Impact of different hydrophobic ion pairs of octreotide on its oral bioavailability in pigs

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Abstract

The objective of this study was to investigate the impact of different hydrophobic ion pairs (HIP) on the oral bioavailability of the model drug octreotide in pigs.

Octreotide was ion paired with the anionic surfactants deoxycholate, decanoate and docusate differing in lipophilicity. These hydrophobic ion pairs were incorporated in self-emulsifying drug delivery systems (SEDDS) based on BrijO10, octyldodecanol, propylene glycol and ethanol in a concentration of 5 mg/ml. SEDDS were characterized regarding size distribution, zeta potential, stability towards lipase, log $D_{SEDDS/release\ medium}$ and mucus diffusion behavior. The oral bioavailability of octreotide was evaluated in pigs via LC-MS/MS analyses.

Most efficient ion pairing was achieved at a molar ratio of 1:3 (peptide : surfactant). SEDDS containing the octreotide-deoxycholate, -decanoate and -docusate ion pair exhibited a mean droplet size of 152 nm, 112 nm and 191 nm and a zeta potential of -3.7, -4.6 and -5.7 mV, respectively. They were completely stable towards degradation by lipase and showed a log $D_{SEDDS/release\ medium}$ of 1.7, 1.8 and 2.7, respectively. The diffusion coefficient of these SEDDS was in the range of 0.03, 0.11 and 0.17 x 10^{-9} cm^2 /sec, respectively. In vivo studies with these HIPs showed no improvement in the oral bioavailability in case of octreotide-decanoate. In contrast, octreotide-deoxycholate and octreotide-docusate SEDDS resulted in a 17.9-fold and 4.2-fold higher bioavailability vs. control.

According to these results, hydrophobic ion pairing could be identified as a key parameter for SEDDS to achieve high oral bioavailability.

Keywords: self-emulsifying drug delivery systems, octreotide, lipase stability, in-vivo study, drug release

1. Introduction

Oral delivery of biologicals is a great challenge due to various physiological barriers including the enzymatic barrier (1), the mucus barrier (2) and the absorption membrane barrier (3). Especially, peptides show low bioavailability after oral administration due to enzymatic degradation and low intestinal permeability. Within recent years, hydrophobic ion pairing (HIP) emerged as valuable tool to improve the lipophilic character of peptide drugs in order to protect them towards enzymatic degradation by intestinal peptidases (4) and to improve their membrane permeability (5). Furthermore, due to hydrophobic ion pairing therapeutic peptides can be incorporated in self-emulsifying drug delivery systems (SEDDS) (6). Lipase stable SEDDS provide an even more pronounced protective effect for incorporated HIPs towards intestinal peptidases (7). Moreover, SEDDS can pass the mucus gel barrier in a comparatively more efficient manner (8) and are known for their permeation enhancing properties (9). So far, promising in vivo results could be obtained by hydrophobic ion pairing of the peptide leuprolide (4) and of the low molecular weight heparin enoxaparin (10) being incorporated into SEDDS. In case of leuprolide an even 17.2-fold increase in oral bioavailability in rats compared to an aqueous solution was achieved (4). However, the full potential of SEDDS containing HIPs for oral delivery of biologicals has by far not been reached, as various key parameters were not addressed within previous studies. One of these key parameters is likely the type of hydrophobic ion pairing that has not yet been investigated in a comparative study at all. Moreover, important in vivo data regarding oral delivery of SEDDS containing a hydrophobically ion paired peptide drug are only available for rodents and a proof of concept in a non-rodent model is still missing.

It was therefore the aim of this study to generate three different ion pairs with a model peptide drug and to compare their performance in vitro and in vivo. Octreotide was chosen as model peptide drug as it is of high clinical relevance (11) and data of phase III clinical trials with orally administered octreotide are already available (12). After hydrophobic ion pairing with the anionic surfactants deoxycholate, decanoate and docusate, octreotide was incorporated into SEDDS. These SEDDS were evaluated in vitro in terms of biodegradability by lipase, drug release, log $D_{SEDDS/release\ medium}$, mucus permeation and permeation enhancing effect. Finally, in vivo studies were performed with these formulations in pigs.

2. Materials and Methods

2.1. Materials

Ammonium acetate, Brij O10, calcium chloride, ethanol 96 % (v/v), fluorescein dextran (FD4), formic acid, glucose, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), heptafluorobutyric acid (HFBA), magnesium sulfate, liquid paraffin, lumogen red, octyldodecanol, potassium chloride, propylene glycol, sodium chloride, sodium deoxycholate, sodium docusate, sodium decanoate and Tween® 80 were obtained from Sigma-Aldrich, Vienna, Austria. Acetic acid, acetonitrile, BD Vaccutainer EDTA tubes, methanol and water HPLC grade were supplied by VWR Austria. Amicon Ultra® centrifugational filters were received from Merck Millipore Darmstadt, Germany. Miglyol 840 was obtained from IOI Oleo GmbH (Hamburg, Germany). Octreotide acetate was purchased from Bachem AG, Bubendorf, Switzerland. The internal standard octreotide-D_8 was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada).

2.2. Methods

2.2.1. HPLC analysis of octreotide

Octreotide acetate was quantified using RP-HPLC. In brief, samples were analyzed on a YMC-Pack C4 column (250 × 4.6 mm, 5 µm, 30 nm pore size) using isocratic elution and a flow rate of 1 ml/min. The mobile phase consisted of 80% A (10% acetonitrile, 89.9% water, 0.1% formic acid, 2 mM ammonium acetate) and 20% B (90% acetonitrile, 9.9% water, 0.1% formic acid, 2 mM ammonium acetate). For quantification, a calibration curve with increasing amounts of octreotide acetate (10-500 µg/ml) was established.

2.2.2. Hydrophobic ion pairing of octreotide

Lipophilicity of octreotide acetate was increased via hydrophobic ion pairing as already described previously (4). For this purpose, the ion pairing agents, namely sodium deoxycholate, sodium decanoate and sodium docusate were dissolved separately in a concentration of 5 mg/ml in demineralized water. Thereafter, the solution of the respective anionic surfactant was added dropwise to a 5 mg/ml solution of octreotide acetate under continuous stirring. The resulting suspensions of precipitated ion pairs were centrifuged at 10000 rpm at 10 °C (Sigma laboratory centrifuge 3-18 KS). The supernatant was analyzed for unbound octreotide acetate via HPLC as described above. The precipitated pellet containing the ion pair was lyophilized at reduced pressure (Christ Gamma 1-16 LSC Freeze dryer) and stored at -20 °C until further use.

2.2.3. Preparation of SEDDS

In order to prepare SEDDS which are likely stable towards enzymatic degradation by lipases, only ingredients without ester substructures were utilized according to a previous study, but with modifications (7). Therefore, Brij™O10, octyldodecanol and paraffin were chosen as emulsifier and oily components, respectively. Furthermore, propylene glycol and ethanol were added as co-solvents. The excipients were mixed in different ratios as listed in Tab. 1, vortexed and incubated at 40 °C under constant shaking at 1000 rpm (Thermomixer comfort, Eppendorf, Germany) for 2 hours, whereby semisolid components were melted in advance. For analytical reasons lumogen red was optionally added in a final concentration of 0.1% (m/v).

Octreotide loaded SEDDS were prepared by incorporating octreotide-deoxycholate in SEDDS B3, octreotide-decanoate in SEDDS B4 and octreotide-docusate SEDDS B6 as listed in Tab. 1. In brief, 5 mg of each ion pair was dissolved in 1 ml of the respective SEDDS pre-concentrate via heating to 40 °C for 5 minutes followed by short ultra-sonication for 10 minutes.

In order to characterize SEDDS, 100 µl of SEDDS pre-concentrates were emulsified in 10 ml 100 mM phosphate buffer pH 6.8 and analyzed regarding mean droplet size and zeta potential via dynamic light scattering (Zeta Potential/Particle Sizer, Nicomp™ 380 ZLS, PSS, Santa Barbara, CA, USA) with laser wavelength of 650 nm and an E-field strength of 5 V. For determination of the zeta potential emulsions were prepared with demineralized water instead of phosphate buffer to exclude effects of the buffer.

2.2.4. Lipase degradation studies

In order to evaluate the stability of SEDDS towards lipases a slightly modified method was used according to Fatouros et al. (13). Briefly, to obtain lipase suspension 50 mg of pancreatic lipase (Type II. 100-400 U/mg) was vortexed for 3 min in 20 ml cooled digestion buffer without bile salts and subsequently centrifuged. The supernatant was recovered and kept at 4 °C to avoid loss of enzymatic activity till use for initiation. SEDDS were emulsified to a final concentration of 1% (m/v) in digestion buffer composed of 2 mM Trizma, 150 mM NaCl, 5 mM $CaCl_2$ and 5 mM bile salts. The temperature of emulsions was set to 37°C and the pH was adjusted to 6.5 with either 1 M NaOH or 1 HCl. To initiate lipolysis freshly prepared lipase suspension was added to emulsion in a ration of 1:10. After the initiation of lipolysis the pH was kept constant at 6.5 during the cause of study by addition of 0.1 M sodium hydroxide and the added amount was computed. Considering pH changes are caused by liberated free fatty acids (FFA) due to hydrolysis process the amount of added 0.1 M sodium hydroxide can be equated with FFA indicating the digestion of SEDDS. The experiment was performed for 120 minutes. As a positive control of digestive activity of pancreatic lipase Miglyol® 840 was used instead of SEDDS.

2.2.5. Drug release studies and log D $_{\text{SEDDS/release medium}}$ determination

Drug release from SEDDS was assessed via ultrafiltration-centrifugation according to a method described previously, but with alterations. For this purpose, 100 µl of each anhydrous SEDDS formulation containing octreotide was emulsified in 10 ml 100 mM phosphate buffer pH 6.8 at 37°C. At predetermined time points, aliquots of

500 µl were removed and replaced with an equal amount of fresh release medium. After centrifugation in Amicon® Ultra centrifugational filters (Ultracel® 10K) for 15 minutes at 6000 rpm and 8 °C, the amount of free octreotide in the filtrate was quantified via HPLC. Quantification occurred according to a standard curve with increasing concentrations of octreotide and cumulative corrections were made for previously removed samples. Before usage, filter inserts and collecting tubes were stored in 0.1 % (v/v) Tween 80 solution and rinsed extensively with demineralized water to avoid adsorption of the peptide to the filter membrane. This procedure was verified by centrifugation of a solution of octreotide acetate (c= 0.05 mg/ml) in 100 mM phosphate buffer and analyzing the content of the peptide in the filtrate.

Log D $_{\text{SEDDS/release medium}}$ representing the likely more informative value regarding the in vivo drug release behavior from SEDDS than in vitro drug release profiles (14) was obtained by determining maximum solubility in the SEDDS preconcentrates and in water. Log D was then calculated by the following equation:

$$\log D_{\text{SEDDS / release medium}} = \log (\text{maximum solubility in SEDDS / maximum solubility in water}) \quad (1)$$

2.2.6. Determination of mucus permeability of SEDDS

Multiple particle tracking (MPT) technique was used to study the diffusivity of different SEDDS through the mucus barrier as described previously (8) (15). Briefly, 25 µl of lumogen red labelled SEDDS aliquots were inoculated onto a 0.5 g native porcine intestinal mucus sample placed in a glass-bottom MatTek imaging dish. These samples were incubated for 2 hours at 37 °C to enable uniform distribution of droplets within the mucus matrix. The transport of fluorescently labelled droplets was

captured in 2-dimensional imaging videos using Epifluorescence microscope (Leica DM IRB wide-field Epifluorescence microscope, 63X magnification oil immersion lens) supplied with high speed camera (20X digital magnification system, Allied Vision Technologies, UK) running at speed rate of 30 frames s^{-1} and video length of 300 frames (10 sec). Within each 0.5 g of mucus sample, around 120 droplets were simultaneously tracked and their movements were converted into trajectories using Fiji ImageJ software. For each SEDDS species, this experiment was carried out in triplicate (three different mucus samples with overall 360 individual droplets were tracked in three mucus samples). Trajectories were converted into metric distances to measure the square displacement of each droplet. The mean square displacement MSD of any single droplet is the mean of its square displacements through entire 30-frame trajectories (1 second). MSD was determined as follows:

$$MSD_{(n)} = (X_{\Delta t})^2 + (Y_{\Delta t})^2 \quad (2)$$

To define the MSD of all droplets within each mucus sample, ‘‘ensemble mean square displacement” (defined by ‹MSD›) was measured by calculating the geometric mean of the MSD of the 120 droplets within each sample. Calculating the ‹MSD› value enables to calculate the effective diffusion coefficient (‹Deff›) for a particular SEDDS species following equation 3:

$$\text{‹Deff›} = \text{‹MSD›}/(4 * \Delta t) \quad (3)$$

where 4 is a constant relating to a 2-dimensional mode of video capture and Δt is the selected time interval.

Proportion of diffusive droplets: Each droplet within the sample is defined as diffusive droplet if its diffusivity factor (DF) is greater than 0.9 (equation 4), i.e., the division of its diffusion at time interval ((*Δt*) of 1 s over 0.2 s is greater than 0.9.

$$DF = Deff_{\Delta t=1\ s} / Deff_{\Delta t=0.2\ s} \quad (4)$$

The proportion of diffusive droplets within a given SEDDS type was then calculated and expressed as % Diffusive droplets.

Heterogeneity in droplet diffusion: Ranking the diffusion coefficients of the 360 droplets in each SEDDS sample can describe the heterogeneity and the presence of outlier sub-populations in each SEDDS species which is indicative of distinctive pathways of diffusion through the matrix. To do so, Deff values of 360 droplets at *Δt* of 1 s were ranked from the highest (90^{th}) to the lowest (10^{th}) percentiles, where for example the 90^{th} percentile is the Deff value below which 90% of the Deff were observed.

Droplet diffusion in water: The droplets' diffusion coefficient (D°) in water was calculated by the Stokes-Einstein equation at 37 C°:

$$[D° = \kappa T / 6\pi\eta r] \quad (5)$$

where κ is Boltzmann constant, *T* is absolute temperature, η is water viscosity, r is radius of the droplet. The diffusion of all droplets was also expressed as the parameter, % ratio [Deff] / [D°].

2.2.7. Permeation through porcine small intestine

In addition to mucus diffusion studies, permeation enhancing effects of SEDDS formulations through the intestinal epithelium were investigated on porcine intestinal mucosa according to a method described by Hintzen et al. (16). For this purpose, jejunal segments of the small intestine of domestic pigs (*sus scrofa domestica*) were removed after sacrificing. The tissue was rinsed with physiological saline to remove luminal contents, cut into pieces of about 1.5 cm in size and mounted on Ussing type chambers with a permeation area of 0.64 cm^2. One milliliter of HEPES buffer (composed of 138 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$ and 10 mM HEPES) served as acceptor medium. The donor compartment was filled with 1 ml of 1% (m/v) SEDDS in 100 mM phosphate buffer, pH 6.8 containing fluorescence labelled dextran (FD4) in a final concentration of 0.05 % (m/v). Thereby, a solution of FD4 (c= 0.05 %) in phosphate buffer was used as control. Every 30 minutes, samples of 100 µl were removed from the acceptor compartment and replaced by fresh buffer. The cumulative permeated amount of FD4 was determined via fluorescence measurement (λ_{ex}=485 nm and λ_{em}=535 nm) using a microplate reader (M200 spectrometer; Tecan infinite, Grödig, Austria). Quantification occurred according to a standard curve using FD4.

2.2.8. In vivo evaluation of octreotide SEDDS in pigs

The protocol for the in vivo study in pigs was approved by the Animal Ethical Committee of Vienna, Austria (ETK-01/01/2016) and adheres to the Principals of Laboratory Animal Care. The in vivo study was performed in 5 male domestic pigs (*sus scrofa domestica*) weighing about 30 kg. Three days prior to the experiment, an

indwelling catheter was implanted into the jugular vein under general anesthesia. Afterwards, the animals were housed separately, but with nose contact to each other. On the day of the experiment, each animal received one formulation containing octreotide and all animals were fasted two hours before and one hour after administration, but with free access to water. Aliquots (10 ml) of SEDDS preconcentrates were administered via a feeding tube followed by 10 ml of apple juice. In case of i.v. administration, the formulation was applied via the catheter, followed by application of 2 ml of heparinized physiological saline (25 U/ml). At predetermined time points, 2 ml of venous blood was removed from the catheter and transferred into BD Vacutainer® EDTA tubes. After blood sampling, 2 ml of heparinized physiological saline (25 U/ml) were administered to fill the dead volume of the cannula and avoid blood clotting within the tube. All blood samples were immediately centrifuged for 10 min at 2000 x g at 4 °C to obtain the plasma. The plasma samples were stored at -80 °C until analysis via LC-MS/MS as described in the following. After a wash out phase of minimum 24 hours, the animals were dosed again, whereby the assignment of formulations and animals occurred in a randomized manner. In total, each animal was dosed four times. An overview over the experimental setup is provided in Table 2.

2.2.9. Quantification of octreotide via LC-MS/MS

Octreotide was quantified in plasma samples from the in vivo study using LC-MS/MS. The analysis was carried out on a 3200 QTRAP (Sciex, Framingham, MA, USA) equipped with a Knauer K-1001 HPLC Pump (Berlin, Germany), a PAL HTC autosampler (CTC Analytics, Zwingen, Switzerland) with a 20 µl loop, and a column

thermostat (Thermotechnic Products GmbH, Langenzersdorf, Austria). The mobile phase was composed of: *A:* 95 % water, 5 % MeOH, 0.5 % acetic acid, 0.05 % HFBA and *B*: 5 % water, 95 % MeOH, 0.5 % acetic acid, 0.05 % HFBA. The samples were separated on a XB-C18 Aeris wide-pore column (3.6 µm, 150 x 210 mm, Phenomenex Aschaffenburg, Germany) using gradient elution with a linear gradient from 0 – 100 % B in 10 minutes at a flow rate of 0.2 ml/min, an oven temperature of 50 °C and an injection volume of 10 µl. Mass spectrometric detection was performed in positive ion mode. The spray voltage was set to 5500 V, Gas flows of 50 arbitrary units for the nebulizer gas and 25 arbitrary units for the turbo gas were used. The temperature of the turbo gas was adjusted to 500 °C. Multiple reaction monitoring was performed using the precursor-to-product ion transitions of the doubly charged octreotide at 510.4 > 120.2 and the doubly charged octreotide-D_8 at 514.4 > 120.2. The collision energy was set to 35 eV, and the dwell time to 100 ms. Chromatograms and mass spectra were recorded on a personal computer with Analyst 1.5 software (AB Sciex).

2.2.10. Standard samples and sample preparation

For the quantification of octreotide, octreotide-D_8 was used as internal standard (IS). For calibrators, stock solutions containing 1.0 mg/ml of octreotide or the IS were prepared in methanol and stored at -20 °C until use. Working standards of octreotide in the concentration range 50-1000 ng/ml were prepared by dilution of the stock solutions with water. The working solution of the IS was prepared by diluting the octreotide-D_8 stock solution with water to a final concentration of 100 ng/ml. Calibration samples were prepared by spiking 250 µl of plasma with 25 µl IS working

solution and aliquots of the octreotide working solutions to obtain ten different concentration levels: 0 ng/ml, 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, 5 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, and 500 ng/ml.

250 µl of in vivo study samples were spiked with 25 µl IS working solution. Samples were mixed with 250 µl of 4 % H_3PO_4 prior to extraction. Octreotide and the IS were isolated from plasma samples via solid phase extraction using a WXC column (1cc, 10 mg sorbent, Waters, Milford, MA, USA). Prior to use, the column was equilibrated with 1 ml methanol followed by 1 ml of distilled water. Then, the sample was loaded onto the column. Afterwards, the column was washed with 500 µl 5 % NH_4OH followed by 500 µl 20 % ACN and the column was vacuum-dried for 5 min. Then, octreotide was eluted with 500 µl of a mixture of 75 % ACN and 25 % water with 1 % TFA. In the end, the solvent was evaporated under nitrogen flow at room temperature and the residue was dissolved in 25 µl of sample solvent (80 % water, 20 % MeOH, 0.5 % acetic acid, 0.05 % HFBA). The samples were analyzed for octreotide and IS via LC-MS/MS as described above.

2.3. Statistical data analyses

Statistical data analysis was performed using one way Anova with Bonferroni post-hoc test and with 95 % confidence interval ($p<0.05$). Results are expressed as the means of at least three experiments ± standard deviation.

3. Results

3.1. Hydrophobic ion pairing of octreotide

Ionic interactions between the positively charged octreotide and the negatively charged surfactants led to formation of water insoluble complexes, visible as white precipitate. Within the investigated range, a molar ratio of 1:3 (peptide to surfactant) was shown to precipitate the highest amount of octreotide. Results of this study are illustrated in Fig. 2.

3.2. Preparation and characterization of SEDDS

In order to avoid degradation of SEDDS droplets by lipases, Brij O10, propylene glycol and octyldodecanol exhibiting no ester structures were chosen. Addition of ethanol to this composition led to a decreased droplet size. By diluting the pre-concentrates with 100 mM phosphate buffer in a ratio of 1:100, dark bluish emulsions were formed. The incorporation of the different hydrophobic ion pairs had a significant impact on droplet size and stability of SEDDS. In order to obtain SEDDS of similar composition, size, zeta potential and stability, numerous ratios of surfactants, solvents and co-solvents were tested (data not shown). Out of these formulations, SEDDS B3, B4 and B6 as listed in Tab. 1 were chosen representing the likely best compromise. In particular, size and zeta potential of these formulations was of minor difference. Furthermore, degradation studies with lipase demonstrated that none of these SEDDS is degraded at all, whereas the control was entirely degraded within 2 hrs.

3.3. In vitro drug release and log D $_{SEDDS/release\ medium}$

In vitro release studies of octreotide out of SEDDS formulations were assessed for the different ion pairs. Formulations containing octreotide-deoxycholate and octreotide-decanoate exhibited a comparatively fast drug release, as the peptide was completely released within the first three hours. In contrast, SEDDS loaded with octreotide-docusate showed a prolonged release for more than six hours. Time dependent release profiles are depicted in Figure 3. During centrifugation drug release caused by mechanical deformation of the SEDDS droplets was not an issue, as no octreotide could be detected in the filtrate at t_0. Log D values of octreotide-deoxycholate, -docusate and –decanoate were determined to be 1.7 ±0.3, 1.8 ±0.4 and 2.7 ±0.3 (n=3).

3.4. Mucus diffusion studies

Interactions between SEDDS and mucus were investigated via multiple particle tracking. Table 3 shows MPT diffusion data [$cm^2\ S^{-1}\ x\ 10^{-9}$] in the native intestinal mucus model <Deff> and in water D° (calculated by Stokes Einstein equation); the ratio of < Deff> to D° expressed as a %; and in the last column, the % of diffusive droplets. The <Deff> is a measure of the absolute diffusion of the droplets through mucus reflecting both surface chemistry and droplet size, while the % ratio <Deff>/D° is a measure of the diffusion of the droplets through mucus with respect to surface chemistry characteristics alone, i.e. normalized against differences in droplet size. Accordingly, Figure 4 shows the effect of the physicochemical properties of SEDDS on their diffusivity. These effects presented as follows: Figure 4A shows the <Deff> versus the droplet size data for the respective SEDDS, Figure 4B shows the<Deff> versus the zeta potential for the respective SEDDS and Figure 4C shows the

%<Deff>/D of each of the SEDDS versus zeta potential. It can be seen that SEDDS B4 showed the highest <Deff> compared with other systems. However, normalizing the droplet size effect by calculating %<Deff>/D° shows that formula B6 is more diffusive through mucus compared with other formulae. Figure 1S describes the DF and the ratio of diffusive droplets which is shown in Table 3. This figure shows that among 20 droplets selected randomly, 7 droplets were diffusive for sample B6 while only 4 droplets were diffusive in sample B3. Figure 5 describes the degree of heterogeneity of droplets diffusion through the mucus. For each droplet types, the Deff of 360 individual droplets at 1sec were ranked into percentiles to allow the comparison of the slowest (10th) percentiles to the fastest (90th) percentiles which is defined as the Deff value below which 90% of the Deff values within the droplet population occur. Figure 5 shows that all systems have a high heterogeneity of droplets diffusion. This can be seen obviously with SEDDS B6 which showed a sharp difference in the Deff of droplets with 40% percentile compared with Deff at 30% percentiles. This high heterogeneity reflects the presence of more than one mechanism of interaction of droplets within the mucus matrix resulted in presence of different populations of droplets with huge difference in their diffusion properties.

3.5. Ex vivo permeation through porcine small intestine

Ex vivo permeation experiments through freshly excised porcine intestinal mucosa were carried out using the model compound FD4 as described previously by Hintzen et al. (16). The cumulative transport of the hydrophilic and macromolecular FD4 is shown in Figure 6. In presence of SEDDS the transport of FD4 could be strongly

improved. These data are in agreement with various previous studies demonstrating a permeation enhancing effect of SEDDS for hydrophilic macromolecules.

3.6. In vivo evaluation of octreotide SEDDS

Results of in vivo studies with different octreotide SEDDS and a solution of the peptide in apple juice in pigs are summarized in Table 4. After oral administration, octreotide was taken up into the systemic circulation from octreotide-deoxycholate SEDDS and SEDDS containing octreotide-docusate. In contrast, administering the peptide in a solution in apple juice and octreotide-decanoate in form of SEDDS resulted in comparatively low plasma concentrations. In case of all oral formulations, the maximum plasma concentration could be observed after 60 minutes as illustrated in Fig. 7. Octreotide-deoxycholate SEDDS and octreotide-docusate SEDDS showed a 17.9-fold and 4.2-higher relative bioavailability, compared to the solution in apple juice. In contrast, oral bioavailability was not increased by octreotide-decanoate SEDDS (Table 4).

4. Discussion

Ion pairing with sodium deoxycholate, sodium decanoate and sodium docusate in different molar ratios (1:1, 1:2, 1:3, 1:4) resulted in a decreased water solubility of the peptide. Within this study, the ratio of 1:3 was shown to be most efficient to prepare ion pairs of octreotide, as nearly quantitative precipitation of octreotide was feasible (Figure 2). As octreotide exhibits two cationic substructures as illustrated in Fig. 1, a molar ratio of 1:2 should already saturate both of these cationic substructures. Our

results, however, showed that a ratio of 1:3 is more efficient. These data are in agreement with previous studies demonstrating that either an equivalent or slightly higher ratio is most efficient for HIP formation (6). Because of its D-amino acids and due to its cyclic structure, octreotide is stabilized against degradation by intestinal proteases (17). In order to exclude also the degradation of SEDDS in the small intestine, lipase stable SEDDS were developed. In general, lipases are endogenous enzymes present in the pancreatic fluid being responsible for the degradation of nutritional fats. However, these enzymes are also capable of degrading ester structures of SEDDS components resulting in a premature release of the incorporated peptide (7) (18). Drug release studies from lipase stable SEDDS revealed a clear correlation between the lipophilicity of the surfactant and the corresponding release kinetic. An increasing lipophilicity of the ion pairing agent resulted in a decelerated drug release. Formulations containing octreotide-decanoate showed the fastest release among the investigated formulations (Figure 3). Exhibiting only 10 carbon atoms, decanoate is considerably less lipophilic compared to deoxycholate with 24 carbon atoms and docusate with 20 carbon atoms, leading to a shorter residence time within the lipophilic SEDDS droplets. As the release from SEDDS with octreotide-deoxycholate was faster than from formulations with the docusate ion pair, also the structure of the utilized compound seems to have an impact. With two branched octyl- residues, docusate might have a greater lipophilic surface area compared to the cyclic bile salt deoxycholate, resulting in an extended retention in the lipophilic phase. These observations are also in good agreement with the determined log D values, as the octreotide-decanoate ion pair exhibiting the fastest drug release showed a log D of 1.7, whereas the octreotide-

docusate ion pair exhibiting the most sustained drug release showed the highest log D of 2.7.

A further requirement for efficient drug delivery systems is the ability to overcome the mucus barrier in the intestinal compartment, as the mucus gel layer represents a major obstacle, drug delivery systems have to overcome it to reach the underlying epithelia (19). Octreotide might pass the mucus barrier within the SEDDS droplets more efficiently, leading to a higher concentration at the absorptive membrane. At an intestinal pH value of around 6.8 octreotide is positively charged due to its basic amino acids. As the mucus layer is negatively charged, ionic interactions between the peptide might restrict permeation. On the other hand, being incorporated into SEDDS droplets and having a neutral charge in form of the respective ion pair, might shield the peptide from ionic interactions and allow passaging the mucus barrier. Accordingly, mucus diffusive properties of SEDDS formulations were studied via multiple particle tracking. In general, SEDDS have two main inter-independent variables that can affect the diffusion of these systems through the mucus. These are: the physicochemical properties of the SEDDS and the ingredients in each system. Figure 4A shows that droplet sizes ranging from 100 up to 250 nm do not correlate with the corresponding <Deff>. Similarly, Figure 4B and 4C failed to reveal any correlation of zeta potential to <Deff> or %ratio <Deff>/D° respectively, where all systems have close zeta potential values but different diffusivities. This finding is in agreement with previous reported work where the physicochemical properties had no effect on the diffusivity of SEDDS (8). Table 3 and Figure 4, however, do show that the SEDDS are affected by their formulation ingredients. Thus, Figure 4C shows that SEDDS B6 which has no ethanol as a co-surfactant within the ingredients has

the highest diffusivity (% Ratio <Deff>/D^o 0.5917) as compared with the other types of SEDDS having ethanol at ratios of 1.5 and 5%.

Moreover, Table 3 presents the % of diffusive droplets of SEDDS within the mucus. As described earlier, DF is the term defines the diffusive droplets as the droplets for which the Deff at 1 second is ≥ 90% of the Deff at the 0.2 second. This definition means that only diffusive droplet will show an increase in the diffusion within time. This is based on the concept that increase of time interval will increase the chances of trapping media to interact with droplets transporting through it. Table 3 shows that 37% of droplets of SEDDS B6 were identified to be diffusive through the mucus while 32% and 24% of droplets were identified to be diffusive for SEDDS B4 and SEDDS B6, respectively. Figure 4 clarifies this behavior for 20 randomly selected droplets from a total of 360 droplets of SEDDS B3 and SEDDS B6. It can be seen that 7 of the randomly selected 20 droplets (35%) of SEDDS B6 showed an increase in the diffusion within time interval increase from lower to higher points. Similarly, for SEDDS B3, 4 droplets of the selected 20 droplets (20%) showed diffusive properties through mucus. It can be seen that the difference in the % Ratio <Deff>/D^o between systems is not directly related to the percent of the diffusive droplets of these systems, for example, the increase of % Ratio <Deff>/D^o by 5 times (from 0.1247 to 0.5917) between systems B3 and B6 is accompanied by 1.5 times increase in % of diffusive droplets for the same systems. This indicates that the droplets which categorised as non-diffusive have higher effect than the diffusive droplets on the final diffusion coefficient of the systems. This is in accordance with Figure 5 which showed almost 60 percentile of droplets of all SEDDS species have much slower diffusion through mucus compared with the fastest 20 percentile.

As limited absorption from the intestinal mucosa is a main reason for the low oral bioavailability, permeation enhancing properties of SEDDS would be favorable (3). Studying the permeation of the hydrophilic and high molecular weight marker FD4 showed that in presence of all SEDDS formulations, the transport through porcine intestinal mucosa was distinctly increased (Figure 6), most likely due to a tight junction opening in presence of SEDDS. Decrease in cell-cell junction activity was already described for self-emulsifying systems containing surfactants with a high HLB value (20), such as Cremophor EL (16) or Tween 80 (21). Similarly, Brij O10 with a HLB value of 12.4 might have caused a loosening of the tight junctions resulting in an increased permeation of FD4.

Finally, SEDDS formulations loaded with octreotide ion pairs were investigated in vivo. Due to anatomical and physiological similarities to humans (22), pigs were chosen for the in vivo study. Oral administration of a solution of octreotide-acetate proved that the peptide is poorly absorbed (C_{max}=4.28 ng/ml) from the gastro-intestinal tract, which is most likely due to a low permeability across the intestinal mucosa. However, applying octreotide ion pairs in SEDDS resulted in a comparatively higher bioavailability (Table 4). Moreover, the absorption was more sustained compared to the solution (Figure 7). These results are in good accordance with a previous in vivo study in rats, in which administration of leuprolide SEDDS resulted in an increased oral bioavailability and an extended absorption compared to an aqueous solution of the peptide (4). Within the current study, however, these findings could only be observed for SEDDS containing octreotide-deoxycholate and octreotide-docusate. Accordingly, oral uptake of peptides from SEDDS into the systemic circulation is strongly dependent on the type of HIP.

On the one hand, the release behavior of the different ion pairs from SEDDS has certainly an important impact on their in vivo performance. SEDDS containing octreotide-deoxycholate showed more than 4-fold higher octreotide plasma levels than SEDDS with octreotide-docusate being comparatively more sustained released. Accordingly, a peptide release from SEDDS within 2-3 hours seems to be favorable in order to achieve a high uptake into the systemic circulation. In contrast, an immediate release seems to be disadvantageous as well, as octreotide-decanoate being most rapidly released from SEDDS could not improve oral bioavailability compared to an aqueous solution.

On the other hand, the intrinsic properties of the anionic surfactants used for ion pairing might have an impact on the performance of HIPs. The inferior performance of SEDDS containing octreotide-docusate might also be attributed to the pharmacological effect of docusate. Stimulating secretion and inhibiting fluid absorption in the jejunum, uptake of octreotide might be reduced in presence of this surfactant. Furthermore, deoxycholate was shown to enhance the oral bioavailability of nanocarriers (23). The responsible mechanism seems to be based on a bile acid pathway allowing overcome of the intestinal epithelium (24).

Comparing the oral bioavailability of 5.2% having been achieved with SEDDS containing the octreotide-deoxycholate ion pair with that of so far established formulations allows an estimation of its efficacy. Using the Intravail®-technology, a relative oral bioavailability of 4.0% vs. subcutaneous administration was achieved in mice (25). In another study a relative oral bioavailability of 2.3% vs. subcutaneous administration was determined in monkeys when an oily suspension formulation containing the well-established permeation enhancer sodium caprylate was used (26). As the subcutaneous bioavailability of octreotide is just around 30% of its

intravenous bioavailability (17), the maximum oral bioavailability obtained with the octreotide-deoxycholate ion pair is even 4.3-fold and 7.5-fold higher. Being aware of that just three different ion pairs were tested within this study and SEDDS containing HIPs have not yet been optimized, demonstrates the likely great potential of this technology for oral peptide delivery.

Conclusions

Within this study, lipase stable SEDDS containing different hydrophobic ion pairs were generated. Due to the stability of the carrier system and the cargo drug against enzymatic degradation, the influence of these ion pairs on oral bioavailability could be elucidated. Deciding over mucus permeability, protection against enzymatic degradation, as well as absorption into the systemic circulation, it could be demonstrated, that the type of hydrophobic ion pairing is a key parameter for the in vivo performance of peptide loaded SEDDS. Moreover, the distinctly improved oral bioavailability of octreotide in pigs in the range of 5%, triggered by an administration via SEDDS, supports findings of previous in vivo studies in rodents. Thus, this study does not only provide evidence for the importance of the type of ion pair but is also a proof of concept that HIPs being incorporated in SEDDS are a promising strategy in the field of oral peptide delivery.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant

agreement no 28076. The authors also wish to thank the team of the University Clinic of Swine of the Vetmeduni Vienna for the support and the performance of the in-vivo study.

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Table 1. Composition, droplet size polydispersity index (PI) and zeta potential of SEDDS

No	Composition in % (v/v)					*HIP in % (m/v)*	*Mean droplet size (nm)*	*PDI*	*Zeta potential (mV)*
	Surfactant	**Co-solvent**		**Oily component**					
	Brij O10	**Propylene glycol**	**Ethanol**	**Paraffin**	**Octyl-dodecanol**				
B1	30	8.5	1.5	30	30	---	*110*	*0.329*	*-3.48 ± 0.83*
B2	50	10	1.5	10	28.5	---	*173*	*0.205*	*-4.33 ± 0.76*
B3	40	10	1.5		48.5	---	*179*	*0.258*	*-4.81 ± 0.44*
B3	40	10	1.5		48.5	0.5 (octreotide-deoxycholate)	*152*	*0.16*	*-3.71 ± 0.43*
B4	40	15	5		40	---	*105*	*0.18*	*-3.54 ± 0.31*
B4	40	15	5		40	0.5 (octreotide-decanoate)	*112*	*0.18*	*-4.56 ± 0.46*
B5	50	10	1.5		38.5	---	*187*	*0.168*	*-3.80 ± 0.30*
B6	60	10			30	---	*151*	*0.151*	*-3.63 ± 0.49*
B6	60	10			30	0.5 (octreotide-docusate)	*191*	*0.15*	*-5.72 ± 0.83*

Table 2. Overview on formulations utilized for in vivo studies in pigs

Formulation	No	Route of administration	Dose	Dosage Form	Volume
Octreotide acetate aqueous solution		intravenous	750 µg	Aqueous solution	5 ml
Octreotide solution in apple juice		oral	50 mg	---	10 ml
Octreotide-deoxycholate SEDDS	B3	oral	50 mg	SEDDS	10 ml
Octreotide-decanoate SEDDS	B4	oral	50 mg	SEDDS	10 ml
Octreotide-docusate SEDDS	B6	oral	50 mg	SEDDS	10 ml

Table 3. Zeta potential, droplet size, Diffusion coefficient in water and mucus and percentage (%) of diffusive droplets of various SEDDS preparations. Mucus diffusion was measured by the MPT technique using the Epifluorescence microscopy while diffusion in water was obtained through Stokes-Einstein equation

SEDDS	D^{o} (water) $cm^{2}.S^{-1} \times 10^{-9}$	<Deff> (mucus) $cm^{2}.S^{-1} \times 10^{-9}$ Mean (±s.e.m)	% Ratio <Deff>/D^{o}	% Diffusive droplets
B3	26.59	0.03315 (±0.00526)	0.1247	24
B4	44.5	0.16793 (±0.03762)	0.3774	32
B6	17.76	0.10509 (±0.02555)	0.5917	37

Table 4. Pharmacokinetic parameters calculated after i.v. and oral administration of investigated octreotide formulations in pigs. Relative bioavailability was calculated with reference to i.v. formulations. Indicated values are means of 4 pigs.

Delivery system	No	AUC (min*ng/ml)	C_{max} (ng/ml)	t_{max} (min)	% Relative bioavailability
i.v. solution		656.01	100.10	-	-
Octreotide-deoxycholate SEDDS	B3	2277.96	74.22	60	5.21
Octreotide-decanoate SEDDS	B4	133.83	3.94	60	0.31
Octreotide-docusate SEDDS	B6	530.62	17.15	60	1.21
Octreotide in apple juice		128.48	4.28	60	0.29

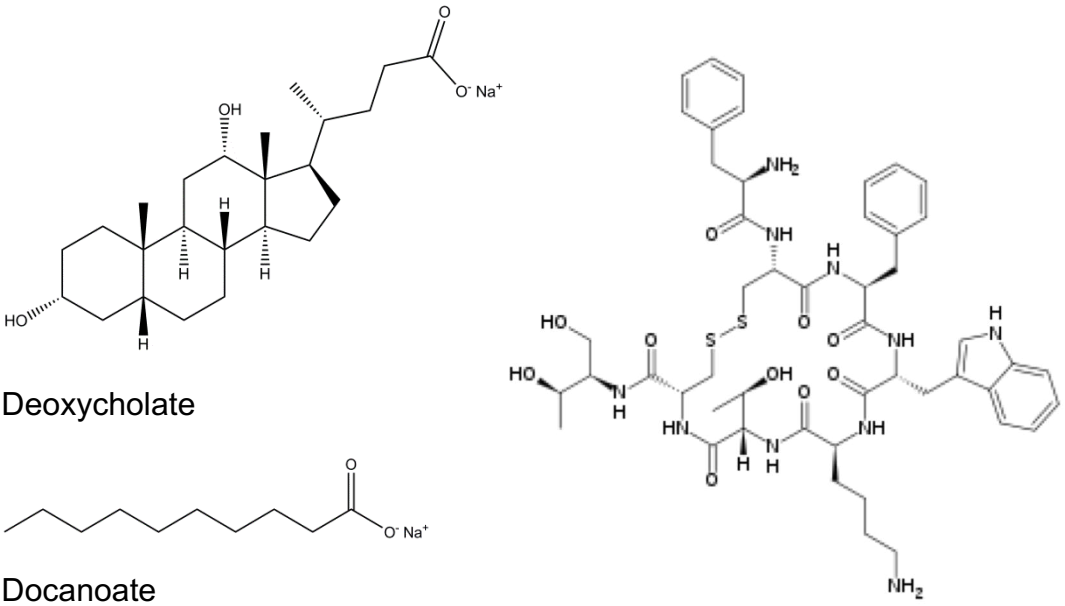


Fig. 1. Chemical structure of octreotide and the anionic counter ions.

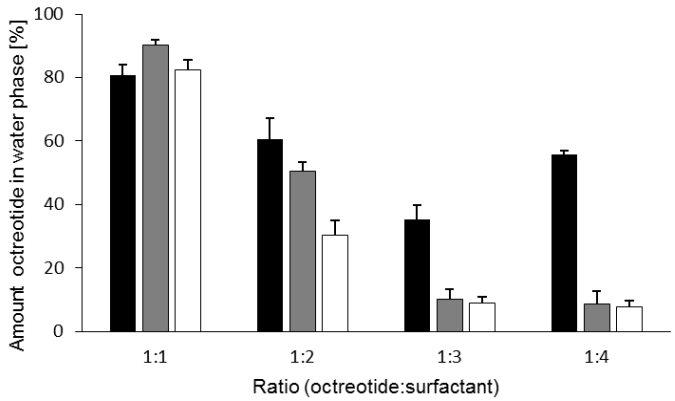


Figure 2. Comparison of remaining octreotide acetate in the water phase in relation to the amount of counter ion (black bars = sodium decanoate; grey bars = sodium deoxycholate; white bars = sodium docusate); indicated values are means ±SD (n=3);

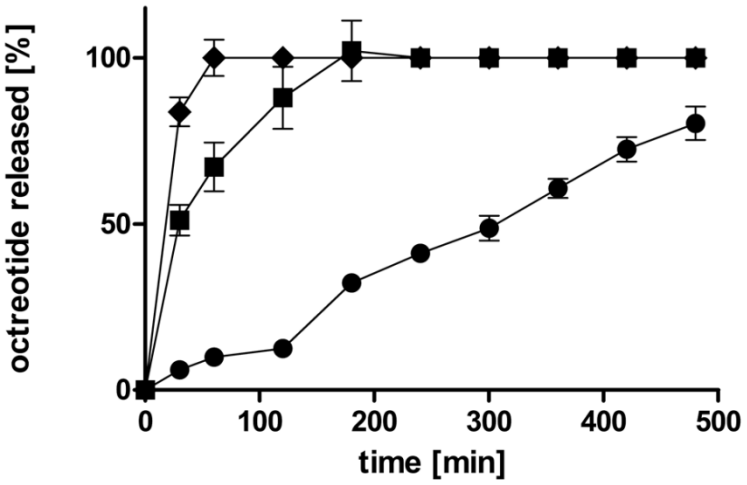


Figure 3. Illustration of release profile for SEDDS containing octreotide ion pairs. Time dependent drug release of octreotide-deoxycholate (■), octreotide-decanoate (♦) and octreotide-docusate (●) in 100 mM phosphate buffer pH 6.8 at 37°C. Depicted values are the means of at least three experiments ± standard deviation.

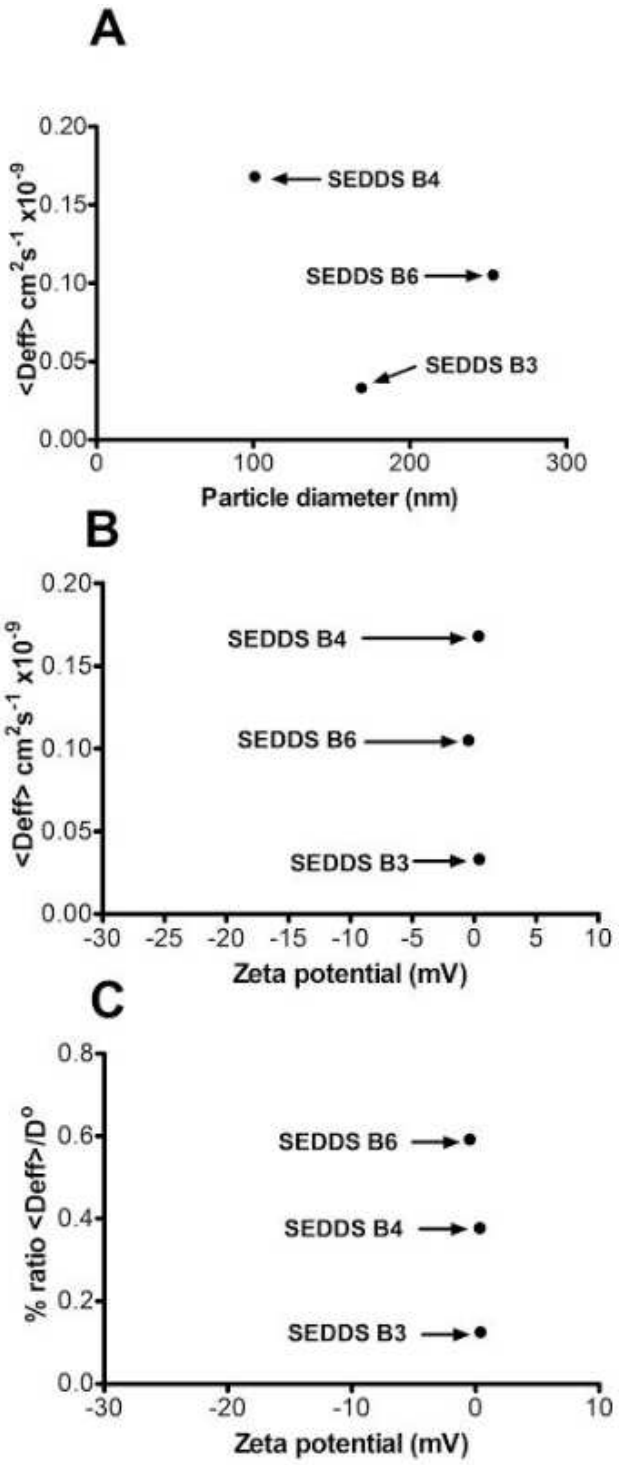


Figure 4. Correlation of droplet size and surface charge of various SEDDS to their mucus diffusion. (A) Correlation of droplet size of various SEDDS versus <Deff>. (B) Correlation of zeta potential of various SEDDS versus <Deff>. (c) Correlation of zeta potential of various SEDDS versus the % ratio of <Deff>/D°. Droplet size is expressed in nm, zeta potential is expressed in mV and Deff is measured in cm^2. s^{-1} $*10^9$.

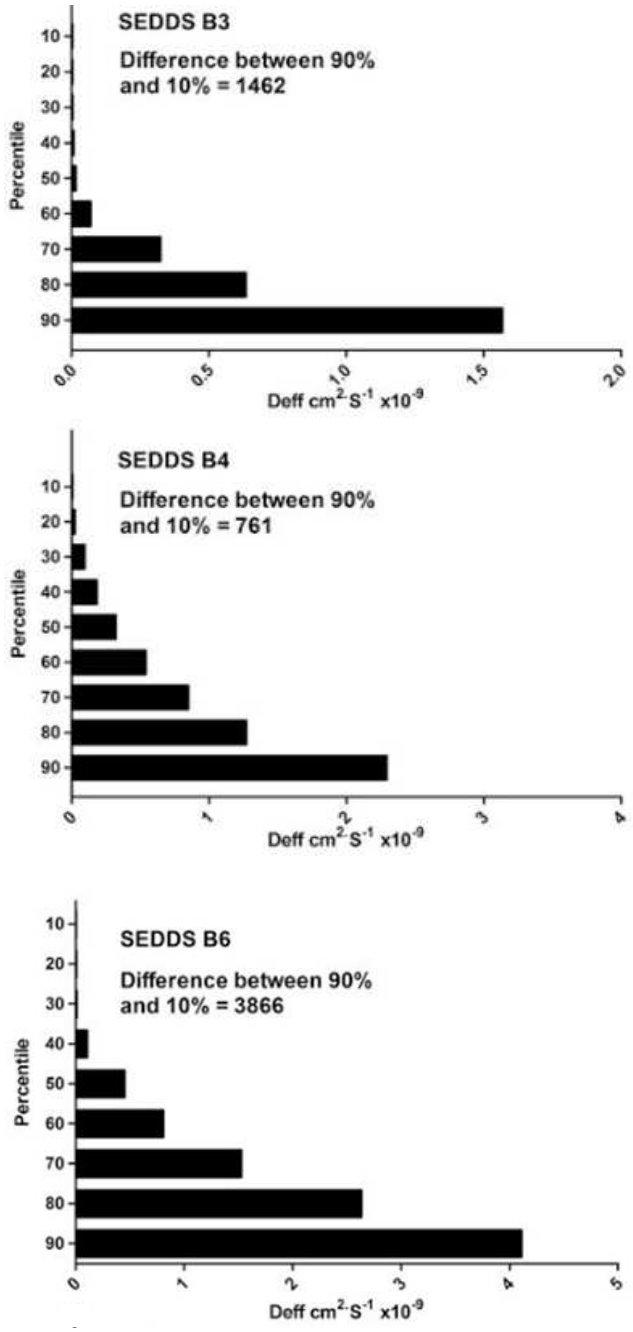


Figure 5. Comparison of average Deff of SEDDS B3, SEDDS B4 and SEDDS B6 at a time scale of 1 sec in mucus of subclasses from the fastest to the slowest percentile. Figure presents data of 3 experiments each with n ≥ 120 droplets.

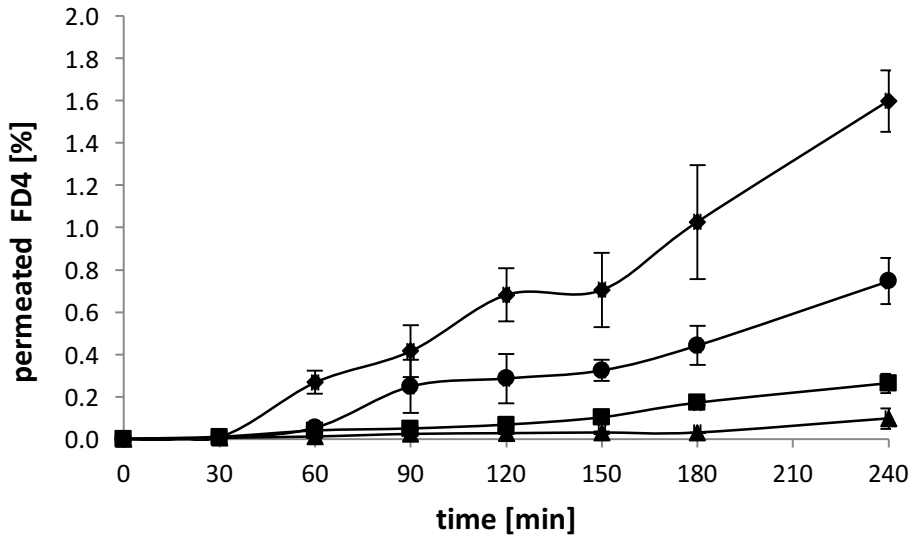
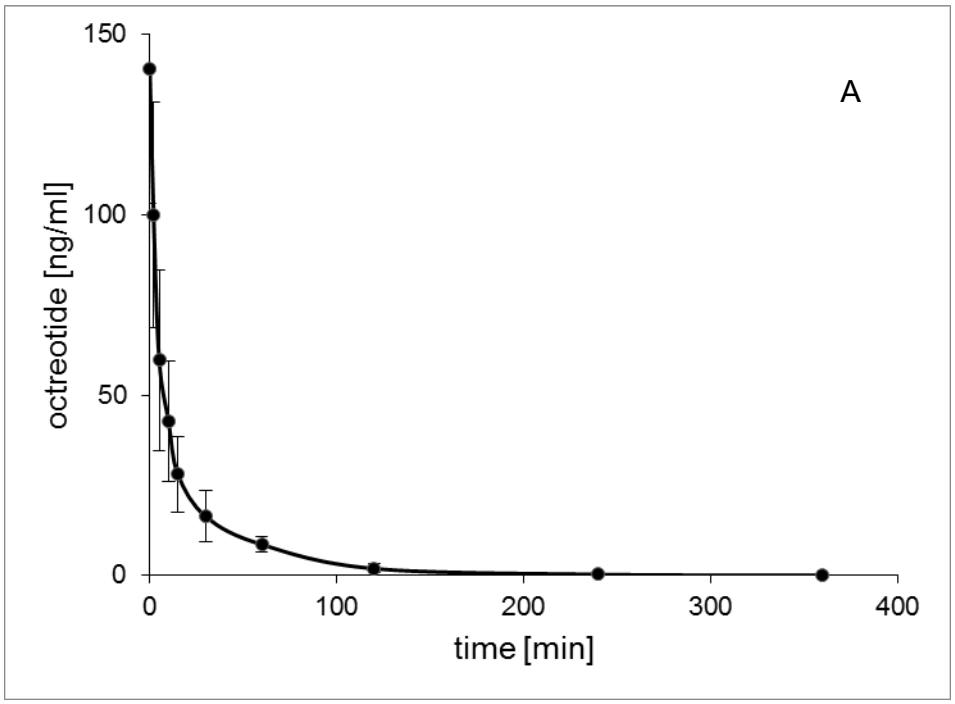


Figure 6. Permeation of FD4 across porcine small intestinal mucosa. Cumulative transport of FD4 in 0.1 M phosphate buffer pH 6.8 at 37°C in presence of 1.0% SEDDS-formulation B3 (■), 1.0% SEDDS-formulation B4 (♦) and 1.0% SEDDS-formulation B6 (●) vs. buffer only (▲). Indicated values are means (± SD, *n*=3)



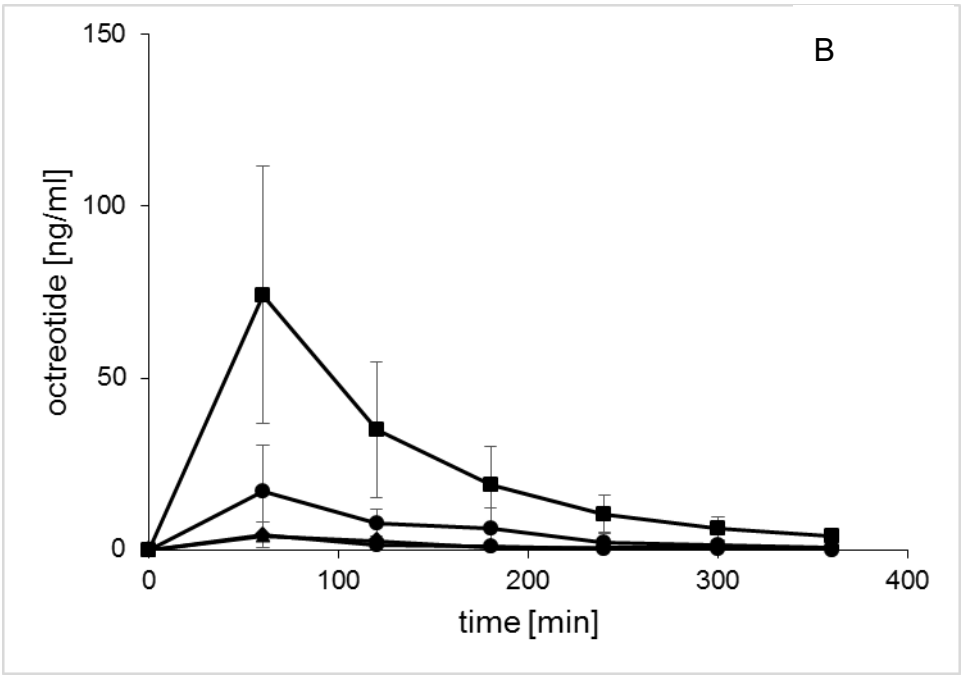


Figure 7. (A) Plasma concentration of octreotide after intravenous injection of octreotide acetate (dose is 750 µg) to pigs. (B) Plasma concentration curves of octreotide after administration of an octreotide acetate solution (▲), octreotide-deoxycholate SEDDS (■), octreotide-decanoate SEDDS (♦) and octreotide-docusate SEDDS (●) to pigs (dose = 50 mg). Indicated values are means from 4 applications ± SD.

Supplementary Figures:

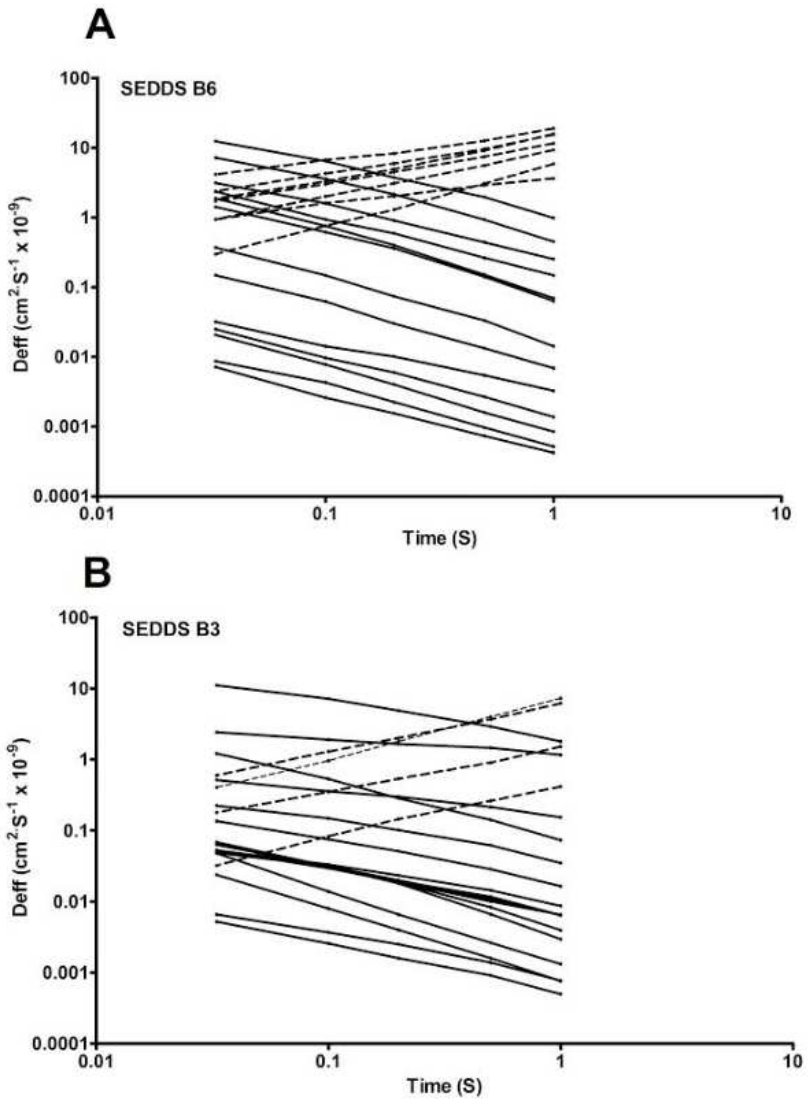


Figure 1S: Effective diffusivities Deff versus time scale of 20 randomly selected droplets selected by (random.org). (A) SEDDS B6: high ratio of droplets shows diffusivities. (B) SEDDS B3: Some droplets are diffusive vs major restricted droplets.