Enhanced Nasal Delivery of Luteinizing Hormone Releasing Hormone Agonist Buserelin by Oleic Acid Solubilized and Stabilized in Hydroxypropyl-β-cyclodextrin

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The potential use of three 2-hydroxypropyl ether derivatives of cyclodextrins (HP-α-, HP-β- and HP-γ-CyDs) as biocompatible solubilizers and stabilizers for oleic acid, a lipophilic absorption enhancer, was assessed in the nasal absorption of buserelin, an agonist of luteinizing hormone-releasing hormone, in rats. HP-CyDs increased the aqueous solubility of oleic acid and protected it against oxidation through the formation of inclusion complexes with the efficacy increasing in the order: HP-γ-CyD ≪ HP-α-CyD < HP-β-CyD. The bend structure due to a *cis*-double bond halfway along the acyl chain of oleic acid provided a better fit into the cavity of HP-β-CyD, in which the double bond appears to be buried, and hence becomes less susceptible to oxidation. The rate and extent of nasal bioavailability of buserelin were remarkably increased by coadministration of oleic acid and HP-β-CyD, compared with the sole use of the enhancer. This enhancement was ascribable to the lowering of both the enzymatic and physical barriers of the nasal epithelium to the peptide, probably through the facilitated transmucosal penetration of oleic acid solubilized in HP-β-CyD.

Key words 2-hydroxypropyl-β-cyclodextrin; oleic acid; solubilizer; stabilizer; buserelin acetate; nasal absorption

Cyclodextrins (CyDs) and their derivatives are capable of forming more or less stable inclusion complexes with both saturated and unsaturated fatty acids in either solution or solid state.[1] The molecular encapsulation of fatty acids with CyDs has been successfully applied in various fields. For instance, the treatment of lipemic serum with CyDs produces an immediate and selective flocculation of lipoproteins, giving an effective means for removal of interfering lipid particles from the serum in clinical diagnosis.[2] Furthermore, the CyD complexes of unsaturated fatty acids can be utilized as either serum substitutes or energy sources in cell cultures.[3]

Oleic acid, a long-chain unsaturated fatty acid, is a well-known percutaneous penetration enhancer.[4] The limited aqueous solubility of oleic acid, however, can become a problem, particularly in an environment of mucosal absorption sites, and can result in decreased adjuvant activity. Several attempts have been made to overcome such drawbacks, including the use of mixed micelles of fatty acids with surfactants[5] or bile salts,[6] and these reportedly resulted in a much greater extent of absorption enhancement compared with that obtained using adjuvants alone.

In a recent study, Yanagi *et al.* described that an inclusion complex of decanoic acid, a medium-chain saturated fatty acid with α-CyD was effective in improving the rectal absorption of cefmetazole from an oleaginous suppository base in rabbits.[7] Our previous studies have shown that 2-hydroxypropyl-β-CyD (HP-β-CyD) enhanced the action of a lipophilic absorption enhancer, 1-[2-(decylthio)ethyl]azacyclopentane-2-one, without causing severe local irritation in nasal formulations of insulin[8] and buserelin acetate, an agonist of luteinizing hormone-releasing hormone.[9]

In these previous studies, HP-β-CyD was chosen as a biocompatible solubilizer because it is well tolerated in long-term nasal administration to humans,[10] shows minimal systemic toxicity even when given parenterally in small animals and humans, and retains excellent solubilizing ability.[11–13] Following on from these studies, we examined whether this advantage of solubilization with HP-CyDs would apply to oleic acid. This paper deals with the cavity size effect of HP-CyDs (HP-α-, HP-β- and HP-γ-CyDs) on the aqueous solubility and chemical stability of oleic acid, in anticipation of optimizing the action of the unsaturated fatty acid in the nasal absorption enhancement of buserelin in rats.

Experimental

Materials Buserelin acetate was a generous gift from Pharma Research Laboratories, Hoechst Japan, Ltd. (Saitama, Japan). HP-α-, HP-β- and HP-γ-CyDs were supplied by Nihon Shokuhin Kako Co. (Tokyo, Japan). The average degrees of substitution of 2-hydroxypropyl groups in the HP-CyDs were confirmed to be 5.2 for HP-α-CyD, 4.0 for HP-β-CyD and 4.9 for HP-γ-CyD by fast atom bombardment mass and nuclear magnetic resonance (NMR) spectrometry.[14] Oleic acid (purity: 99% (by gas chromatography), stable in oil at 4 °C, Nacalai Tesque Co., Kyoto, Japan) and its trans isomer, elaidic acid (Nacalai Tesque Co., Kyoto, Japan), sodium oleate (Nofable BO-99N, Nippon Oil & Fat Co., Ltd., Tokyo, Japan) and fluorescein isothiocyanate (FITC)-dextran (FD-4, with an average molecular mass of 4000 Da) and polyoxyethylene 9 lauryl ether (Laureth-9) (Sigma Chemical Co., MO, U.S.A.) were used as supplied. All other materials and solvents used were of analytical reagent grade and deionized double-distilled water was used.

Solubility Measurements A constant and excess amount of oleic acid or elaidic acid was added to an isotonic phosphate buffer solution (pH 7.4) containing a given concentration of HP-CyDs. These were mixed by a reciprocal shaker at 25 °C and the head space was replaced by nitrogen gas to minimize the oxidation of the unsaturated fatty acids. After being shaken vigorously for 2 d, an aliquot was filtered through a cellulose acetate membrane, Advantec Dismic 25CS045AN (Toyo-Roshi Co., Tokyo, Japan). The fatty acids in the filtrate were assayed by high-performance liquid chromatography (HPLC)[8] under the following conditions: UV monitor, Hitachi L-4000 (Tokyo, Japan) at 204 nm; pump, Hitachi L-6000 (Tokyo, Japan); column, Inertsil ODS-2 (4.6 i.d. × 150 mm; GL-Science Co., Tokyo, Japan); mobile phase, acetonitrile–water (9 : 1 v/v); flow rate, 1.5 ml/min. The stability constant of the complexes of the fatty acids with HP-CyDs ($K_{1:n}$), assuming that $1:n$ higher-order complexes occur in a stepwise reaction, was calculated from

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the ascending curvatures of the solubility diagrams according to the optimization technique described previously.[15]

Stability of Oleic Acid against Oxidation One hundred microliters of ethanol containing oleic acid (500 μM) was added to the buffer solution (10 ml, pH 7.4) containing HP-CyDs (500 μM) and incubated at 25 °C for 3 h while oxygen gas was bubbled through the solution at a pressure of 0.5 kg/cm^2 in the dark. In a separate experiment, oleic acid (1% w/v) emulsified or dissolved in the phosphate buffer (pH 7.4) in the absence and presence of HP-β-CyD (20% w/v) was stored in the dark in a glass-stoppered tube at 25 °C or 40 °C for 7 d. After being re-emulsified, an aliquot (30 μl) was withdrawn from the sample, dissolved in the HPLC mobile phase (3 ml), and filtered through a cellulose acetate membrane, Advantec Dismic 25JP045AN (Toyo-Roshi Co., Tokyo, Japan). The remaining oleic acid in the sample was assayed by the HPLC method as described above.

NMR Studies ^{1}H-NMR spectra were recorded at ambient temperature on a JNM-α 500 spectrometer (Jeol, Tokyo, Japan), operating at 500 MHz. Deuterium oxide was used as a solvent and the water signal as an internal reference. In this study, sodium oleate was used instead of oleic acid because of the limited solubility of the free form in D_2O. Chemical shifts were given as parts per million (ppm) relative to that of the HOD signal (4.65 ppm), with an accuracy of about $\pm$0.005 ppm. The NMR signals of sodium oleate and β-CyD were assigned according to previous reports.[16,17]

Stability of Buserelin in Nasal Homogenates Male Wistar rats weighing 220—280 g were fasted overnight, anesthetized with diethyl ether and decapitated. The nasal mucosa on the septal cartilage was isolated from the frontal bone and homogenized in a 10-fold volume of cold isotonic phosphate buffer (pH 7.4) using a blade homogenizer (Physcotron® NS-50, Niti-On Co. Ltd., Chiba, Japan). The homogenates were centrifuged at 9000 $\times$ *g* for 10 min at 4 °C and the resulting supernatant (0.2 ml, 2.5 mg/ml proteins) was added to the buffer solution (0.8 ml, pH 7.4) containing buserelin acetate (0.125 mg/ml), oleic acid (1.25% w/v) and/or HP-β-CyD (25% w/v), and incubated at 37 °C. At appropriate intervals, an aliquot of the mixture (0.1 ml) was withdrawn and added to 0.1 N hydrochloric acid solution (1 ml, 0 °C) to terminate the enzymatic reaction. The residual buserelin in the mixture was determined by HPLC under the following conditions: monitor, Shimadzu RF-550A fluorescence spectrophotometer (Kyoto, Japan) at excitation wavelength 280 nm and emission wavelength 350 nm; pump, Hitachi L-6000 (Tokyo, Japan); column, GL-Science Inertsil ODS-2, 6.0 i.d. $\times$ 150 mm (Tokyo, Japan); mobile phase, 0.8% KH_2PO_4 (pH 6.2)–acetonitrile (2 : 1 v/v); flow rate, 1.0 ml/min.[17]

Nasal Absorption Studies The nasal absorption studies were performed according to the method of Hirai *et al.*,[18] and the experimental procedures were essentially the same as those described previously.[19] Male Wistar rats weighing 220—280 g were fasted for 16 h and anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). During the experiment the rats were kept lying on their back on a thermostated rug at 37 °C, and anesthesia was maintained with subsequent injections of the anesthetic (15 mg/kg) every 1 to 2 h. Ten milligrams of buserelin acetate was dissolved in the isotonic phosphate buffer (1 ml, pH 7.4) with or without oleic acid (1.5% w/v) and HP-β-CyD (20% w/v). Each solution was administered to the nostril with a micropipette at a dose of 0.1 mg/kg as buserelin acetate. In comparison, buserelin acetate in the phosphate buffer was administered intravenously to the jugular vein of the rats at a dose equivalent to that by nasal administration. Blood samples (0.4 ml) were collected periodically from the jugular vein, centrifuged to obtain plasma and stored at -30 °C for later analysis. The concentration of buserelin in plasma was determined by the double-antibody radioimmunoassay method.[20]

The permeability of rat nasal mucosa was assessed by measuring the extent of nasal absorption of FD-4, an inert and poorly permeable marker, for 3 h post-administration. The concentration of FD-4 in plasma was measured by fluorescence spectrometer (Hitachi F-4010, Tokyo, Japan) at excitation wavelength 495 nm and emission wavelength 519 nm.[21]

Data Analysis *In vivo* data were analyzed statistically by one-way analysis of variance, using Duncan's multiple comparison test, and *P* values of <0.01 were viewed as statistically significant.

Results and Discussion

Complexation of Oleic Acid with HP-CyDs Figure 1 shows the phase solubility diagrams obtained for oleic acid with the three HP-CyDs in isotonic phosphate buffer (pH 7.4) at 25 °C. The solubility of oleic acid increased with a rise in the HP-CyD concentrations, showing a positive deviation from linearity. These solubility curves can be classified as type A_P,[22] suggesting the formation of higher-order complexes. The ascending curvatures in Fig. 1 were quantitatively analyzed according to the optimization technique[15] to obtain the stability constants of higher-order complexes ($K_{1:n}$), and the results are summarized in Table 1. As judged from Akaike's information criterion for non-linear regression equations, the 1 : 1 and 1 : 2 complexes of oleic acid with the HP-CyDs were assumed to have formed. The $K_{1:1}$ values were remarkably greater than the $K_{1:2}$ values for all the complexes, suggesting that the 1 : 1 complexes are the predominant species under the present conditions.

It is well known that the acyl chain of free fatty acids and their CoA derivatives fits tightly into the hydrophobic cavity of α-CyD and more loosely into the larger inner space of β- and γ-CyDs.[23] Nevertheless, the inclusion ability of the HP-CyDs for oleic acid increased in the order: HP-γ-$\ll$HP-α-$<$HP-β-CyD; the β-size cavity was found to be the most efficient. Recent studies have shown that within a fatty acid molecule as the number of *cis*-double bonds increases, the compound assumes a non-linear structure and more compact geometry, resulting in the formation of a more stable inclusion complex with β-CyD derivatives.[3,16,24] Since oleic acid has a *cis*-double bond halfway along the acyl chain, its bent structure may provide a better fit into the cavity of HP-β-CyD rather than HP-α-CyD. This view is supported by the fact that the smallest cavity of HP-α-CyD is the most appropriate partner for elaidic acid, a linear trans-isomer of oleic acid, as indicated by the magnitude of the stability constants of the complexes in Table 1.

Insight into the inclusion mode of oleic acid with HP-β-CyD was gained by employing ^{1}H-NMR spectroscopy. In a recent study, the structure of linoleic acid, an unsaturated fatty acid having two *cis*-double bonds, with β-CyD has been characterized by ^{1}H spin-lattice relaxation

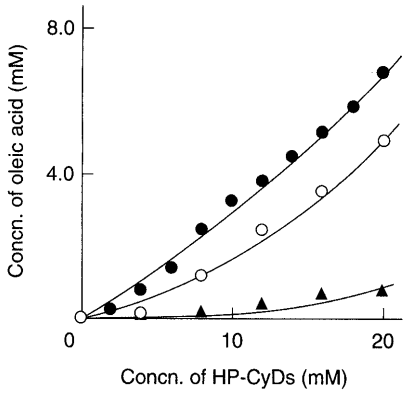

Fig. 1. Phase Solubility Diagrams of Oleic Acid/HP-CyD Systems in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

○: HP-α-CyD, ●: HP-β-CyD, ▲: HP-γ-CyD. Each point represents the mean of 2 experiments.

Table 1. Stability Constants of Complexes of Oleic Acid and Elaidic Acid with HP-CyDs in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

System	Stability constant ($K_{guest:host}$, M^{-1})			
	Oleic acid		Elaidic acid	
	$K_{1:1}$	$K_{1:2}$	$K_{1:1}$	$K_{1:2}$
HP-α-CyD	4600	100	3900	50
HP-β-CyD	15000	20	2000	70
HP-γ-CyD	1400	30	30	150

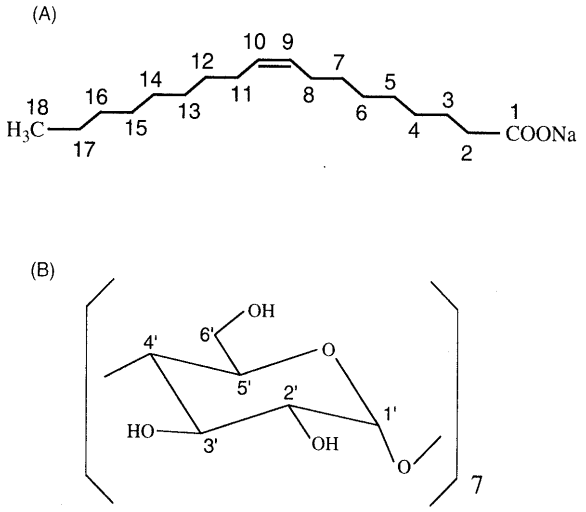

Fig. 2. Chemical Structures of Sodium Oleate (A) and β-CyD (B)

time measurements and one-dimensional difference nuclear Overhauser enhancement experiments, in which the double bond at position 9—10 in linoleic acid is partly buried in the CyD cavity.[16] In this study, a sodium salt of oleic acid (Fig. 2) was used because of the limited solubility of the free form in D_2O. Table 2 summarizes the ^{1}H-NMR chemical shift changes for the sodium oleate/β-CyD system and the sodium oleate/HP-β-CyD system. Because of the structural heterogeneity of HP-β-CyD, the proton signals were broadening and could not be assigned. The H3′ and H5′ protons of β-CyD, which are directed towards the interior of the cavity, showed a marked upfield shift, in contrast, the shifts of the other protons of β-CyD, which are located outside the cavity, were small. Upon binding to either β-CyD or HP-β-CyD, the proton signals (H8-11 and H18) of oleic acid were largely shifted downfield, while the shifts of the proton signals (H2-3) were small. Furthermore, in the two-dimensional nuclear Overhauser effect spectrum, the cross peaks connecting the intermolecular protons between the resonances of β-CyD (H3′ and H5′) and the resonances of oleic acid (H5-6 or H13-17) were observed. These cross peaks are due only to the nuclear Overhauser effect, because they arise between two different molecules; they suggest the close contact (<4—5 Å) of the interacting protons. These results suggest that the methyl-end arm (C10-18) of oleic acid is present within the hydrophobic cavity of HP-β-CyD, while the carboxyl arm (C1-9) is located outside of the cavity. The *cis*-double bond of oleic acid appears to be buried within the CyD cavity.

Table 2. ^{1}H-NMR Chemical Shift Displacement of Sodium Oleate (SOA, 10 mM) and β-CyD for SOA/β-CyD (20 mM) System and SOA/HP-β-CyD (20 mM) System in D_2O

Proton	Chemical shift of SOA (ppm)				
	δ_0 [a]	$\delta_{\beta\text{-CyD}}$ [b]	$\Delta\delta_{\beta\text{-CyD}}$ [c]	$\delta_{\text{HP-}\beta\text{-CyD}}$ [d]	$\Delta\delta_{\text{HP-}\beta\text{-CyD}}$ [e]
2	2.078	2.064	−0.014	2.089	0.011
3	1.463	1.434	−0.029	1.474	0.011
8, 11	1.951	2.009	0.058	1.958	0.007
9, 10	5.283	5.402	0.119	5.355	0.072
18	0.797	0.858	0.061	0.833	0.036

Proton	Chemical shift of β-CyD (ppm)		
	δ_0 [f]	δ_{SOA} [g]	$\Delta\delta_{SOA}$ [h]
1′	4.983	4.969	−0.014
2′	3.563	3.509	−0.054
3′	3.876	3.782	−0.094
4′	3.496	3.536	0.040
5′	3.768	3.615	−0.153
6′	3.791	3.802	0.011

a) Chemical shift of SOA alone. *b*) Chemical shift of SOA with β-CyD. *c*) $\delta_{\beta\text{-CyD}}-\delta_0$. *d*) Chemical shift of SOA with HP-β-CyD. *e*) $\delta_{\text{HP-}\beta\text{-CyD}}-\delta_0$. *f*) Chemical shift of β-CyD alone. *g*) Chemical shift of β-CyD with SOA. *h*) $\delta_{SOA}-\delta_0$.

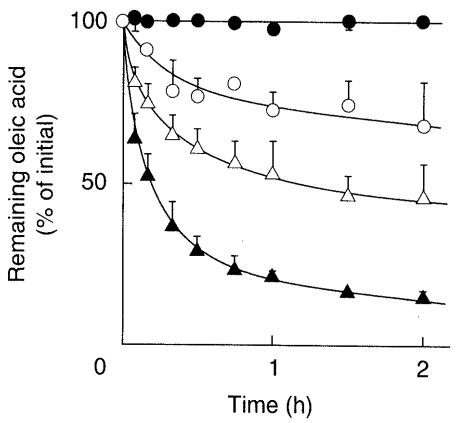

Fig. 3. Effects of HP-CyDs (0.5 mM) on Oxidative Degradation of Oleic Acid (5 μM) in Isotonic Phosphate Buffer (pH 7.4) Containing Ethanol (1% v/v) at 25 °C

▲: without HP-CyDs, ○: with HP-α-CyD, ●: with HP-β-CyD, △: with HP-γ-CyD. Each point represents the mean ± S.E. of 2—3 experiments.

The susceptibility of oleic acid to oxidation may limit its practical use as an adjuvant in nasal formulations. CyDs are known to inhibit either enzymatic or non-enzymatic peroxidation of unsaturated lipids.[3] Figure 3 shows the effects of the three HP-CyDs on the oxidative degradation of oleic acid in isotonic phosphate buffer (pH 7.4) containing ethanol (1% v/v) under an oxygen-saturated condition. The HP-CyDs protected oleic acid against oxidative degradation in the order of HP-γ- < HP-α- < HP-β-CyD, which clearly fits the sequence of their inclusion abilities for oleic acid. In particular, the degradation of oleic acid was almost completely suppressed by HP-β-CyD within the time employed. We also examined the chemical stability of oleic acid in a typical example of the nasal formulations, in which oleic acid (1% w/v) was emulsified

or dissolved in the phosphate buffer (pH 7.4) in the absence and presence of HP-β-CyD (20% w/v). After being stored at 25 °C for 7 d in a glass-stoppered tube in the dark, the remaining percentage of oleic acid with HP-β-CyD was 97.3 ± 2.3%, while oleic acid without HP-β-CyD had completely disappeared, probably due to oxidation through the radical reactions initiated by oxygen in the head space of the tube or in solution. As expected from the ^{1}H-NMR results (Table 2), oleic acid orients within the cavity of HP-β-CyD, and the double bond appears to be buried, and hence becomes less susceptible to oxidation. Based on the above results, HP-β-CyD which has the highest binding capacity was selected as the preferred solubilizer and stabilizer for oleic acid and was used in the following studies.

Protection of Buserelin against Proteolysis Various types of aminopeptidases are present in nasal mucosa, which appears to be the major metabolic barrier to the nasal absorption of peptide and protein drugs.[25] In the chemical structure of buserelin acetate, a D-amino acid derivative and proline-ethylamide substitute for the glycine residue in position 6 and for the C-terminal sequence proline-glycinamide, respectively, of the native luteinizing hormone releasing hormone (LHRH) sequence. Although these modifications considerably reduce the susceptibility of the LHRH agonist to proteolytic enzymes, the peptide is still inactivated in nasal mucosa. Figure 4 shows the effects of oleic acid and HP-β-CyD on the enzymatic degradation of buserelin in rat nasal mucosal homogenates. Buserelin was rapidly degraded and almost completely disappeared within 1 h. This degradation was inhibited by the addition of either oleic acid (1% w/v, in emulsion) or HP-β-CyD (20% w/v), the former being more effective. Both may protect buserelin from proteolytic degradation in a differential manner. Because of the limited interaction between buserelin and oleic acid, it is likely that oleic acid reduces the activities of mucosal or cytosolic peptidases, possibly by denaturing the enzymes and preventing the formation of the enzyme-substrate complex.[26] In sharp contrast, CyDs interact preferentially with hydrophobic amino acid residues (tryptophan, tyrosine and *tert*-butyl-D-serine) in buserelin, as recently confirmed by NMR and fluorescence techniques.[27] Since these hydrophobic side chains are located near the enzymatic cleavage sites of the peptide, these interactions may restrict the formation of a catalytic complex of enzyme with buserelin. As shown in Fig. 4, the combination of oleic acid with HP-β-CyD showed no synergism with regard to the protection of buserelin against the proteolysis. Compared with the sole use of oleic acid, the rather weak protection afforded by the combination of oleic acid with HP-β-CyD can be ascribable largely to the formation of the oleic acid–HP-β-CyD complex that is less effective in reducing the activities of peptidases. Furthermore, HP-β-CyD, whose cavity is occupied with oleic acid, may no longer act as a stabilizer for buserelin against the enzymatic degradation.

Increased Permeability of Nasal Mucosa The permeability barrier of nasal mucosa is the resistance of the membrane to diffusion of solutes, which in the cases of

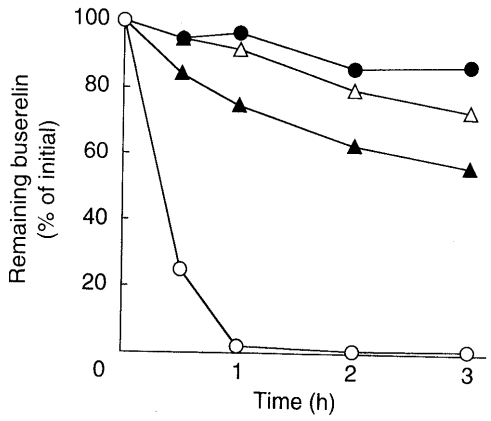

Fig. 4. Effects of Oleic Acid (1% w/v) and HP-β-CyD (20% w/v) on Degradation of Buserelin Acetate (0.01% w/v) in Rat Nasal Homogenates in Isotonic Phosphate Buffer (pH 7.4) at 37 °C

○: without additives, ●: with oleic acid, ▲: with HP-β-CyD, △: with oleic acid and HP-β-CyD. Each point represents the mean ± S.E. of 3 experiments.

peptides and proteins may be related to their large molecular size and hydrophilicity.[28] Since FD-4 is enzymatically stable before or while crossing the nasal epithelium and poorly permeable into the membrane, the extent of nasal absorption of FD-4 co-administered with oleic acid and HP-β-CyD may provide a reliable measure for estimating their effects on the permeability barrier of nasal mucosa.

Figure 5 shows the plasma profiles of FD-4 after the nasal administration of FD-4 with oleic acid at various concentrations and HP-β-CyD (20% w/v) to rats. HP-β-CyD itself did not affect the permeability of the nasal mucosa. In the absence of HP-β-CyD, oleic acid exists mainly as oil droplets. Hamilton has described that oleic acid can be absorbed directly from oil droplets without passing through an intermediary micellar phase in the rat jejunum.[29] A gradual increase in the nasal absorption of FD-4 over the concentration ranges of oleic acid (Fig. 5A) indicates that oleic acid is transferred *via* oil droplets as well as aqueous phase into the nasal mucosa. As shown in Fig. 5B, HP-β-CyD (20% w/v) reduced the activity of oleic acid when applied in concentrations below 1.0% w/v of oleic acid. In our previous studies, maximum transfer of the enhancer into the nasal mucosa was achieved when just enough HP-β-CyD was used to maintain all the enhancer in solution.[8] The critical concentration of oleic acid to be solubilized by HP-β-CyD (20% w/v) was *ca.* 1.5% w/v. The presence of excess amounts of HP-β-CyD in the solution may reduce the free fraction of oleic acid, which is in an equilibrium with the complexed form, and thereby reduce the availability of the enhancer at the mucosal surface, because the complexed form of the guest molecule is generally less permeable through biological membranes due to its bulky and hydrophilic nature.[9]

On the other hand, when oleic acid was applied at concentrations above 1.5% w/v in conjunction with HP-β-CyD (20% w/v), the nasal absorption of FD-4 was significantly increased, compared with that for the sole use of the enhancer (Fig. 5B). HP-β-CyD may enhance the absorption promoting activity of oleic acid by

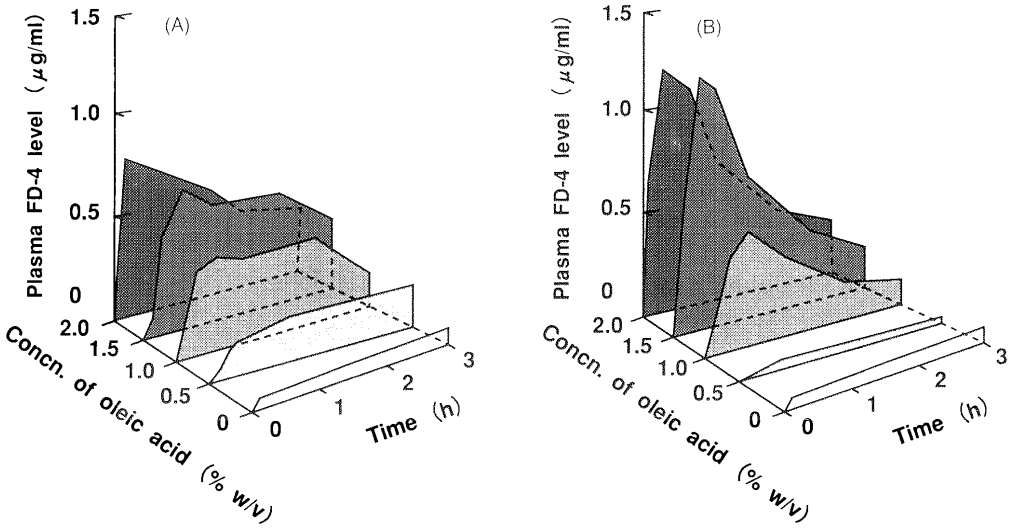

Fig. 5. Plasma Levels of FD-4 after Nasal Administration of FD-4 (7.5 mg/kg) with Oleic Acid and HP-β-CyD (20% w/v) to Rats

(A): with oleic acid, (B): with oleic acid and HP-β-CyD. Each point represents the mean of 4—9 rats.

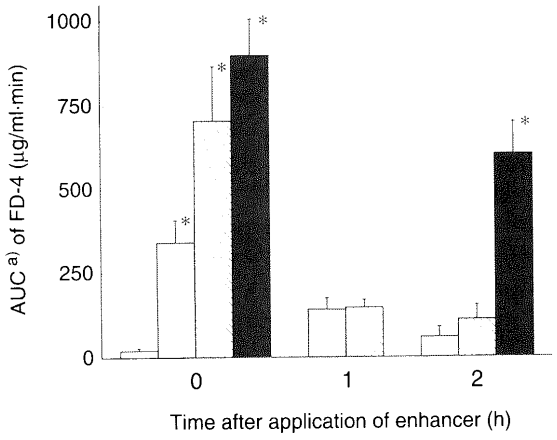

Fig. 6. Recovery from Hyperpermeable State of Nasal Mucosa as Indicated by AUC Value of FD-4 after Nasal Administration of FD-4 with Oleic Acid (1.5% w/v), HP-β-CyD (20% w/v) and Laureth-9 (1% w/v) to Rats

[ ]: without additives, [▒]: with oleic acid, [▨]: with oleic acid and HP-β-CyD, [■]: with Laureth-9. Each point represents the mean ± S.E. of 3—6 rats. $*p<0.01$ *vs.* without additives. a) The area under the plasma FD-4 concentration-time curve up to 2 h post-administration.

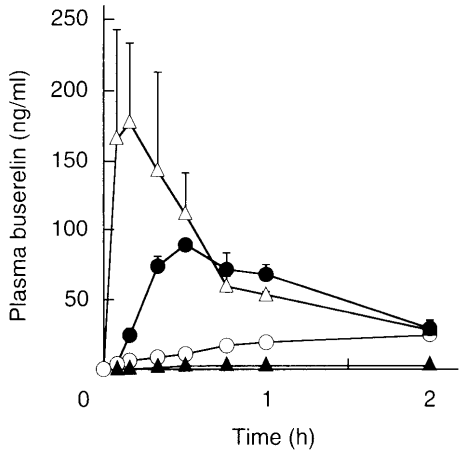

Fig. 7. Plasma Levels of Buserelin after Nasal Administration of Buserelin Acetate (0.1 mg/kg) with Oleic Acid (1.5% w/v) and HP-β-CyD (20% w/v) to Rats

○: buserelin acetate alone, ●: with oleic acid, ▲: with HP-β-CyD, △: with oleic acid and HP-β-CyD. Each point represents the mean ± S.E. of 3—6 rats.

solubilizing, thus making it more available at the mucosal surface for subsequent penetration into the nasal epithelium, a site of action. In other words, the rate of overall processes involving the dissociation of oleic acid from its HP-β-CyD complex and subsequent uptake of the free form of oleic acid into the mucosa seems to be much faster than that of the transfer of oleic acid from oil droplets into the mucosa, consequently leading to the increased rate and extent of the FD-4 absorption.

From a safety point of view, we evaluated the rate of recovery from the hyperpermeable state of the nasal mucosa induced with the combined use of oleic acid and HP-β-CyD. In this experiment, oleic acid and HP-β-CyD were administered to the nasal cavity of non-anesthetized rats, and after predetermined time intervals the nasal absorption of FD-4 without the adjuvants was determined. As shown in Fig. 6, the hyperpermeable state of the nasal mucosa mediated by either sole or combined use of oleic acid and HP-β-CyD returned to normal physiological level within 2 h after the nasal application. The recovery from the hyperpermeable state caused by this combination was significantly faster than that caused by Laureth-9, a membrane irritating surfactant.

Enhanced Nasal Absorption of Buserelin As mentioned above, the combined use of oleic acid with HP-β-CyD reduced the metabolic and physical barriers existing in the nasal epithelium, a situation which would improve the nasal bioavailability of buserelin. Figure 7 shows the effects of oleic acid (1.5% w/v) and HP-β-CyD (20% w/v) on the plasma levels of buserelin after the nasal administration of buserelin acetate (0.1 mg/kg) in anaesthetized rats. The pharmacokinetic parameters for buserelin were calculated from the plasma profiles up to 2 h post-administration and the results are summarized in Table 3. HP-β-CyD reduced the nasal absorption of buserelin, probably through the formation of a complex that was less permeable through the nasal epithelium due to its bulky and highly hydrophilic nature. The rate and extent

Table 3. Pharmacokinetic Parameters[a] of Buserelin after Nasal Administration of Buserelin Acetate (BLA, 0.1 mg/kg) with Oleic Acid (1.5% w/v) and HP-β-CyD (20% w/v) to Rats

System	C_{max}[b] (ng/ml)	T_{max}[c] (h)	MRT[d] (h)	AUC[e] (ng/ml·h)	F[f] (%)
BLA alone	24.7± 4.0	1.5±0.2[h]	1.2±0.0[h]	33.2± 5.5[h]	11.9±2.0[h]
With oleic acid	89.5± 3.8	0.5±0.0[g]	0.9±0.0[g]	109.0±11.4[g]	39.1±4.1[g]
With HP-β-CyD	3.4± 0.4[h]	1.4±0.4[h]	1.2±0.1[h]	4.1± 0.4[h]	1.5±0.1[h]
With oleic acid and HP-β-CyD	229.3±68.3[g,h]	0.1±0.0[g]	0.7±0.0[g,h]	144.3±26.9[g,h]	51.8±9.6[g,h]

a) Each value represents the mean±S.E. of 3—6 rats. *b*) Maximum plasmalevel. *c*) Time required to reach the maximum plasma level. *d*) Mean residence time in plasma. *e*) Area under the plasma level-time curve up to 2 h post-administration. *f*) Extent of bioavailability compared with the *AUC* value of BLA administered intravenously (0.1 mg/kg). *g*) $p<0.01$ *vs.* BLA alone. *h*) $p<0.01$ *vs.* with oleic acid.

of nasal bioavailability of buserelin were remarkably increased by coadministration of oleic acid and HP-β-CyD, compared with the sole use of the enhancer. This combination provided an approximately 5-fold increase in the extent of nasal bioavailability of buserelin, reaching *ca.* 50% of that based on intravenous administration.

Conclusion

HP-β-CyD can serve as a biocompatible solubilizer and stabilizer to expand the usefulness of lipophilic absorption enhancers. The combination of oleic acid with HP-β-CyD at the appropriate ratio appears to be an effective and probably safe adjuvant in promoting the nasal absorption of buserelin. This combination can provide a valuable tool for designing aqueous *trans*-mucosal formulations of peptide and protein drugs with a required balance between efficacy and safety.

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References

1) Schlenk H., Sand D. M., *J. Am. Chem. Soc.*, **83**, 2312 (1961).
2) Sharma A., Anderson D., Baker J. W., *Clinical Chem.*, **36**, 529 (1990).
3) Szente L., Szejtli J., Szemán J., Kató L., *J. Incl. Phenom.*, **16**, 339 (1993).
4) Barry B. W., *J. Controlled Release*, **6**, 85 (1987).
5) Yoshikawa H., Takada K., Muranishi S., Satoh Y., Naruse N., *J. Pharmacobio-Dyn.*, **7**, 59 (1984).
6) Tengamnuay P., Mitra A. K., *Pharm. Res.*, **7**, 127 (1990).
7) Yanagi H., Ishikura N., Kenmotsu H., Yagi N., Goto K., Kato S., Sumiya S., Takada M., *Yakugaku Zasshi*, **111**, 65 (1991).
8) Irie T., Abe K., Adachi H., Uekama K., Manako T., Yano T., Saita M., *Drug Delivery System*, **7**, 91 (1992).
9) Abe K., Irie T., Adachi H., Uekama K., *Int. J. Pharmaceut.*, **123**, 103 (1995).
10) AL-Nakib W., Higgins P. G., Barrow G. I., Tyrrell D. A. J., Andries K., Bussche G. V., Taylor N., Janssen P. A. J., *Antimicrobial Agents and Chemotherapy*, **33**, 522 (1989).
11) Brewster M. E., Estes K. S., Bodor N., *Int. J. Pharmaceut.*, **59**, 231 (1990).
12) Mesens J.L., Putteman P., Verheyen P., "New Trends in Cyclodextrins and Derivatives," ed. by D. Duchêne, Editions de Santé, Paris, 1991, pp. 369—407.
13) Pitha J., Irie T., Sklar P. B., Nye J. S., *Life Sci.*, **43**, 493 (1988).
14) Pitha J., Milecki J., Fales H., Pannell L., Uekama K., *Int. J. Pharmaceut.*, **29**, 73 (1986).
15) Uekama K., Horiuchi Y., Kikuchi M., Hirayama F., *J. Incl. Phenom.*, **6**, 167 (1988).
16) Jyothirmayi N., Ramadoss C. S., Divakar S., *J. Agric. Food Chem.*, **39**, 2123 (1991).
17) Uekama K., Arima H., Irie T., Matsubara K., Kuriki T., *J. Pharm. Pharmacol.*, **41**, 874 (1989).
18) Hirai S., Yashiki T., Mima H., *Int. J. Pharmaceut.*, **9**, 165 (1981).
19) Irie T., Wakamatsu K., Arima H., Aritomi H., Uekama K., *Int. J. Pharmaceut.*, **84**, 129 (1992).
20) Saito S., Saito H., Yamasaki R., Hosoi E., Komatsu M., Iwahana H., Maeda T., *J. Immunol. Methods*, **79**, 173 (1985).
21) Ohtani T., Murakami M., Yamamoto A., Takada K., Muranishi S., *Int. J. Pharmaceut.*, **77**, 141 (1991).
22) Higuchi T., Connors K. A., *Adv. Anal. Chem. Instr.*, **4**, 117 (1965).
23) Bergeron R., Machida Y., Bloch K., *J. Biol. Chem.*, **250**, 1223 (1975).
24) Asakura K., Tanaka N., Kanauchi T., Matsumura S., Yoshikawa S., *Jpn. Oil. Chem. Soc.*, **41**, 203 (1992).
25) Stratford R. E., Jr., Lee V. H. L., *Int. J. Pharmaceut.*, **30**, 73 (1986).
26) Lee V. H. L., *CRC Crit. Rev. Ther. Drug Carrier Systems*, **5**, 69 (1988).
27) Irie T., Arima H., Abe K., Uekama K., Matsubara K., Kuriki T., "Minutes of the 6th International Symposium on Cyclodextrins", ed. by A.R. Hedges, Editions de Santé, Paris, 1992, pp. 503—508.
28) Lee V. H. L., Yamamoto A., *Advanced Drug Delivery Reviews*, **4**, 171 (1990).
29) Hamilton J. D., *Biochim. Biophys. Acta*, **239**, 1 (1971).