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Cyclodextrins and chitosan derivatives in sublingual delivery of low solubility peptides: A study using cyclosporin A, α-cyclodextrin and quaternary chitosan *N*-betainate

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ABSTRACT

Systemic drug delivery through intraoral membranes may offer a promising administration route for lipophilic peptide drugs. The aim of the present study was to investigate the effect of α-cyclodextrin (α-CD) and a novel chitosan derivative, chitosan *N*-betainate (CH), on sublingual absorption of a hydrophobic model peptide cyclosporin A (CsA), and the effect of temperature on the complexation of CsA with α-CD.

Complexation of CsA with α-CD was studied using the phase-solubility method.

Sublingual absorption of CsA was studied by administration of solid CsA/α-CD complex (with and without CH solution), solid CsA/α-CD/CH formulation and solid plain CsA to rabbits.

The solubility of CsA in aqueous α-CD solution (14%) increased with decreasing temperature; the solubility of CsA at room temperature, +5 and +1 °C was 1.2, 12 and 19 mg/ml, respectively. The bioavailability of CsA after administration of plain CsA, solid CsA/α-CD and solid CsA/α-CD/CH (0.6 ± 0.5, 1.4 ± 0.7 and 1.7 ± 0.8%, respectively; mean ± S.D.) was further increased when solid CsA/α-CD was administered together with CH solution (3.2 ± 2.2%).

The present study shows that decreased temperature can be effectively utilized to produce CsA/α-CD complexes. It was also shown that α-CD and CH may be advantageous in sublingual delivery of lipophilic peptides, although the absolute bioavailability remains low.

1. Introduction

Successful therapeutic utilization of therapeutically active peptides depends on the ability to administer them in an efficient and patient-compliant manner. Oral delivery of peptides is rarely possible due to their enzymatic and acidic instability in the gastro-intestinal (GI)-tract, limited absorption through biological membranes as well as pre-systemic metabolism (Lee and Yamamoto, 1990). As a result, to date many peptides have to be administered parenterally either via intravenous, subcutaneous or intramuscular route.

Systemic drug delivery through intraoral membranes would circumvent some of the conditions of the GI-tract as well as pre-systemic metabolism (Rathbone et al., 1996). Other benefits of intraoral route include easy access, robustness to tolerate absorption enhancers and low enzymatic activity (Harris and Robinson, 1992). However, intraoral delivery of drug compounds has strict requirements; the compound must first dissolve and disperse into the saliva, then cross the unstirred water layer consisting of mucin network, and finally, the oral membrane (Veuillez et al., 2001; Loftsson et al., 2007). Most of the peptides are large and hydrophilic and thus, absorption enhancers are often needed in order to improve systemic absorption (Shojaei, 1998; Loftsson et al., 2007). Some peptides are, however, relatively small and lipophilic and may need dissolution enhancers to achieve absorption.

Cyclodextrins (CDs) are a group of excipients which can improve the permeation of hydrophobic drugs through biological membranes by enhancing the dissolution and transport through the unstirred water layer (Shojaei, 1998). CDs have previously been shown to be useful in the development of intraoral drug formulations of small lipophilic drug molecules such as Δ^9-tetrahydrocannabinol, cannabidiol, nicotine (β-CD) (Molander and Lunell, 2001; Mannila et al., 2006, 2007) and testosterone (hydroxypropyl-β-CD) (Salehian et al., 1995). However, only few studies have dealt with the use of CDs in intraoral peptide delivery; e.g. hydroxypropyl-β-CD (HP-β-CD) has been shown to improve sublingual absorption of insulin (Cui et al., 2005). To our knowledge, there are no studies dealing with the advantages of CDs in intraoral delivery of lipophilic peptides.

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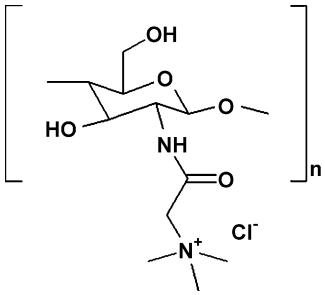

Fig. 1. Chemical structure of chitosan *N*-betainate chloride.

Another potential group of excipients in transmucosal peptide delivery is chitosan and its derivatives (Illum, 1998; Di Colo et al., 2008). Chitosans are suggested to enhance absorption of drugs through mucoadhesion, improved aqueous solubility of a drug and an increase in permeability of biological membranes by loosening the tight junctions or interfering with the lipid organization (Illum, 1998; Senel et al., 2000). The effects of chitosans in intraoral delivery of peptide or protein drugs are not widely studied; it has been reported that chitosan can improve the flux of transforming growth factor-β across buccal mucosa (Senel et al., 2000). In addition, thiolated chitosan derivative has been shown to increase the buccal bioavailability of pituitary adenylate cyclase-activating polypeptide (Langoth et al., 2006).

The aim of the present study was to examine the possible advantages of CDs and chitosans in sublingual delivery of a lipophilic model peptide, cyclosporin A (CsA). A novel complexation method with improved complexation efficiency of CsA with α-CD was developed, and the bioavailability of CsA was studied after sublingual administration of solid CsA/α-CD complex. In addition, the effects of a novel water-soluble chitosan derivative, chitosan *N*-betainate (CH), on sublingual absorption of CsA was studied after sublingual administration CsA/α-CD.

2. Materials and methods

2.1. Materials

Chitosan *N*-betainate (Fig. 1) was prepared by a 5-step synthetic route as reported earlier, by protection and deprotection of the amino- and primary hydroxyl groups (Holappa et al., 2004, 2006). Chitosan *N*-betainate was measured to have a degree of substitution of 0.05 and molecular weight of 42.1 kDa. CsA was obtained from Fluka Biochemica (Buchs, Switzerland) and cyclosporin D (CsD) was purchased from R&S Pharmchem Co. (Hangzhou, Zhejiang, China). The cyclodextrins α-CD and hydroxypropyl-α-CD (HP-α-CD; 1297 g/mol) were purchased from Wacker-Chemie (Burghausen, Germany). All other reagents were of pharmaceutical grade and were used as received.

2.2. Analytical methods

2.2.1. HPLC

High performance liquid chromatography (HPLC) was used for the quantification of CsA in phase-solubility and dissolution studies. The HPLC system consisted of a Merck Hitachi L-7400 UV-detector (wavelength 214 nm), D-7000 interface module, L-7250 autosampler, L-7100 pump (Hitachi ltd, Tokyo, Japan), column oven (MetaTherm Technologies, Torrance, CA, USA) and HPLC System Manager software (Hitachi, ltd., 1996). Purospher RP-18 (125 mm × 4 mm i.d., 5 μm) column was purchased from Merck KGaA (Darmstadt, Germany). Chromatographic conditions were as follows—column temperature: 50 °C; injection volume: 20 μl; isocratic flow rate: 1.0 ml/min; mobile phase: 80% (v/v) acetonitrile in water with 0.1% (v/v) formic acid. The range of the HPLC method was 0.1–100 μg/ml.

Table 1
The precision and accuracy of the HPLC–MS method used for the analysis of CsA.

Concentration (ng/ml)	Within-day precision (%R.S.D.; $n=3$)	Between-day precision (%R.S.D.; $n=6$)	Accuracy (%; $n=3$)
0.2[a]	8.0	8.4	92
0.5	4.6	11.2	105
50	5.7	4.7	97

[a] Lower limit of quantitation.

2.2.2. HPLC–MS

HPLC equipped with mass spectrometer (MS) was used for the quantification of CsA from rabbit plasma. HPLC consisted of a Finnigan Surveyor MS Pump and a Finnigan Surveyor Autosampler (Thermo Electron, San Jose, CA, USA) with a Xterra C8 column (50 mm × 2.1 mm, 3.5 μm; Waters, Milford, MA, USA) and a Zorbax XDB-C8 Narrow-Bore Guard Column (Agilent Technologies, Palo Alto, CA, USA). Column temperature was maintained at 60 °C. Separation was achieved by gradient elution at a flow rate of 200 μl/min: the mobile phase consisted of 10 mM ammonium acetate in water (A) and 10 mM ammonium acetate in water–acetonitrile (B; 5:95; v/v). The gradient program was as follows—0–5 min: 40% B → 100% B, 5–9.5 min: 100% B, 9.5–10 min: 100% B → 40% B, 10–12 min: 40% B.

The mass analysis was carried out with a Finnigan LTQ linear ion trap mass spectrometer equipped with Finnigan Ion Max electrospray ionization source operating in the positive ion mode (Thermo Electron, San Jose, CA, USA). The following instrumental conditions were used: nitrogen sheath and auxillary gas flow rates 25 and 0 arbitrary (instrument) units, respectively. Spray voltage of 4.5 kV, capillary temperature of 200 °C, collision induced dissociation energy in the ion source region of 100 V, collision energy of 40%, capillary voltage of 20 V and tube lens offset of 100 V. Collection time for the ion trap was set at 30 ms and the following transitions were monitored: m/z 1203 → 1072 + 1185 for CsA and 1217 → 1086 + 1199 for CsD (internal standard, IS). The eluent was allowed to flow into mass spectrometer from 4 to 8.4 min of each run. Data acquisition was performed by using Xcalibur 1.4.SR1 software. For quantitation, analyte to IS peak ratios were calculated as a function of concentration of the analyte using Lquan 2.0 software. The HPLC–MS method is specific, linear, accurate and precise over the range 0.2–100 ng/ml (Table 1).

2.2.3. Sample preparation

On the day of analysis, the rabbit plasma samples were thawed to ambient temperature. A total of 50 μl of the IS working solution and 50 μl of methanol (80%) was added to 500 μl of plasma and samples were vortexed (1 s). To each sample 1 ml of ACN was added leading to precipitation of plasma proteins. The samples were then vortexed (1 s), centrifuged (10,000 × *g*; 3 min) and put to freezer (−20 °C) for 30 min. The organic phase was then removed and evaporated to dryness under nitrogen. Samples were reconstituted with 100 μl of 80% (v/v) MeOH.

Calibration curve and quality control (QC) samples were analysed with each set of samples. Both calibration standards and QC samples were freshly prepared on the day of analysis by spiking rabbit plasma (500 μl) with CsA and IS solutions (50 μl each). The samples were then treated identically to the plasma samples.

2.3. Phase-solubility studies

The phase-solubility studies were carried at room temperature (RT) and at +5 °C. Excess amounts of CsA were added to aqueous

solutions containing 0–14% (w/v) α-CD (0–0.14 M) and the suspensions were shaken in the dark for 72 h (room temperature) or 7 days (+5 °C) in order to reach equilibrium. After equilibration, the suspensions were filtered (Millex HV 0.45 μm, Millipore, USA), diluted by MeOH (80%; v/v) and analysed by HPLC.

According to Higuchi and Connors (1965), the binding constants in A_P-type phase-solubility behaviour can be calculated according to Eq. (1):

$$\frac{[S_t]-[S_0]}{[L_t]} = K_{1:1}[S_0] + K_{1:1}K_{1:2}[S_0][L_t] \quad (1)$$

where $[S_t]$ is the total CsA concentration at total α-CD concentration $[L_t]$, $[S_0]$ is the solubility of CsA in the absence of CD, and $K_{1:1}$ and $K_{1:2}$ represent the binding constants for the 1:1- and 1:2-complex, respectively. The histogram of $([S_t]-[S_0])/[L_t]$ vs. $[L_t]$ results in a linear plot with a slope of $K_{1:1}K_{1:2}[S_0]$ and an intercept of $K_{1:1}[S_0]$.

2.4. Preparation of CsA formulations

2.4.1. Formulations for in vitro studies

The solid complex of CsA with α-CD for dissolution studies was prepared by adding excess CsA into an aqueous 14% (w/v) α-CD-solution. After 7 days of shaking at +1 °C, the suspension was filtered (Millex HV 0.45 μm, Millipore, USA) and freeze-dried (FTS® Systems, Inc., NY, USA). The solid CsA/α-CD complex was then filled into gelatine capsules (size 1). The amount of CsA in all capsules, as determined by HPLC, was equivalent to 1.0 mg (corresponding to 6.5 ± 1.4 mg (mean ± S.D.; $n = 5$) of freeze-dried CsA/α-CD complex powder).

For the preparation of capsules containing plain CsA, plain CsA was weighed directly into the capsule.

2.4.2. Formulations for in vivo studies

The solid CsA/α-CD complex was prepared by adding excess CsA into an aqueous 14% (w/v) α-CD-solution; after 7 days of shaking at +1 °C, the suspension was filtered and freeze-dried. The solid of CsA/α-CD/CH formulation was prepared by adding excess CsA into an aqueous solution of α-CD (14%; w/v) and CH (0.5%; w/v); after 7 days of shaking at +1 °C, the suspension was filtered and freeze-dried. The ethanolic CsA solution was prepared by allowing approximately 11 mg of CsA to dissolve into approximately 100 μl of ethanol (96%; v/v). A fraction of very fine CsA particles of less than 15 μm in diameter was obtained by sieving the purchased CsA manually through a 15 μm sieve.

2.5. Dissolution studies

Dissolution studies were carried out in a 50 ml beaker (Schott, Mainz, Germany) containing 20 ml of dissolution medium (0.16 M phosphate buffer, pH 6.6, ionic strength 0.5 M). α-CD (6%; w/v) was added to the dissolution medium in order to ensure sink conditions for CsA. The beaker was placed into a shaking water bath (Certomat® WR, B. Braun, Melsungen, West-Germany) (140 agitations min^{-1}, 37 °C). The capsule formulation (containing 1.0 mg CsA/capsule) was inserted into a ⌀ 7 mm × 30 mm cylinder, prepared from a 0.5 mm steel mesh. This cylinder was placed in the beaker at the beginning of the experiment. Aliquots of 1 ml were carefully withdrawn between 0 and 75 min, and the CsA concentration was measured by HPLC. The samples were immediately replaced with fresh dissolution medium of the same temperature after each aliquot was removed. The time required to reach 30% dissolution ($t_{30\%}$) was graphically determined for each experiment by a time vs. percent dissolved curve. The differences in values of $t_{30\%}$ for the two formulations (plain CsA vs. CsA/α-CD complex prepared at 1 °C) were statistically compared by the Mann–Whitney test. A value of $p < 0.05$ was considered as statistically significant.

2.6. Pharmacokinetic studies

New Zealand white male rabbits ($n = 7$; 2.7 ± 0.2 kg; mean ± S.D.) were purchased from National Laboratory Animal Center in Kuopio, Finland. The rabbits were allowed to eat commercial food pellets and drink water *ad libitum*, except during the test, when they were under anaesthesia. All procedures with animals were reviewed and approved by the Animal Ethics Committee at the University of Kuopio.

The rabbits were anaesthetised with combination of medetomidine (0.5 mg/kg; Domitor®; Orion Ltd.; Espoo, Finland) and ketamine (25 mg/kg; Ketalar®; Pfizer Ltd.; Espoo, Finland). Anaesthetized rabbits were positioned on a table, with the lower jaw supported in a horizontal position. CsA was given at a dose of 1 mg/kg intravenously or at a target dose of 3 mg/kg sublingually.

Blood was withdrawn into Venoject® (Terumo, Leuven, Belgium) tubes from either a central artery or marginal vein of the ear prior to CsA administration and over period of 10–360 min after administration. Blood samples were centrifuged at 3700 × *g* within 30 min, and the recovered plasma was immediately frozen to −20 °C. Samples were stored at −80 °C until analysis.

In the intravenous (i.v.) formulation, HP-α-CD was used instead of α-CD because of the better safety profiles of hydroxyalkylated CD derivatives in i.v. drug delivery (Irie and Uekama, 1997). The i.v. formulation was 20% (w/v) HP-α-CD solution containing 0.9 mg/ml of CsA. Before administration, the i.v. solution was made isotonic by adding NaCl and filtered using a sterile membrane filter (pore size 0.22 μm). The i.v. solution (target dose 1.0 mg/kg) was injected directly into the marginal ear vein. Sublingual formulations (target dose 3 mg/kg) were solid CsA/α-CD complex (average amount 70 mg) containing 1 mg CsA in approximately 8 mg of material, solid CsA/α-CD/CH formulation (average amount 50 mg) containing 1 mg CsA in approximately 6 mg of material, an ethanolic solution (average amount 80 μl) containing approximately 11 mg of CsA in 100 μl ethanol and micronized solid powder (average amount 8 mg) of plain CsA. Sublingual administration was as follows: the rabbits' tongues were carefully lifted with tweezers and the appropriate amount of solid plain CsA, solid CsA/α-CD complex powder, solid CsA/α-CD/CH powder formulation or ethanolic solution of CsA was placed under the tongue. For solid formulations 50 μl of either saline or 1% CH solution were administered under the tongue 15 min before the administration, and every 5 min after the administration, to ensure wetting of the formulation. The formulations were observed to remain in the sublingual cavity without escaping down the GI-tract.

2.7. In vivo data analysis

The maximum plasma concentration of CsA (C_{max}) and the time required to reach the maximum concentration (t_{max}) were obtained directly from the actual plasma profiles. The area under the curve between 0 and 360 min ($AUC_{0-360\,min}$) was calculated by linear trapezoidal method (0 min concentrations for the i.v. administration studies were extrapolated by the WinNonlin program (Version 4.0.1)). The elimination rate constants (k_{el}) and elimination half lives ($t_{1/2}$) were determined from i.v. data for each rabbit using the WinNonlin program. The mean k_{el} was used for the determination of $AUC_{360\,min-\infty}$ for each experiment according to Eq. (2):

$$AUC_{360\,min-\infty} = \frac{C(360\,min)}{k_{el}} \quad (2)$$

where C (360 min) represents the concentration of CsA at 360 min. $AUC_{0\,min-\infty}$ is a sum of $AUC_{0-360\,min}$ and $AUC_{360\,min-\infty}$. Absolute

bioavailabilities (F, %) of sublingually administered CsA formulations were calculated according to Eq. (3):

$$F = \frac{AUC_{EV} \times D_{IV}}{AUC_{IV} \times D_{EV}} \times 100\% \quad (3)$$

where AUC_{EV} is $AUC_{0\,min-\infty}$ for sublingual administration and AUC_{IV} is $AUC_{0\,min-\infty}$ for i.v. administration and D_{IV} and D_{EV} are doses (mg/kg) of intravenous and extravascular administration, respectively. In order to statistically compare individual values for C_{max}, $AUC_{0-360\,min}$ and $AUC_{0\,min-\infty}$, the values were normalized to the dose of 3 mg/kg by using Eq. (4):

$$X_N = X_{Obs} \times \frac{3\,mg/kg}{D_{EV}} \quad (4)$$

where X_N is the dose-normalized value and X_{Obs} is the observed value.

The differences of dose-normalized values of C_{max}, $AUC_{360\,min-\infty}$ and $AUC_{0-360\,min}$ between groups were statistically compared by the one way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Complexation of CsA with α-CD

One of the main demands for intraoral drug delivery is the small volume of the formulation. This may cause a problem especially with solid formulations containing CDs because CD complexation always increases the formulation bulk. Earlier studies have shown that CsA forms complexes with unmodified α-CD (Kanai et al., 1989; Sasamoto et al., 1991) and α-CD derivatives (Miyake et al., 1999; Okada et al., 1999; Fukaya et al., 2003). The complexation efficiency of CsA with unmodified α-CD is, however, rather low. For example, Kanai et al. (1989) have shown that 0.75 mg/ml solubility of CsA can be achieved in 8% (w/v) α-CD solution. From this data it can be calculated that after freeze-drying, 106 mg of unmodified CD is needed to complex 1 mg of CsA.

In the present study it was shown that CD complexation of CsA can be increased significantly by decreasing the temperature. Fig. 2 shows CsA forms A_P-type phase-solubility diagrams with α-CD at room temperature and at +5 °C, however, the complexation efficiency is much higher at +5 °C. The A_P-type phase-solubility behaviour generally suggests formation of complexes that are of apparent first order with respect to the drug and of first and/or higher order with respect to cyclodextrin (Higuchi and Connors, 1965). The stability constants for 1:1 and 1:2 complexes between CsA and α-CD were 1300 M^{-1} ($K_{1:1}$) and 730 M^{-1} ($K_{1:2}$) at room temperature, and 2500 M^{-1} ($K_{1:1}$) and 51 M^{-1} ($K_{1:2}$) at +5 °C (Table 2).

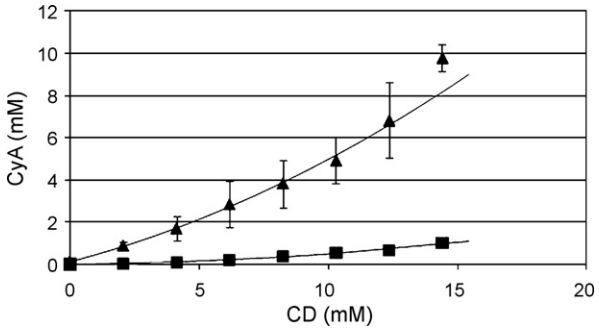

Fig. 2. Phase-solubility diagrams of CsA in 0–0.144 M α-CD at RT (■) (mean ± S.D.; $n = 3$) and at +5 °C (▲) (mean ± S.D.; $n = 3$–8). The dots represent the experimental values and the lines represent the calculated values.

Table 2
Calculated stability constants ($K_{1:1}$; $K_{1:2}$) between CsA and α-CD and intrinsic solubility (S_0) of CsA at +5 °C and at room temperature (RT).

Temperature (°C)	S_0 (M)	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
+5	1.25×10^{-4}	2500	51
RT	4.43×10^{-6}	1300	730

The aqueous solubilities of CsA at room temperature and at +5 °C were 5.3 and 150 μg/ml, respectively, implying that the complexation efficiency is mainly enhanced as a result of increased intrinsic solubility of CsA. It has been reported earlier that the aqueous solubility of CsA is inversely proportional to the solution temperature, possibly due to changes in inter-molecular hydrogen bonding (Ismailos et al., 1991); however, to our knowledge, decreased medium temperature has not been previously utilized when preparing CsA/CD complexes.

In the present study, the solubilities of CsA in 14% (w/v) α-CD at RT and at +5 °C were 1.2 and 12 mg/ml, respectively, and the solubility of CsA in 14% α-CD was further increased to 19 mg/ml at +1 °C. Thus, based on the data presented in this study, it can be concluded that the amounts of α-CD needed to solubilize 1 mg of CsA would be 120, 13 and 6.5 mg at room temperature, +5 and +1 °C, respectively, and that decreased temperature can be effectively utilized to produce CsA/α-CD complexes.

3.2. Dissolution studies

Fig. 3 shows the effect of α-CD complexation on dissolution rate of CsA: the value of $t_{30\%}$ for CsA/α-CD complex capsules (3.4 ± 1.3 min; mean ± S.D.) was significantly ($p < 0.05$) lower than $t_{30\%}$ of plain CsA in capsules (50.1 ± 5.5 min). The dissolution medium contained 6% (w/v) α-CD to ensure sink conditions were met. It is important, that although the complex was prepared at +1 °C, it was able to rapidly dissolve at 37 °C.

3.3. In vivo pharmacokinetic studies

In order to determine the systemic absorption of CsA, four solid and one liquid formulations of CsA were administered sublingually to rabbits. CsA was also administrated i.v. (in 20% HP-α-CD solution) to allow the determination of elimination rate constant for CsA and furthermore the absolute bioavailability of sublingually administered CsA. Rabbit was selected as an animal model for absorption studies due to its convenient size, which allows for sublingual administration and blood sample volumes that are sufficient for quantitative analysis. In addition, the intraoral tissue of a rabbit closely resembles human sublingual and buccal tissues and

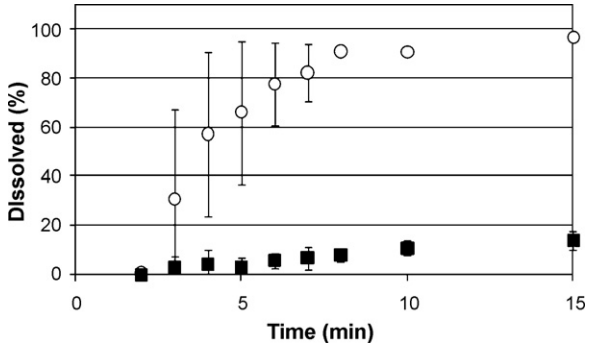

Fig. 3. Dissolution profiles of CsA capsule formulations (CsA 1.0 mg/capsule) at 37 °C (dissolution medium: 0.16 M phosphate buffer, pH 6.6; ionic strength 0.5; 6% (m/v) α-CD): the CsA/α-CD complex prepared at +1° (O) and plain CsA (■) ($n = 4$; mean ± S.D.).

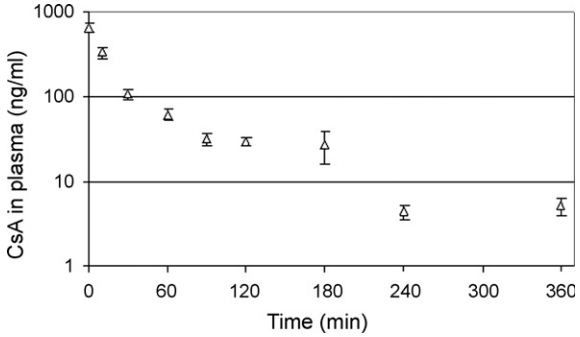

Fig. 4. Mean plasma concentrations of CsA after i.v. administration of CsA (1 mg/kg) in 20% (w/v) HP-α-CD solution to rabbits (mean ± S.E.M.; $n = 4$).

thus is an applicable model for determining sublingual absorption (Rathbone et al., 1996; Odou et al., 1999).

Fig. 4 shows the mean plasma concentration after i.v. administration of CsA in semi-logarithmic scale. The curve suggests biphasic kinetics with first order elimination phase. The representative kinetic values were determined for each rabbit individually by WinNonlin program, using a 2-compartment IV-bolus model with 1st order elimination. The values (mean ± S.D.) for k_{el}, $AUC_{0\,min-\infty}$, CL (clearance), V_{ss} (volume of distribution at steady-state) and $t_{1/2}$ were $0.009 \pm 0.003\ min^{-1}$, $19{,}000 \pm 1700\ min \times ng/ml$, 160 ± 10 ml/min, 12 ± 2 l and 82 ± 22 min, respectively.

Fig. 5a and b shows the mean plasma concentrations of CsA after sublingual administration of plain micronized CsA, solid CsA/α-CD complex and solid CsA/α-CD/CH formulation. C_{max}, t_{max}, $AUC_{0-300\,min}$, $AUC_{0\,min-\infty}$ and F values of CsA after i.v. and sublingual administrations are summarized in Table 3. The bioavailability of CsA from solid micronized CsA formulation and from ethanolic CsA formulation was 0.6 ± 0.5% (mean ± S.D.) and 0.8 ± 0.9% (mean ± S.D.), respectively. Thus, ethanolic CsA did not improve the bioavailability of CsA, and it is possible that CsA may have precipitated after dilution of ethanol in the oral cavity.

The bioavailability of CsA tended to increase when it was administered as solid complexes with α-CD (Table 3). It can be assumed that the improvement in sublingual bioavailability of CsA in the presence of α-CD is mainly explained by enhanced dissolution of CsA (Figs. 2 and 3) at the absorption site. In addition, the possibility that α-CD may have altered the permeability of sublingual mucosa cannot be ruled out. Previously it has been reported that when CsA was delivered via the lungs with maltosyl-α-CD, its effectiveness was improved, probably because of the increased aqueous solubility of CsA in the presence of CDs (Fukaya et al., 2003). Similarly,

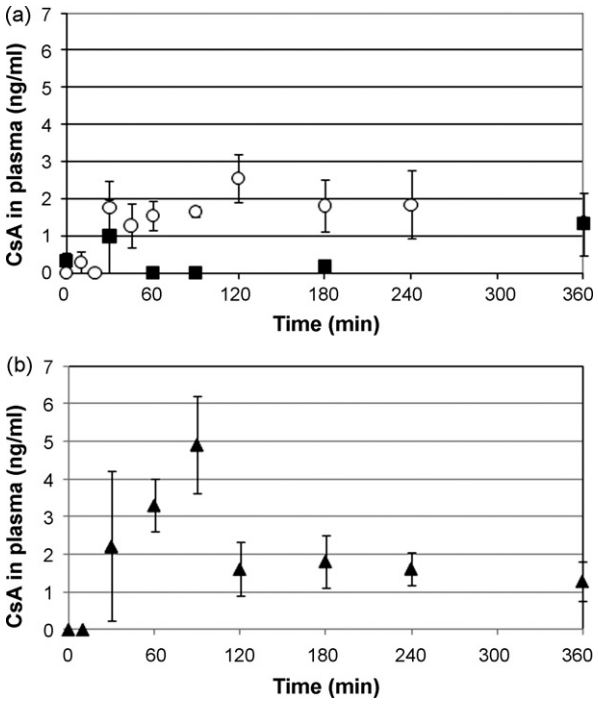

Fig. 5. Mean plasma concentrations of CsA after sublingual administration of plain micronized CsA (a; ■), solid CsA/α-CD complex (a; O) and solid CsA/α-CD/chitosan formulation (b; ▲) (doses normalized to 3 mg/kg) to rabbits (mean ± S.E.M.; $n = 4$).

co-administration of β-CD with a poorly water-soluble cyclic peptide, FK224, led to an increase in pulmonary bioavailability of FK224 (Nakate et al., 2003). The effect was suggested to be attributable to enhanced dissolution of FK224 in the alveolae in the presence of β-CD.

In earlier studies, the systemic absorption of CsA after peroral delivery has been shown to improve when CsA is administered in chitosan hydrochloride nanoparticles (El-Shabouri, 2002). In addition, chitosan hydrochloride nanoparticles have been shown to be effective in delivering CsA topically to the ocular surface (De Campos et al., 2001). However, the present study was the first to show the potential of a novel water-soluble quaternary derivative of chitosan, chitosan *N*-betainate (CH), in systemic sublingual delivery of a poorly water-soluble peptide. The results obtained in the present study indicate that the addition of CH in solid CsA/α-CD formulation by freeze-drying a saturated CsA solution containing α-CD and CH results in an improvement in the sublingual bioavailability

Table 3
C_{max}, t_{max}, $AUC_{0-300\,min}$, $AUC_{0\,min-\infty}$ and absolute bioavailability values (mean ± S.D.) of CsA after intravenous and sublingual administration of CsA formulations in rabbits ($n = 4$–5). The CsA dose was 1 mg/kg in intravenous administration; target dose of 3 mg/kg was used in extravascular administration.

Route of administration	Formulation	C_{max} (ng/ml)	t_{max} (min)	$AUC_{0-360\,min}$ (ng/ml × min)	$AUC_{0\,min-\infty}$ (ng/ml × min)	F (%)
Intravenous	CsA/HP-α-CD[c] complex	660 ± 70[a]	0[a]	17000 ± 800	19000 ± 1700	100
Sublingual	Solid micronized CsA (<15 μm)	2.1 ± 1.6[b]	190 ± 200	240 ± 160[b]	350 ± 370[b]	0.6 ± 0.5
Sublingual	Ethanolic CsA	3.1 ± 2.6[b]	150 ± 160	220 ± 230[b]	420 ± 480[b]	0.8 ± 0.9
Sublingual	Solid CsA/α-CD complex	3.0 ± 1.4[b]	100 ± 40	600 ± 340[b]	620 ± 450[b]	1.4 ± 0.7
Sublingual	Solid CsA/α-CD complex with chitosan solution[d]	16 ± 11[b,#]	120 ± 70	1500 ± 1100[b,*]	1700 ± 1200[b,$]	3.2 ± 2.2[$]
Sublingual	Solid CsA/α-CD/chitosan formulation	5.0 ± 2.6[b]	83 ± 15	730 ± 380[b]	910 ± 460[b]	1.7 ± 0.8

[a] Extrapolated by using WinNonlin.
[b] Value normalized to the dose of 3 mg/kg by using Eq. (4).
[c] HP-α-CD was used due its better i.v. safety profile.
[d] 50 μl of 1% chitosan solution was administered under the tongue 15 min before the administration and every 5 min after the administration.
[#] Significantly ($p < 0.05$) different from 'sublingual ethanolic CsA', 'sublingual solid CsA/α-CD complex' and 'sublingual solid micronized CsA'.
[*] Significantly ($p < 0.05$) different from 'sublingual ethanolic CsA' and 'sublingual solid micronized CsA'.
[$] Significantly ($p < 0.05$) different from 'sublingual solid micronized CsA'.

of CsA when compared to either solid micronized CsA, ethanolic CsA or solid CsA/α-CD complex formulation. Most prominent effect was, however, observed when 1% CH solution was added under the tongue 15 min before the administration of solid CsA/α-CD complex and henceforth every 5 min; the addition of CH solution resulted in significant changes in, e.g. the C_{max} when compared to formulations devoid of CH (Table 3). The results obtained in the present study indicate that quaternary chitosan *N*-betainate is a potential absorption enhancer in sublingual delivery of CsA.

4. Conclusions

In the present study, the effects of α-CD with and without a quaternary chitosan *N*-betainate on sublingual absorption of a model hydrophobic peptide CsA were studied. A novel method to produce CsA/CD complexes with enhanced complexation efficiency was described. CsA/α-CD complexes increased dissolution rate of CsA when compared to plain CsA. Also, sublingual bioavailability of CsA in rabbits tended to improve as a consequence of complexation with α-CD. When a quaternary chitosan *N*-betainate was included in solid CsA/α-CD formulation, no significant enhancement of sublingual bioavailability of CsA was observed when compared to solid CsA/α-CD complex formulation. Instead, the sublingual bioavailability of CsA was significantly improved when solid CsA/α-CD complexes were administered with a quaternary chitosan *N*-betainate solution.

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References

Cui, C.Y., Lu, W.L., Xiao, L., Zhang, S.Q., Huang, Y.B., Li, S.L., Zhang, R.J., Wang, G.L., Zhang, X., Zhang, Q., 2005. Sublingual delivery of insulin: effects of enhancers on the mucosal lipid fluidity and protein conformation, transport and in vivo hypoglycemic activity. Biol. Pharm. Bull. 28, 2279–2288.

De Campos, A.M., Sanchez, A., Alonso, M.J., 2001. Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. Int. J. Pharm. 224, 159–168.

Di Colo, G., Zambito, Y., Zaino, C., 2008. Polymeric enhancers of mucosal epithelia permeability: synthesis, transepithelial penetration-enhancing properties, mechanism of action, safety issues. J. Pharm. Sci. 97, 1652–1680.

El-Shabouri, M.H., 2002. Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. Int. J. Pharm. 249, 101–108.

Fukaya, H., Iimura, A., Hoshiko, K., Fuyumuro, T., Noji, S., Nabeshima, T., 2003. A cyclosporin A/maltosyl-alpha-cyclodextrin complex for inhalation therapy of asthma. Eur. Respir. J. 22, 213–219.

Harris, D., Robinson, J.R., 1992. Drug delivery via the mucous membranes of the oral cavity. J. Pharm. Sci. 81, 1–10.

Higuchi, T., Connors, K.A., 1965. Phase-solubility techniques. In: Reilley, C.N. (Ed.), Advances in Analytical Chemistry and Instrumentation. Interscience Publisher/John Wiley&Sons, New York, pp. 117–212.

Holappa, J., Hjalmarsdottir, M., Masson, M., Runarsson, Ö., Asplund, T., Luttikhedde, T., Soininen, P., Nevalainen, T., Jarvinen, T., 2006. Antimicrobial activity of chitosan *N*-betainates. Carbohydr. Polym. 56, 114–118.

Holappa, J., Nevalainen, T., Savolainen, J., Soininen, P., Elomaa, M., Safin, R., Suvanto, S., Pakkanen, T., Masson, M., Loftsson, T., Jarvinen, T., 2004. Synthesis and characterization of chitosan *N*-betainates having various degrees of substitution. Macromolecules 37, 2784–2789.

Illum, L., 1998. Chitosan and its use as a pharmaceutical excipient. Pharm. Res. 15, 1326–1331.

Irie, T., Uekama, K., 1997. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J. Pharm. Sci. 86, 147–162.

Ismailos, G., Reppas, C., Dressman, J.B., Macheras, P., 1991. Unusual solubility behaviour of cyclosporin A in aqueous media. J. Pharm. Pharmacol. 43, 287–289.

Kanai, A., Alba, R.M., Takano, T., Kobayashi, C., Nakajima, A., Kurihara, K., Yokoyama, T., Fukami, M., 1989. The effect on the cornea of alpha cyclodextrin vehicle for cyclosporin eye drops. Transplant. Proc. 21, 3150–3152.

Langoth, N., Kahlbacher, H., Schoffmann, G., Schmerold, I., Schuh, M., Franz, S., Kurka, P., Bernkop-Schnurch, A., 2006. Thiolated chitosans: design and in vivo evaluation of a mucoadhesive buccal peptide drug delivery system. Pharm. Res. 23, 573–579.

Lee, V.H.L., Yamamoto, A., 1990. Penetration and enzymatic barriers to peptide and protein absorption. Adv. Drug Deliv. Rev. 4, 171–207.

Loftsson, T., Vogensen, S.B., Brewster, M.E., Konradsdottir, F., 2007. Effects of cyclodextrins on drug delivery through biological membranes. J. Pharm. Sci. 96, 2532–2546.

Mannila, J., Jarvinen, T., Jarvinen, K., Jarho, P., 2007. Precipitation complexation method produces cannabidiol/beta-cyclodextrin inclusion complex suitable for sublingual administration of cannabidiol. J. Pharm. Sci. 96, 312–319.

Mannila, J., Jarvinen, T., Jarvinen, K., Tervonen, J., Jarho, P., 2006. Sublingual administration of Delta9-tetrahydrocannabinol/beta-cyclodextrin complex increases the bioavailability of Delta9-tetrahydrocannabinol in rabbits. Life Sci. 78, 1911–1914.

Miyake, K., Hirayama, F., Uekama, K., 1999. Solubility and mass and nuclear magnetic resonance spectroscopic studies on interaction of cyclosporin A with dimethyl-alpha- and -beta-cyclodextrins in aqueous solution. J. Pharm. Sci. 88, 39–45.

Molander, L., Lunell, E., 2001. Pharmacokinetic investigation of a nicotine sublingual tablet. Eur. J. Clin. Pharmacol. 56, 813–819.

Nakate, T., Yoshida, H., Ohike, A., Tokunaga, Y., Ibuki, R., Kawashima, Y., 2003. Improvement of pulmonary absorption of cyclopeptide FK224 in rats by co-formulating with beta-cyclodextrin. Eur. J. Pharm. Biopharm. 55, 147–154.

Odou, P., Barthelemy, C., Chatelier, D., Luyckx, M., Brunet, C., Dine, T., Gressier, B., Cazin, M., Cazin, J.C., Robert, H., 1999. Pharmacokinetics of midazolam: comparison of sublingual and intravenous routes in rabbit. Eur. J. Drug Metab. Pharmacokinet. 24, 1–7.

Okada, Y., Matsuda, K., Hara, K., Hamayasu, K., Hashimoto, H., Koizumi, K., 1999. Properties and the inclusion behavior of 6-O-alpha-D-galactosyl- and 6-O-alpha-D-mannosyl-cyclodextrins. Chem. Pharm. Bull. (Tokyo) 47, 1564–1568.

Rathbone, M.J., Ponchel, G., Ghazali, F.A., 1996. Systemic oral mucosal drug delivery and delivery systems. In: Rathbone, M.J. (Ed.), Oral Mucosal Drug Delivery. Marcel Dekker, Hamilton.

Salehian, B., Wang, C., Alexander, G., Davidson, T., McDonald, V., Berman, N., Dudley, R.E., Ziel, F., Swerdloff, R.S., 1995. Pharmacokinetics, bioefficacy, and safety of sublingual testosterone cyclodextrin in hypogonadal men: comparison to testosterone enanthate—a clinical research center study. J. Clin. Endocrinol. Metab. 80, 3567–3575.

Sasamoto, Y., Hirose, S., Ohno, S., Onoe, K., Matsuda, H., 1991. Topical application of ciclosporin ophthalmic solution containing alpha-cyclodextrin in experimental uveitis. Ophthalmologica 203, 118–125.

Senel, S., Kremer, M.J., Kas, S., Wertz, P.W., Hincal, A.A., Squier, C.A., 2000. Enhancing effect of chitosan on peptide drug delivery across buccal mucosa. Biomaterials 21, 2067–2071.

Shojaei, A.H., 1998. Buccal mucosa as a route for systemic drug delivery: a review. J. Pharm. Pharm. Sci. 1, 15–30.

Veuillez, F., Kalia, Y.N., Jacques, Y., Deshusses, J., Buri, P., 2001. Factors and strategies for improving buccal absorption of peptides. Eur. J. Pharm. Biopharm. 51, 93–109.