan solutions on the changes in plasma calcium after nasal administration of sCT (10 IU/kg) to rats. (a) sCT with 1% CS J; (b) sCT with 1% CS G. Each point represents mean ± S.D. ($n = 5$ rats/group).

3.2. Effect of concentration on the absorption enhancing activities of chitosans

The effect of varying chitosan concentration was subsequently studied at their corresponding optimal pH. Table 1 shows %*D* after nasal administration of sCT in the presence of various concentrations of chitosans. The effect of both chitosans appeared to be concentration dependent from 0.25 to 1.0% and level off after this concentration since the value of %*D* at 1.25% was not much different from 1.0%, indicating that their enhancing activity was saturable. This was in agreement with previous reports which suggested that the mechanism(s) of absorption enhancement of chitosan may be different from typical membrane-disrupting enhancers like sodium

Table 1
Pharmacodynamic parameters (plasma calcium data) of various concentrations of chitosans in comparison with 5% DM-β-CD and 5% HP-β-CD after nasal administration of sCT (10 IU/kg) to rats

Adjuvants	C_{min} (% of initial value)	T_{min} (min)	$AUC_{0-240\,min}$ (% min)	%*D*
0.25% CS J (pH 4.0)	81.49 ± 1.73	56.00 ± 8.00	21974.5 ± 239.1	7.72 ± 1.00
0.50% CS J (pH 4.0)	82.55 ± 1.11	68.00 ± 19.39	21818.6 ± 175.3	8.47 ± 0.68
0.75% CS J (pH 4.0)	76.73 ± 3.24	48.00 ± 9.80	21731.7 ± 180.5	8.75 ± 0.76
1.00% CS J (pH 4.0)	73.47 ± 1.47	44.00 ± 8.00	21469.0 ± 282.9	9.85 ± 1.19
1.25% CS J (pH 4.0)	75.64 ± 0.89	48.00 ± 9.80	21400.6 ± 401.9	10.14 ± 1.69
0.25% CS G (pH 6.0)	84.84 ± 1.91	62.00 ± 16.00	22528.9 ± 123.1	6.28 ± 0.52
0.50% CS G (pH 6.0)	83.14 ± 1.13	44.00 ± 8.00	22388.4 ± 140.2	6.86 ± 0.58
0.75% CS G (pH 6.0)	81.89 ± 1.33	44.00 ± 8.00	22255.5 ± 112.6	7.41 ± 0.47
1.00% CS G (pH 6.0)	79.38 ± 1.82	44.00 ± 8.00	22009.0 ± 162.2	8.44 ± 0.68
1.25% CS G (pH 6.0)	80.74 ± 1.19	44.00 ± 8.00	22063.4 ± 90.3	8.21 ± 0.38
5.0% DM-β-CD (pH 7.4)	77.51 ± 1.57	44.00 ± 8.00	21810.4 ± 74.4	9.68 ± 0.31
5.0% HP-β-CD (pH 7.4)	82.83 ± 1.24	44.00 ± 8.00	22203.7 ± 110.9	8.05 ± 0.46

Each value = mean ± S.D. ($n = 5$ rats/group).

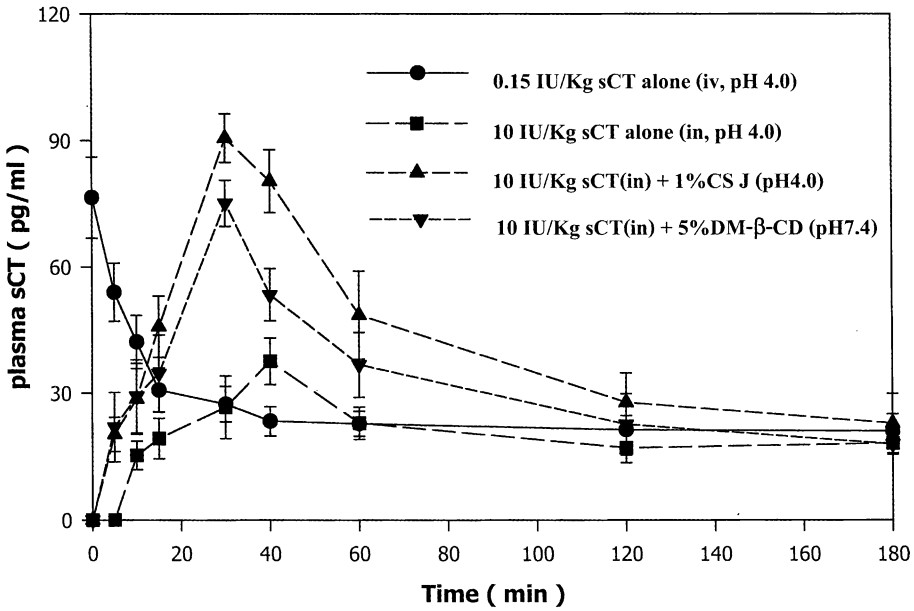

Fig. 3. Plasma sCT concentration time-profiles following nasal (in) administration of sCT (10 IU/kg), with and without enhancers, in comparison with intravenous (i.v.) administration (0.15 IU/kg) to rats. Data = mean ± S.D. ($n = 3$ rats/group).

taurodihydrofusidate, synthetic surfactants, and bile salts (Artursson et al., 1994; Illum et al., 1994). Thus, based on the data obtained in this part, the concentration of 1.0% appeared to be optimal for both CS J and CS G to enhance the in vivo nasal absorption of sCT in rats.

3.3. Comparative hypocalcemic effect and nasal bioavailability in rats

The nasal absorption enhancing activity of the two chitosans at their optimal concentration and pH was also compared with that of HP-β-CD and DM-β-CD. The two cyclodextrins, at conventional concentration of 5.0% w/v, were selected as reference enhancers due to their enhancing efficacy profiles (Gill et al., 1994; Merkus et al., 1999; Verhoef et al., 1994; Shao et al., 1992). Statistical comparison of %D revealed that the enhancing activity of 1% CS J was equivalent to 5% DM-β-CD and 1% CS G to 5% HP-β-CD under their respective optimum pH ($P > 0.05$) (Table 1). Furthermore, the enhancing activity of DM-β-CD and CS J appeared to be greater than HP-β-CD and CS G ($P < 0.05$).

The separate study was conducted to monitor changes in plasma sCT following nasal administration

Table 2
Pharmacokinetic parameters of plasma sCT after nasal (in) administration of sCT to rats, with and without enhancers, in comparison with i.v. administration

Dose and route of adjuvant administration		C_{max} (pg/ml)	$AUC_{0-180\,min}$ (min pg/ml)	$\%F_{abs}$ [a]	$\%F_{rel}$ [b]
0.15 IU/kg, i.v.	None	76.47 ± 7.85	4488.3 ± 180.5	100.0	–
10 IU/kg, in	None	37.53 ± 4.50	3643.9 ± 423.1	1.22	100.0
10 IU/kg, in	1% CS J (pH 4.0)	90.59 ± 4.71	7330.9 ± 1054.5	2.45	201.2
10 IU/kg, in	5% DM-β-CD (pH 7.4)	75.16 ± 4.47	5702.1 ± 890.5	1.91	156.5

Each value = mean ± S.D. ($n = 3$ rats/group).
[a] Absolute nasal bioavailability.
[b] Relative nasal bioavailability.

of the peptide with and without enhancers, in comparison with i.v. sCT. As seen from Fig. 3, i.v. injection of sCT solution resulted in immediate appearance of peptide in the blood with biphasic elimination showing a rapid decline during the first 20 min followed by a slow elimination phase. On the other hand, nasal administration of sCT solution alone resulted in a much lower plasma sCT level. Inclusion of both 1% CS J and 5% DM-β-CD in this solution further increased both the rate and extent of sCT nasal absorption. F_{abs} was calculated to be 1.22, 2.45, and 1.91% for the control, 1% CS J and 5% DM-β-CD, respectively. The value for control was in agreement with the result of Lee et al. (1994), who reported that sCT administered intranasally (in) in healthy volunteers without enhancer at pH 4.0 was minimally absorbed, with bioavailability of only 1.16%. Although the absolute nasal bioavailability seemed to be low when compared to i.v. administration, the C_{max} and $AUC_{0–180}$ values of both 1% CS J and 5% DM-β-CD were significantly greater than the control intranasal group ($P < 0.05$, Table 2). Inclusion of the two enhancers also induced faster absorption as t_{max} was significantly reduced ($P < 0.05$, Fig. 3). Furthermore, Duncan's test revealed that the effect of 1% CS J on C_{max} was significantly greater than 5% DM-β-CD ($P < 0.05$). However, the $AUC_{0–180}$ values were not significantly different from each other ($P > 0.05$).

4. Conclusion

The in vivo absorption data indicated the potential of CS J and CS G as effective nasal absorption enhancers of peptides like sCT. Their enhancing effects were equivalent to β-cyclodextrins, particularly 1% CS J which exhibited strong activity comparable to 5% DM-β-CD under their corresponding optimum pH. However, our previous study found that DM-β-CD was more irritating to the rat nasal mucosa than CS J and CS G (Tengamnuay et al., 2000). Perfusion of the rat nasal cavity with 1.25% DM-β-CD for 30 min induced the release of LDH, an intracellular enzyme used as a marker of membrane irritation, nearly two-fold greater than 0.5% CS J and 0.5% CS G. DM-β-CD was also reported to be extremely hemolytic (Yoshida et al., 1988) as opposed to chitosans (Natsume et al., 1999). Thus, judging from both the safety and efficacy viewpoints, the chitosans may possess superior characteristics to DM-β-CD as a safe and effective nasal absorption enhancer.

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