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RESEARCH PAPER

Bioavailability of Cinnarizine in Dogs: Effect of SNEDDS Loading Level and Correlation with Cinnarizine Solubilization During In Vitro Lipolysis

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ABSTRACT

Purpose To investigate the effect of increasing the loading level of the poorly soluble drug cinnarizine in a self-nanoemulsifying drug delivery system (SNEDDS) both *in vitro* and *in vivo*.

Methods A fixed dose of cinnarizine was administered orally to dogs in solution in different amounts of SNEDDS vehicle. Furthermore, the SNEDDSs were characterised using the dynamic *in vitro* lipolysis model.

Results Statistical differences in bioavailability were not obtained between the different amounts of SNEDDS vehicle, in spite of differences in the tendency of cinnarizine to precipitate during *in vitro* lipolysis of the treatments. Use of the SNEDDS concept decreased the variation in cinnarizine exposure observed between dogs as compared to administering cinnarizine in an aqueous suspension.

Conclusions Optimization of SNEDDSs towards keeping the drug compound in solution upon *in vitro* lipolysis of the SNEDDSs may not be as important as previously suggested.

KEY WORDS bioavailability · cinnarizine · lipolysis · oral drug delivery · self-nanoemulsifying drug delivery systems

ABBREVIATIONS

CYP	Cytochrome P450
PC	Phosphatidyl Choline
SIM	Simulated Intestinal Media
SNEDDS	Self-Nanoemulsifying Drug Delivery System
SNP	Single Nucleotide Polymorphism
TC	Sodium Taurocholate

INTRODUCTION

Self-nanoemulsifying drug delivery systems (SNEDDSs) serve as an option to increase the bioavailability of poorly water soluble drug compounds (1–3). However, rational development of SNEDDSs is hampered by the lack of understanding of the critical quality attributes for this formulation type. The relevance of current methods applied to testing these systems *in vitro* for prediction of the *in vivo* performance of SNEDDSs is unclear. Only a few publications exist where different SNEDDS formulations have been compared and therefore only a limited amount of data on the link between the *in vitro* and *in vivo* performance of SNEDDSs is available (4,5). Typically the basis for SNEDDS development is a study of the solubility of the drug compound in excipients relevant for obtaining SNEDDSs. The SNEDDS is then developed from the excipients with the highest drug compound solubility (6,7). Furthermore, the ratio between the excipients is often optimized towards the smallest nanoemulsion droplet size (8).

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The impact of halofantrine loading level in SNEDDS has previously been investigated. It should be emphasized that when testing drug loading level in a SNEDDS either the dose of the drug compound is altered or the SNEDDS vehicle amount is altered. In one case 3 mg halofantrine dissolved in either 0.1 g or 1 g, at either 56.7% or 5.7% of saturation solubility, respectively, in a long chain lipid SNEDDS was dosed to rats, and no difference in bioavailability was observed (9). In another study both long chain lipid and medium chain lipid SNEDDS containing halofantrine were tested in dogs. For the medium chain SNEDDS the dogs received 56.4 mg halofantrine and for the long chain SNEDDS 76.8 mg. In both cases halofantrine was dosed at either 75% or 150% of saturation solubility in the given SNEDDS and no significant differences in the bioavailability was found (10). In a similar study in dog, a 150% supersaturated SNEDDS containing simvastatin as a model drug compound resulted in significant higher bioavailability of simvastatin in compared with dosing of the same SNEDDS vehicle containing only 75% of saturation (11). The dose of simvastatin was kept constant, whereas the amount of vehicle was 0.8 and 1.6 g in the former and latter treatment, respectively (11). However, the SNEDDSs with the low amounts of vehicle were supersaturated with respect to the drug in contrast to the SNEDDSs with the higher amount of vehicle in the studies in dog. These studies indicate that a higher loading level of drug compound in SNEDDS does not have a negative impact on the obtained bioavailability, but when comparing different loadings in non saturated SNEDDSs the effect of loading may differ.

The purpose of the present study was to investigate the impact of loading degree in SNEDDSs on oral bioavailability of poorly water soluble cinnarizine in dog and to study during *in vitro* lipolysis, how the drug loading affects the distribution of cinnarizine between the different physical digestion phases obtained after ultracentrifugation. Furthermore, the aim was to compare the oral bioavailability of cinnarizine from SNEDDSs with an aqueous suspension in order to assess whether the investigated SNEDDSs were superior to traditional solid dosage forms.

MATERIALS AND METHODS

Materials

Oleic acid (composition from certificate of analysis: 0.4% C16, 2.5% C18, 89.2% C18-1, 5.9% C18-2, 1.0% C18-3) was a gift from Danisco (Copenhagen, Denmark). Phosphatidylcholine SPC (≥98%) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cremophor RH40 was obtained from BASF (Ludwigshafen, Germany). Brij 97, Polysorbate 80, cinnarizine (≥99%), TRIS maleate, pancreatin at least 3xUSP, porcine bile extract and sodium taurocholate (≥95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyethylene glycol 400 (Ph. Eur.), sesame oil (Ph. Eur.) and 4-bromobenzene boronic acid were obtained from Fluka (Buchs, Switzerland). Ethanol (Ph. Eur. grade) and acetonitrile (HPLC grade) were purchased from VWR (Herlev, Denmark). Methocel was obtained from Dow Chemical Company (Midland, MI, USA). Hard white gelatine capsules (size 000 with Coni-SNAP) were purchased from Apodan-Nordic PharmaPackaging (Copenhagen, Denmark).

Preparation of SNEDDSs

A previously developed SNEDDS was slightly modified with respect to the loading of cinnarizine and hereafter utilized in the present study (4). The composition of the SNEDDSs is shown in Table I. The SNEDDSs were prepared by weighing the appropriate amount of excipient in glass containers and mixed by magnetic stirring. Cinnarizine was weighed in a second glass container and the SNEDDS preconcentrate was added to prepare a final concentration of cinnarizine of 50 ($SNEDDS_{HIGH}$), 25 ($SNEDDS_{MEDIUM}$), or 12.5 mg/g ($SNEDDS_{LOW}$). After stirring overnight protected from light the SNEDDSs were obtained. A 100 fold dilution of the SNEDDS in pH 6.5 100 mM tris maleate buffer resulted in a droplet size of 26.9 ± 0.9 nm (hydrodynamic diameter, mean ± SD, $n = 3$) measured using dynamic light scattering (4).

Preparation of Aqueous Cinnarizine Suspensions

Two different suspensions were used in the present study, SUSP I (5 mg/ml) and SUSP II (25 mg/ml). The suspensions were prepared by weighing the desired amount of cinnarizine

Table I Composition of SNEDDSs

	$SNEDDS_{HIGH}$	$SNEDDS_{MEDIUM}$	$SNEDDS_{LOW}$
Composition of formulations (w/w %)			
Sesame oil	20.6	20.6	20.6
Oleic acid	15.4	15.4	15.4
Cremophor RH 40	45	45	45
Brij 97	9	9	9
Ethanol	10	10	10
Cinnarizine content			
mg cinnarizine per g SNEDDS mg/g)	50	25	12.5
Percent of saturation solubility in SNEDDS (%)	85.0	42.5	21.3

in volumetric flasks and then adding the dispersion medium. The dispersion medium was an aqueous solution of 0.5% Methocel and 0.1% Polysorbate 80 (w/w). Hereafter the suspensions were stirred overnight. An ultrasonic probe was used in order to reduce the particle size of SUSP I whereas SUSP II was used without further reduction in particle size. The particle sizes of the suspensions were measured by laser diffraction using the Frauenhofer theory on a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). The suspensions were diluted prior to size measurements in purified water.

Composition of Simulated Intestinal Media

Simulated Intestinal Media (SIM) were prepared as previously described (12). The compositions of the SIM included in the present study are shown in Table II. The SIM contained different amounts of sodium taurocholate (TC), phosphatidylcholine (PC), oleic acid and monooleate. Additionally the SIM also contained 100 mM tris maleate to buffer the media and 3 mM NaN_3 to prevent microbial growth. NaCl was added to the media to give the same osmolality of 270 mosm/l in the different SIM and pH was adjusted to 6.5 in all the SIM used in the present study.

Solubility of Cinnarizine in SIM

Cinnarizine in excess (approx 50 mg) was added to 10 ml freshly prepared SIM ($n=3$). The suspensions were mounted on an end-over-end rotation apparatus with constant rotation and placed in a heating cabinet at 37°C. Samples were withdrawn after 24, 48, and 68 h of incubation. The sample tubes were centrifuged at 4,500 rpm for 10 min in a Labofuge 400R centrifuge (Hereaues Instruments, Langenselbold, Germany). Approximately 1 ml of the supernatant was transferred to a small centrifuge tube and centrifuged at 15,000 rpm for 15 min in a Biofuge 15 (Hereaus-Sepatech, Oterode, Germany). The content of cinnarizine in the supernatant was quantified by use of an HPLC method with fluorescence detection. The sample tubes were vortexed before resumption of incubation. The solubility was defined as the concentration reached, when the results from two consecutive samples varied by less than 5%. To ensure that pH was kept constant, the pH was measured in the samples after 24 and 68 h and pH adjustment was not necessary.

Table II Content of Sodium Taurocholate, Phosphatidylcholine, Oleic Acid and Monooleate in SIM

	Sodium taurocholate (mM)	Phosphatidylcholine (mM)	Oleic acid (mM)	Monooleate (mM)
SIM 1	5	1.25	–	–
SIM 2	10	2.5	–	–
SIM 3	15	3.75	–	–
SIM 4	20	5	–	–
SIM 3-A	15	3.75	5	2.5
SIM 3-B	15	3.75	10	5
SIM 3-C	15	3.75	15	7.5
SIM 3-D	15	3.75	20	10

Quantification of Cinnarizine in SIM

The supernatants obtained from the second centrifugation step mentioned above were diluted to appropriate concentrations with mobile phase (acetonitrile: ammonium phosphate monobasic buffer 10 mM (50:50 v/v)). The dilutions ranged between 100 and 4000 times. The concentration of cinnarizine in the diluted samples was determined by a reversed-phase HPLC method with fluorescence detection. The method used was slightly modified from a previously described method (13). A Hitachi HPLC system from Merck (Darmstadt, Germany) was used; D-7000 interface, L-7200 autosampler, L-7100 pump, L-7350 column oven (35°C) and a L-7480 fluorescence detector. Data was processed by use of the software HPLC System Manager Version 4.0 (Merck, Darmstadt, Germany). The chromatographic separation was achieved using a Luna C18 4.60×150 mm, 5 µm column and a C18 3.0 mm×4 mm, guard column both purchased from Phenomenex (Torrance, CA, USA). A quantity of 25 µl was injected into the chromatographic system and excitation at 249 nm and emission at 311 nm were used. Standards were prepared in mobile phase. Quantitation was performed from peak areas of samples and external standards solutions using a six point linear standard curve ranging from 5–160 ng/ml and limit of quantitation and limit of detection was 3.17 ng/ml and 1.04 ng/ml, respectively, based on 6 replicates. The intra-day and inter-day variation expressed as relative standard deviation was 0.58% and 3.04% (3 days), respectively, measured at a cinnarizine concentration of 80 ng/ml and based on 6 replicates.

Dynamic *In Vitro* Lipolysis Study

The SNEDDSs with the different loadings of cinnarizine were tested in the dynamic *in vitro* lipolysis model. The dynamic *in vitro* lipolysis experiments were conducted as previously described (14) using continuous addition of calcium (15). A solution of bile salt and phosphatidylcholine (PC) in purified water was mixed overnight (approx. 24 h) at 37°C. The bile salt and PC containing medium was mixed

with buffer in a thermostatically controlled vessel (37 ± 0.5°C). The amount of formulation applied to the *in vitro* lipolysis model was 1.5 g, 3 g and 6 g for $SNEDDS_{HIGH}$, $SNEDDS_{MEDIUM}$ and $SNEDDS_{LOW}$, respectively. As a result, the cinnarizine dose administered to the lipolysis model was kept constant at 75 mg for all the treatments. The pH was adjusted to 6.5 with 1.00 M NaOH and after an equilibration time of 3 min, the lipolysis process was initiated by addition of lipase and the continuous addition of 0.5 M Ca^{2+} solution (0.09 ml/min). The hydrolysis was followed by titration keeping pH at 6.5 with addition of 1.00 M sodium hydroxide. The titration was carried out on an 842 Titrando titrator fitted with a Unitrode pH electrode with a Pt1000 temperature sensor, two Dosino 10 ml dosing units, and a rod stirrer with a 96 mm stirrer propeller (Metrohm AG, Herisau, Switzerland). The background lipolysis was determined from a lipolysis experiment without adding SNEDDS nor drug. The initial composition of the lipolysis medium, and the calcium addition rate, in the lipolysis studies is shown in Table III.

At specific times, quantities of 3 ml (for separation by ultracentrifugation) and 100 µl (for determining of the total content of cinnarizine) were withdrawn, and the lipase activity in the 3 ml sample was inhibited with 4-bromobenzene boronic acid solution as previously described (15). Lipase activity in the 100 µl sample was inhibited by addition of 900 µl acetonitrile. The 3 ml samples were separated in three digestion phases, upper, aqueous and pellet phase by means of ultracentrifugation in thick-walled polycarbonate ultracentrifuge tubes 13 × 56 mm. Centrifugation was conducted at 37°C at $5.4 \times 10^5 g$ for 30 min (100,000 rpm) in a Beckman Optima™ MAX benchtop ultracentrifuge with a TLA-110 fixed angle rotor (Beckman-Coulter, Fullerton, CA, USA). In order to quantify the cinnarizine partition into the 3 phases the following procedure was employed: the entire sample together with the tube was weighed, the upper phase was collected with a handheld peristaltic Ismatech pump from IDEX Health & Science GmbH (Wertheim-Mondfeld, Germany), the remaining sample was weighed (aqueous phase + pellet), a 100 µl sample of the aqueous phase was sampled from the middle of the centrifugation tube, the rest of the aqueous phase was removed, and the remaining sample (pellet) was weighed. Quantification of cinnarizine in the three digestion phases were determined by HPLC as previously described (5).

Table III *In Vitro* Lipolysis Parameters Used

Substance	Initial concentration
Bile salt [mM]	5
PC [mM]	1.25
Pancreatic lipase [USP units/ml]	800
Trizma-maleate [mM]	2
Na^+ [mM]	150
Initial total volume [ml]	300
Ca^{2+} addition rate [mmole/min[a]]	0.045

[a] Dispensing rate (0.09 ml/min of a 0.5 M Ca^{2+} solution)

Transport of Cinnarizine in the Caco-2 Cell Culture Model

Caco-2 cells (HTB-37) obtained from the American Type Culture Collection (ATCC) at passage 20 were grown in T-75 culture flasks in an atmosphere of 5% CO_2 and 95% O_2 (v/v) at 37°C. The cells were maintained in Dulbecco's modified eagle medium supplemented with 10% (v/v) FBS, 90 U/ml penicillin 90 µg/ml streptomycin and 1% non-essential amino acids and L-glutamine and used in passage numbers 27–29. Caco-2 cells were sub-cultured when reaching 80–90% confluence. Cells were seeded at a density of 1×10^5 cells/filter and were cultured for 27 days before initiation of the experiments. Culture medium was replaced in both the apical and basolateral compartment every second day.

Transepithelial electrical resistance (TER) was measured prior to the transport experiments and after finishing the experiment in a tissue resistance measurement chamber (Endohm, World Precision Instruments, Berlin, Germany) with a voltohmmeter (EVOM, World Precision Instruments). TER values were measured at ambient temperature.

Transepithelial transport of cinnarizine in the absence or presence of verapamil, was investigated in both the apical to basolateral direction and the basolateral to apical direction. Caco-2 cells were conditioned prior to the transport study by 30 min incubation with 0.5 and 1 ml buffer on the apical and basolateral side, respectively. The cells were circularly and continuously shaken at 95 rpm on a KS15 Edmund Bühler Compact Shaker (Holm and Halby, Broendby, Denmark) at 37°C. The used buffer was Hank's buffered salt solution supplemented with 0.05% bovine serum albumin and 10 mM MES (pH 6). Transport of cinnarizine was initiated by replacing apical buffer with fresh buffer containing either 0.7 µM cinnarizine or cinnarizine and 100 µM verapamil. For transport of cinnarizine in the presence of verapamil, the transport buffer of both the apical and basolateral compartments contained 100 µM verapamil. Cinnarizine was added to the buffer in DMSO resulting in a final DMSO concentration of 1%. For apical to basolateral transport 100 µl samples were taken from the basolateral compartment and for basolateral to apical transport 50 µl was taken from the apical compartment. Samples were taken at $t = 0$, 15, 30, 45, 60, 90, 120 min and immediately replaced with fresh buffer. The samples were diluted twofold with acetonitrile and centrifuged. The content of cinnarizine in the supernatants was determined by LC-MS utilizing a previously published method (5).

Apical to basolateral transport of ^{14}C-mannitol was measured immediately after each cinnarizine transport study.

The Caco-2 cell monolayer was washed twice with buffer. Transport of ^{14}C-mannitol was initiated by replacing the apical buffer with buffer containing 17 μM ^{14}C-mannitol. Basolateral samples (100 μl) were taken at t=0, 30 and 60 min and transferred to scintillation vials, to which 2 ml of scintillation fluid was added and the radioactivity was counted in a liquid scintillation analyzer (Packard Tri-Carb 2100 TR, Canberra, Dreich, Germany).

The apparent permeability coefficients (P_{app}) of transepithelial transport of cinnarizine and ^{14}C-mannitol were calculated using Eq. 1:

$$P_{app} = \frac{J}{C_0} = \frac{Q_t}{C_0 \cdot A \cdot t} \quad (1)$$

where J is the steady state flux (nmol cm^{-2} min^{-1}), C_0 is the initial concentration in the donor compartment, A is the area of the filter support (1.13 cm^2), and Q_t is the accumulated amount of compound (nmol) in the receiver compartment at time t (min).

In Vivo Study

The *in vivo* study in Labrador dogs took place at AstraZeneca in Mölndal (Sweden), and the protocol was approved by the animal ethical committee of Gothenburg (ethical license number 208–2009) and all animal procedures were carried out in compliance with EC Directive 86/609/EEC and with the Swedish laws regulating experiments on animals. Four male Labrador dogs, age 4–6 years and weighing between 28.4 and 32.9 kg at the days of administration were included in the experiment. The dogs were fed standard food once daily and had access to water *ad libitum*. Dogs were fasted for 20–24 h before dosing and water was available *ad libitum* during the study. A neutral pH in the stomach of the dogs was achieved using a method described previously (16). The purpose of keeping the pH neutral was to avoid cinnarizine from distributing out of the nanoemulsion at low gastric pH. Cinnarizine is a weak base and has a significantly higher solubility at acidic pH compared to a neutral pH. Briefly, esomeprazole (1 mg/kg) was administered by intravenous infusion into a foreleg vein over a 3 min period and hereafter 75 ml of water was given through an orogastric tube. Ninety minutes after the esomeprazole administration the SNEDDSs or aqueous suspensions were administered orally to the dogs. A 50 mg dose of cinnarizine was used in all treatments. The SNEDDSs and SUSP II were loaded into size 000 hard gelatine capsules immediately prior to the administration. For SUSP I (5 mg/ml) 10 ml was administered through an orogastric tube followed by 20 ml water and for SUSP II (25 mg/ml) two capsules containing 1 ml each was orally administered to the dogs followed by 20 ml water. The capsules containing 1 ml SUSP II were prepared by pipetting 1 ml SUSP II into the body of the capsules and hereafter the cap of each capsule was snapped on. The capsules containing SNEDDSs contained 1 g each and were prepared by individual weighing of 1 g formulation into the body of the capsules utilizing an analytical balance and hereafter the cap was snapped on. For $SNEDDS_{HIGH}$, $SNEDDS_{MEDIUM}$ and $SNEDDS_{LOW}$ each dog received 1 (50 mg/g), 2 (25 mg/g) and 4 (12.5 mg/g) capsules each, respectively. After the oral administration of the capsules in fasting condition 20 ml of water was administered. Four hours after administration of the SNEDDSs or suspensions the dogs were fed. Each dog received each formulation in a cross-over design with a 6-day wash-out in between dosing. Blood samples (approx. 2 ml) were collected, prior to, 30 min, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 h after administration from the front leg vein in vacutainer heparin sampling tubes from BD (Franklin Lakes, NJ, USA). Within 30 min upon blood sampling the sample tubes were centrifuged at 3,000*g* for 10 min at 4°C and the plasma was transferred to 2 ml micro tubes from Sarstedt AG & Co. (Nümbrecht, Germany) and kept at −20°C until analysis. The content of cinnarizine in the plasma samples were determined by LC-MS using a previously described method (5).

Pharmacokinetic Data Analysis

Pharmacokinetic parameters were determined using WinNonLin version 4.1 from Pharsight Corp. (Mountain View, CA, USA). The peak plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were obtained directly from the individual plasma concentration *versus* time profiles. Area under the curve of cinnarizine from 0 to 48 h ($AUC_{0\text{-}48\ h}$) for individual dogs were determined by the linear trapezoidal method.

Statistical Analysis

The data for AUC and C_{max} were logarithmically transformed in order to normalize the variations. Hereafter, the statistical analysis was performed using one-way repeated measures analysis of variance for the obtained AUC, T_{max} and C_{max}. Pairwise comparisons between means were performed by the Fishers least significant difference method. A 5% level of significance was used for concluding statistically significant differences. All calculations were carried out using SigmaStat for Windows version 3.5 (Systat Software Inc., Richmond, CA, USA).

RESULTS

Solubility of Cinnarizine in Simulated Intestinal Media

In the simulated intestinal media (SIM) used, the concentration ratio of TC to PC was kept constant at 4:1 (TC:PC). Furthermore, when mimicking the fed state by adding lipid

digestion products to the SIM in this case oleic acid and monooleate the concentration ratio of oleic acid to monooleate was kept constant (2:1). Increasing the level of TC and PC increased the solubility of cinnarizine (Fig. 1a). A 3–4 fold increase was seen going from low TC:PC level (5:1.25 mM) to high TC:PC level (20:5 mM). A more dramatic increase (19 fold) was observed from plain buffer to SIM containing 5 mM TC and 1.25 mM PC. The effect of adding lipid digestion products on increasing the solubility of cinnarizine was much more pronounced as compared to with the effect of increasing the TC level (Fig. 1b). When increasing the oleic acid level from 5 mM to 20 mM in SIM3 containing 15:3.75 mM TC:PC an 8–9 fold increase in the cinnarizine solubility was observed.

Dynamic *In Vitro* Lipolysis

The amount of free fatty acids titrated during the dynamic *in vitro* lipolysis tests are shown in Fig. 2a. The results shown are with the blank background lipolysis subtracted and therefore represent solely the lipolysis of the SNEDDSs. The lipolysis profiles are not starting at 0-mmol fatty acids titrated because oleic acid (excipient in the SNEDDSs) is titrated 3 min before initiation of the lipolysis, when the system pH is adjusted to 6.5. It is seen that when the amount of vehicle is increased, the amount of free fatty acids titrated as a result of lipolysis is also increased. From Fig. 2b and c it is seen that $SNEDDS_{HIGH}$ cannot keep the cinnarizine dose solubilized in the aqueous phase throughout the lipolysis experiment and cinnarizine starts to precipitate immediately. Cinnarizine from $SNEDDS_{MEDIUM}$ also starts to precipitate but not until after 50 min of lipolysis. Precipitation as a function of lipolysis was not observed from $SNEDDS_{LOW}$. From Fig. 2d it is seen that $SNEDDS_{MEDIUM}$ and $SNEDDS_{LOW}$ eventually has a higher content of cinnarizine in the upper phase compared with $SNEDDS_{HIGH}$, but initially the content of cinnarizine in the upper phases was similar (Fig. 2d).

Transport of Cinnarizine in the Caco-2 Cell Culture Model

Figure 3 shows the obtained apparent permeabilities of cinnarizine. No statistically significant difference between the apical to basolateral ($4.2\pm0.3\times10^{-6}$ cm/s) and basolateral to apical ($4.1\pm0.8\times10^{-6}$ cm/s) transport of cinnarizine was observed. Likewise, no difference between the apical to basolateral ($4.2\pm0.8\times10^{-6}$ cm/s) and the basolateral to apical ($4.4\pm0.9\times10^{-6}$ cm/s) permeability of cinnarizine was observed when the cells were incubated with cinnarizine and the P-glycoprotein inhibitor verapamil (17). The TER values measured before and after incubation of cinnarizine with and without verapamil are shown in Fig. 4. The TER values were either higher or similar after the transport studies. The permeability of mannitol measured after termination of the cinnarizine transport studies was $1.1\times10^{-6}\pm2.2\times10^{-7}$ and $1.1\times10^{-6}\pm2.1\times10^{-7}$ cm/s after incubation with cinnarizine in the apical to basolateral and basolateral to apical direction, respectively, and it was $1.1\times10^{-6}\pm2.2\times10^{-7}$ and $1.2\times10^{-6}\pm5.0\times10^{-7}$ cm/s after incubation with cinnarizine and verapamil in the apical to basolateral and basolateral to apical direction, respectively.

In Vivo Study

Figure 5 shows the plasma concentration-time profiles obtained after administration of the SNEDDSs containing cinnarizine to dogs, Table IV shows the related pharmacokinetic parameters. Significant differences were not found for

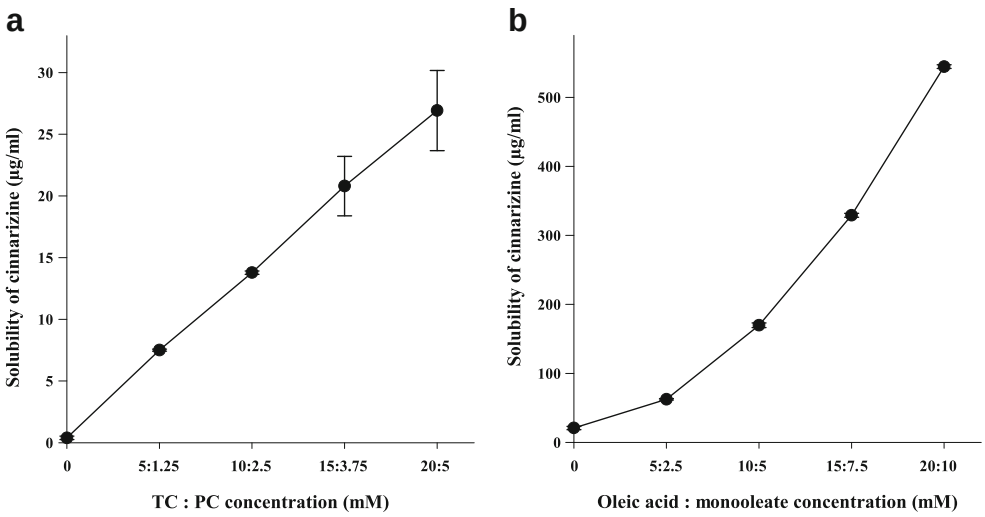

Fig. 1 (**a**) Cinnarizine solubility as a function of the sodium taurocholate (TC):phosphatidylcholine (PC) concentration in the simulated intestinal media. (**b**) Cinnarizine solubility as a function of the oleic acid:monooleate concentration in the simulated intestinal medium containing 15 mM sodium taurocholate and 3.75 mM phosphatidylcholine at pH 6.5 and 37°C.

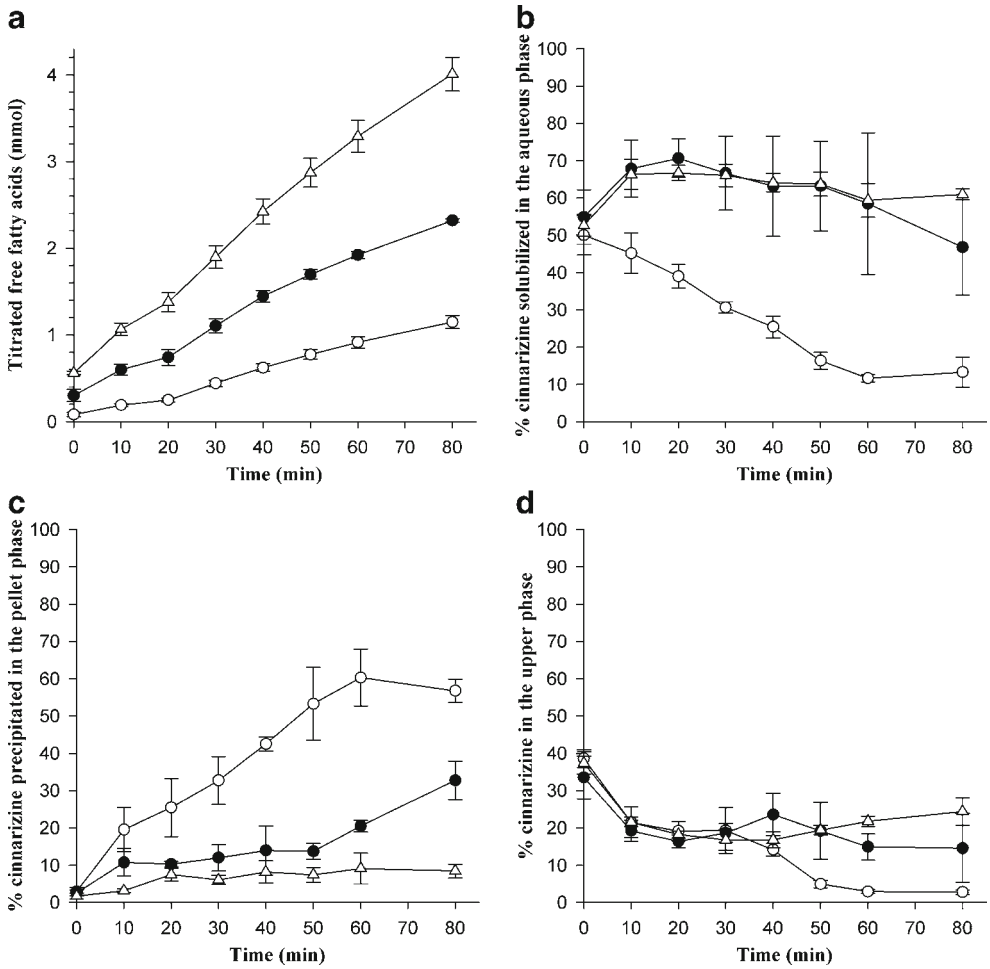

Fig. 2 Dynamic *in vitro* lipolysis of $SNEDDS_{HIGH}$, $SNEDDS_{MEDIUM}$ and $SNEDDS_{LOW}$ containing 75 mg cinnarizine. (**a**) The amount of free fatty acids titrated during the lipolysis experiment. (**b**) The percentage of the 75 mg dose that is distributed to the aqueous phase. (**c**) The percentage of the cinnarizine dose which precipitates into a pellet phase during lipolysis. (**d**) The percentage of cinnarizine distributed to the upper phase. *Open circles* are 1.5 g $SNEDDS_{HIGH}$ (50 mg/g), *filled circles* are 3 g $SNEDDS_{MEDIUM}$ (25 mg/g), and *open triangles* are 6 g $SNEDDS_{LOW}$ (12.5 mg/g).

the obtained AUC, C_{max} and T_{max} values. The obtained AUC values ranged from 355 ($SNEDDS_{HIGH}$) to 485 ng/ml*h^{-1} ($SNEDDS_{MEDIUM}$). Even though there was no significant difference between the obtained T_{max} values, a trend towards longer T_{max} values with higher amounts of SNEDDSs administered was observed. T_{max} was 1.8±0.14 h, 2.3±0.64 h and

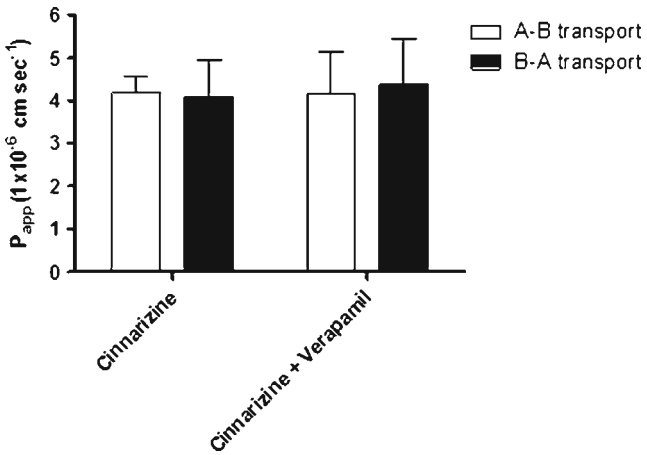

Fig. 3 Apparent permeability coefficients (P_{app}) of transepithelial transport of cinnarizine across Caco-2 cells with and without verapamil. *A-B*: apical to basolateral transport. *B-A*: basolateral to apical transport.

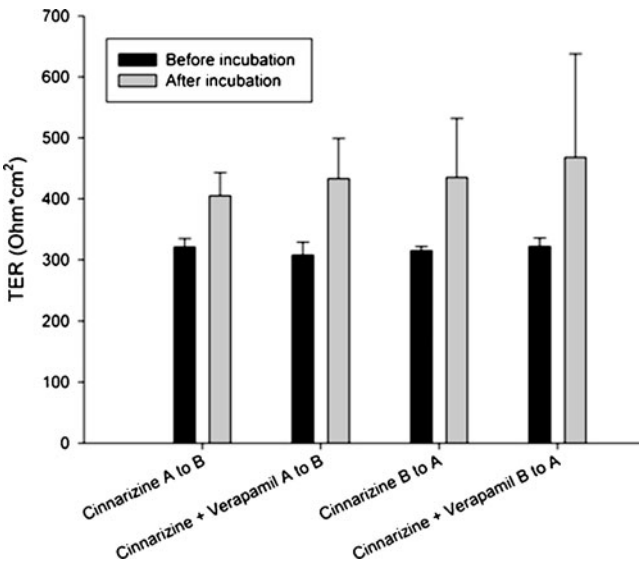

Fig. 4 Transepithelial electrical resistance values measured before and after cinnarizine and verapamil incubation in Caco-2 cells.

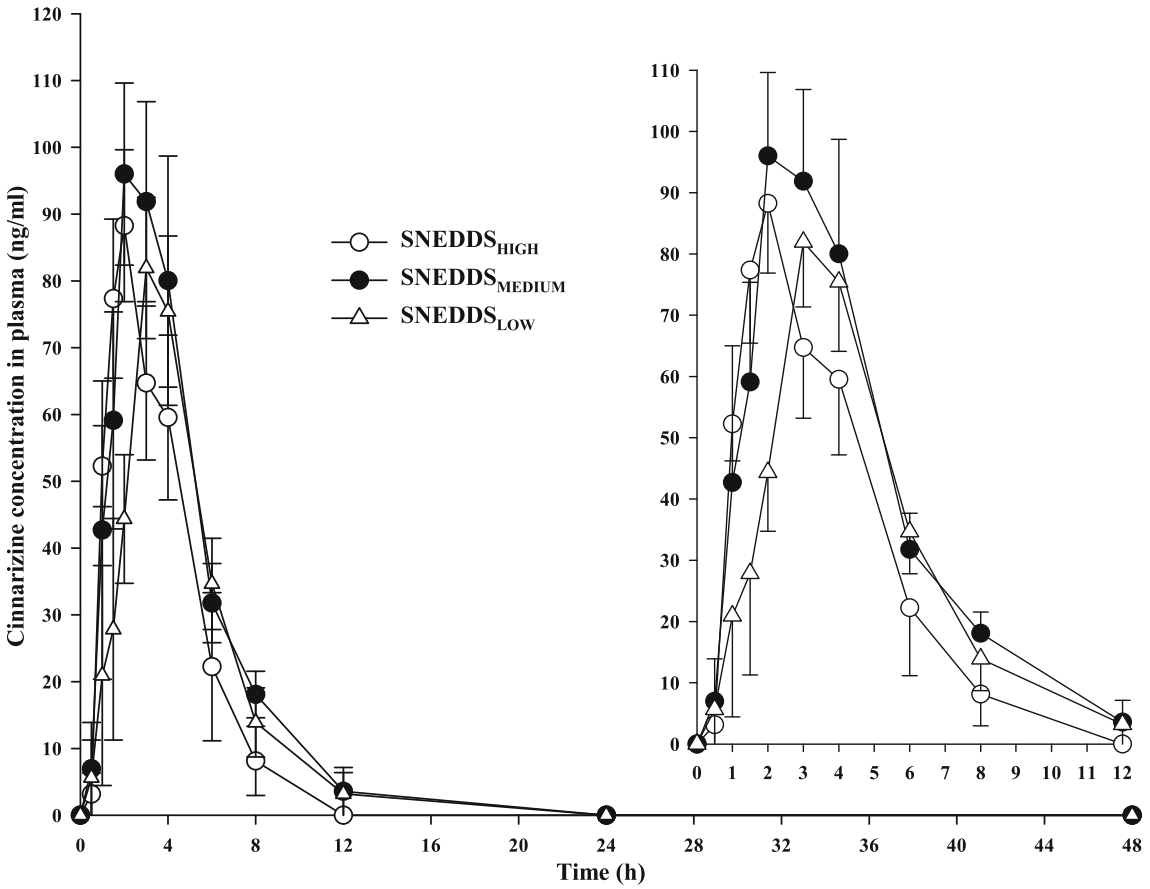

Fig. 5 Plasma concentration-time profiles (mean ± SE, $n=4$) for cinnarizine following oral administration of 50 mg cinnarizine in SNEDDS$_{HIGH}$, SNEDDS$_{MEDIUM}$ and SNEDDS$_{LOW}$ to fasted male Labrador dogs with elevated gastric pH. The insert shows enlargement of the profiles between 0 and 12 h. The amount of SNEDDS vehicle administered to the dogs differed. *Open circles* are 1 g SNEDDS$_{HIGH}$ (50 mg/g), *filled circles* are 2 g SNEDDS$_{MEDIUM}$ (25 mg/g), and *open triangles* are 4 g SNEDDS$_{LOW}$ (12.5 mg/g).

3.0±0.71 h after administration of 1 g SNEDDS$_{HIGH}$, 2 g SNEDDS$_{MEDIUM}$ and of 4 g SNEDDS$_{LOW}$, respectively.

Figure 6 shows the plasma concentration-time profiles obtained after administration of cinnarizine in the two different suspensions and the pharmacokinetic parameters are shown in Table IV. The particle sizes of the two suspensions were 6.79 μm (2.09) and 11.17 μm (2.79) for SUSP I and SUSP II, respectively. The values in brackets show the obtained span values. SUSP I was administered on one occasion to four dogs and it was observed that only dog 2 and dog 4 obtained cinnarizine plasma concentrations above the limit of quantification (10 ng/ml). The AUC levels obtained for the two other dogs were in the same range as the values obtained after administration of the SNEDDSs. To investigate the obtained results further, an additional administration of an aqueous suspension (SUSP II) was given to the same four dogs (Fig. 6). Again only the same two dogs obtained cinnarizine plasma levels above the limit of quantification. Since AUC values were only obtained from two dogs statistic comparisons were not made between the two suspensions and between the suspensions and the SNEDDSs.

DISCUSSION

In Vitro Characterisation of SNEDDS

The solubility of cinnarizine increased in SIM when the sodium taurocholate concentration increased. Also, the addition of long chain lipid digestion products to SIM containing 15 mM sodium taurocholate had a pronounced effect on the cinnarizine solubility (Fig. 1). Increasing the level of long chain lipid digestion products in SIM results in a higher increase in cinnarizine solubility, compared with the effect of increasing the TC level. This is in accordance with previously published solubility data for cinnarizine where a mixture of bile salts were used when preparing the biorelevant media (18). The increase observed is not only a result of the increase in overall surfactant content, but also a result of the mixed micelle structures generated being better solubilizers for cinnarizine compared with micelles composed of only bile salt and phospholipid (18).

The dynamic *in vitro* lipolysis model was utilized to determine the effect of lipid digestion of the SNEDDSs and its impact on cinnarizine solubilization (Fig. 2). Long chain lipid

Table IV Individual and Mean Pharmacokinetic Parameters Following Oral Administration of 50 mg Cinnarizine in 1 g, 2 g or 4 g SNEDDSs or from Aqueous Suspensions to Fasted Labrador Dogs with Elevated Gastric pH in a Cross Over Study Design

	Dog 1	Dog 2	Dog 3	Dog 4	Mean ± SE
AUC 0–48 (ng/ml*h^{-1})					
$SNEDDS_{HIGH}$ (50 mg/g)	279	544	206	393	355 ± 74
$SNEDDS_{MEDIUM}$ (25 mg/g)	448	537	338	615	485 ± 60
$SNEDDS_{LOW}$ (12.5 mg/g)	391	526	278	374	392 ± 52
SUSP I (5 mg/ml)	0	527	0	593	NC
SUSP II (25 mg/ml)	0	37	0	245	NC
C_{max} (ng/ml)					
$SNEDDS_{HIGH}$ (50 mg/g)	81	120	75	96	93 ± 10
$SNEDDS_{MEDIUM}$ (25 mg/g)	127	127	97	110	115 ± 7
$SNEDDS_{LOW}$ (12.5 mg/g)	88	110	69	83	88 ± 9
SUSP I (5 mg/ml)	0	112	0	108	NC
SUSP II (25 mg/ml)	0	12	0	50	NC
T_{max} (h)					
$SNEDDS_{HIGH}$ (50 mg/g)	2	2	1.5	1.5	1.8 ± 0.14
$SNEDDS_{MEDIUM}$ (25 mg/g)	2	4	1	2	2.3 ± 0.63
$SNEDDS_{LOW}$ (12.5 mg/g)	4	3	1	4	3.0 ± 0.71
SUSP I (5 mg/ml)	0	3	0	4	NC
SUSP II (25 mg/ml)	0	4	0	2	NC

NC not calculated

digestion products are released from the SNEDDSs during digestion and as shown in SIM this has a pronounced effect on the solubility of cinnarizine. For the used SNEDDSs it is mainly the sesame oil that is available as a substrate for

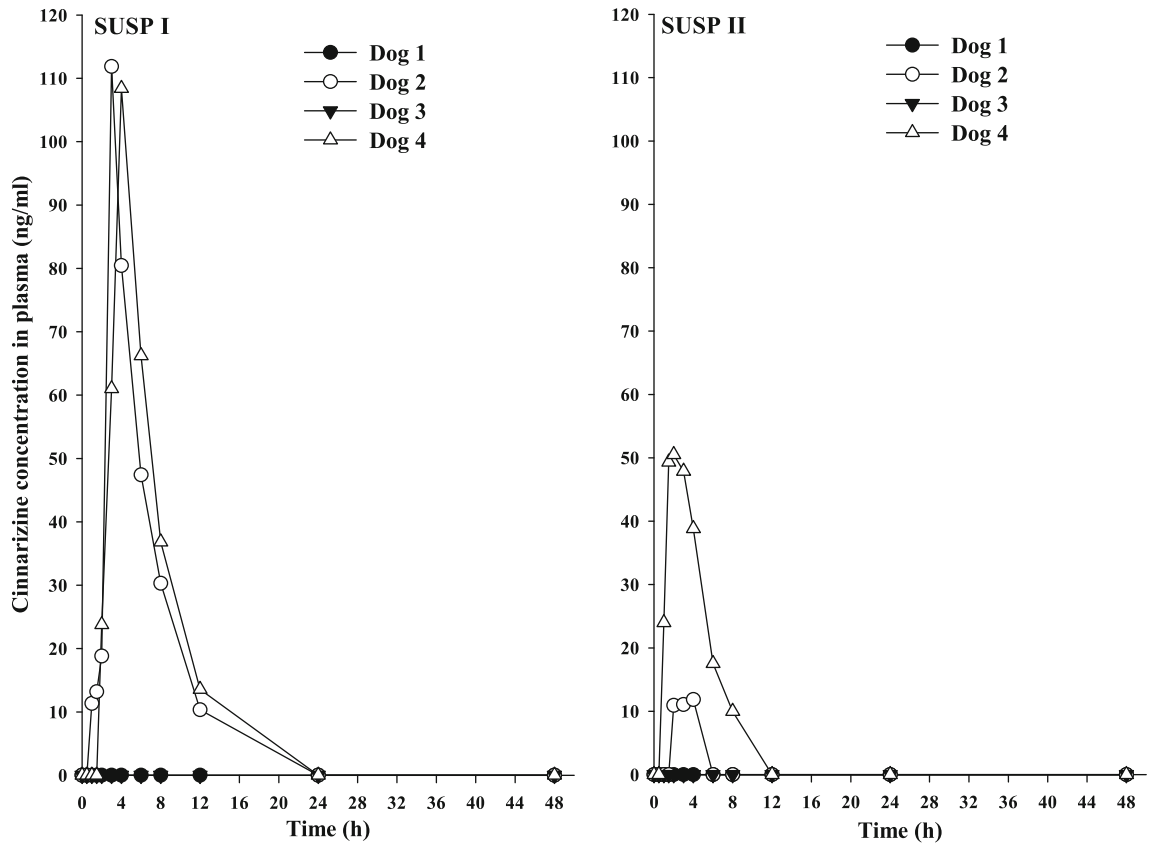

Fig. 6 Plasma concentration-time profiles for cinnarizine following oral administration of 50 mg cinnarizine from SUSP I (5 mg/ml) or SUSP II (25 mg/ml) to fasted male Labrador dogs with elevated gastric pH. Each profile comes from one dog and the same dogs were used for both suspensions.

lipases and to a minor extent Cremophor RH40, meaning that the surfactants will not lose their solubilization capacity during digestion (4).

The solubility of cinnarizine in the SNEDDS vehicle is 58.8 mg/g (4) and the cinnarizine loading in the vehicle is 85.0%, 42.5% and 21.3% of saturation solubility for $SNEDDS_{HIGH}$, $SNEDDS_{MEDIUM}$ and $SNEDDS_{LOW}$, respectively. The ethanol content contributes to the solubility of cinnarizine in the SNEDDSs, but when formulations are dispersed, the ethanol is dissolved in the dispersion medium and loses its solubility enhancing effect. Already at a minor degree of lipolysis of $SNEDDS_{HIGH}$ the SNEDDS itself is losing its capacity to keep the cinnarizine dose dissolved. The release of lipid digestion products during lipolysis was not sufficient to compensate for the loss of solubilization capacity of the SNEDDS vehicle and as a consequence cinnarizine started to precipitate right after lipolysis was initiated. Therefore, in $SNEDDS_{HIGH}$ the amount of surfactant in the formulation is substantially lower than what is needed to solubilize cinnarizine upon dispersion in the lipolysis medium. For $SNEDDS_{MEDIUM}$ precipitation of cinnarizine did not occur until after 60 min of digestion, whereas for $SNEDDS_{LOW}$ precipitation was not observed, probably due to sufficient solubilization capacity of the surfactant present. However, solubilization is facilitated by the lipolysis mediated release of long chain fatty acids that get incorporated into bile salt and phosphatidylcholine mixed micelles and thereby increase cinnarizine solubility in the digestion medium. The ranking of the precipitation observed ($SNEDDS_{HIGH} > SNEDDS_{MEDIUM} > SNEDDS_{LOW}$) is explained by the following; when higher amounts of vehicle are administered together with the same dose of cinnarizine, more of the SNEDDS formulation has to be digested before the SNEDDS itself will lose its solubilization capacity and more vehicle present results in a higher release of lipid digestion products, which aid in increasing the solubilization of cinnarizine as a function of lipolysis.

To the best of the authors' knowledge there are no publications examining the affinity of cinnarizine towards P-glycoprotein. Therefore, a bi-directional transport study in the intestinal epithelial cell line Caco-2 was conducted. The transport of cinnarizine in the Caco-2 cell model was determined and cinnarizine did not appear to be a substrate for P-glycoprotein or other efflux transporters expressed by the Caco-2 cells as no difference between apical to basolateral and basolateral to apical permeability was found. Furthermore, incubation with the known P-glycoprotein inhibitor verapamil did not affect the permeability of cinnarizine in any direction. The observed apical to basolateral permeability of 4.2×10^{-6} cm/s indicates that cinnarizine readily permeates across epithelial cells. A permeability in this range translates into a predicted complete absorption in humans (19). Thus, supporting classification of cinnarizine as a Biopharmaceutical Classification System Class II compound (20,21). The mannitol permeability data and TER values before and after cinnarizine transport indicate that the barrier function of the Caco-2 cells was not compromised during the cinnarizine transport experiments.

In Vivo Study

In a previous study, the bioavailability of cinnarizine from $SNEDDS_{MEDIUM}$ was determined and a bioavailability comparable to the present study was obtained; 515 ± 133 and 485 ± 60 ng/ml*h^{-1}, respectively. In the previous study, $SNEDDS_{MEDIUM}$ was compared with three other SNEDDSs having slightly different compositions and loaded with cinnarizine at 88.3%, 68.7% and 37.1% of the saturation solubility (5). The bioavailability of cinnarizine from the SNEDDSs with the highest loading was increased compared to $SNEDDS_{MEDIUM}$, indicating that the closer to the dissolving capacity the loading is, the higher the bioavailability (5). However, other factors, like SNEDDS composition, dispersion rate and nanoemulsion droplet size, also varied between the SNEDDSs tested in the previous study and therefore no final conclusion of the determining factors for bioavailability could be made.

When comparing different amounts of SNEDDS dosed as in the present study, the loading factor investigated can be biased by an excipient dose dependent efflux transporter interaction. Some excipients used in lipid based drug delivery systems have been shown to inhibit the efflux transporter P-glycoprotein (22,23). Studying the effect of loading using a drug which is a P-glycoprotein substrate may be confounded by differences in P-glycoprotein inhibition by the different amounts of dosed vehicle. However, knowing that cinnarizine is not a P-glycoprotein substrate the different *in vivo* performances between SNEDDS formulations with varying compositions and varying levels of vehicle containing cinnarizine cannot be ascribed to excipient P-glycoprotein inhibition.

In the present study, oral administration of the same dose of cinnarizine in varying amounts of SNEDDS vehicle and thereby different loadings resulted in the same bioavailability of cinnarizine. This finding is in agreement with previous studies where two different levels of loading have been tested for SNEDDSs containing the poorly soluble drug compound halofantrine. In one study, 3 mg of halofantrine was dosed to rats in SNEDDS with halofantrine loading of either 56.7% or 5.7% resulting in a tenfold increase in the amount of vehicle and this did not impact the bioavailability of halofantrine (9). In another study a twofold increase in SNEDDS loading did not result in significant differences in the bioavailability of halofantrine from medium or long chain lipid SNEDDS in dogs (10). In a dog study the poorly soluble simvastatin was dosed from long chain lipid SNEDDS loaded with 75% or 150% (supersaturated SNEDDS) where the amount of vehicle was 1.6 and 0.8 g respectively. The 150% loaded SNEDDS

resulted in a significantly increased bioavailability of simvastatin compared with the 75% loaded SNEDDS (11). Based on the present findings and recently published data about drug compound loading in SNEDDS vehicles, there are no indications towards high loadings decreasing the bioavailability due to too low amounts of vehicle.

As a reference, the bioavailability of cinnarizine from aqueous suspensions was determined. The administration of the aqueous suspension was repeated because on the first occasion two out of the four dogs, plasma levels were below the limit of quantitation. This was not expected and furthermore the two other dogs had bioavailabilities in the same range as was observed from the SNEDDS formulations. The two dogs (2,4) with the high exposure of cinnarizine also seemed to have higher AUC values after administration of the SNEDDSs compared with the two other dogs (1,3). Surprisingly, for the repeated experiment, the same outcome was obtained. The same two dogs had no exposure of cinnarizine above the limit of quantitation in plasma (1,3). SUSP II seemed to have lower exposure in the two dogs compared to SUSP I, however this can be ascribed to the larger particle size of SUSP II compared to SUSP I. Furthermore, SUSP I was dispersed in a larger amount of aqueous medium (10 ml) compared with SUSP II (2 ml).

In order to explain the repeatably different exposure in the dogs, it was speculated that it could be related to the metabolism of cinnarizine, as cinnarizine is a known CYP2D6 substrate (24,25). Among 10 kinds of CYP (Cytochrome P450) enzymes examined, only CYP2D6 catalyses the p-hydroxylation of the cinnamyl phenyl ring of cinnarizine, and only CYP2B6 exhibits activity for p-hydroxylation of the diphenylmethyl group of cinnarizine (24). Polymorphism in the CYP2D6 enzyme can result in poor, intermediate, efficient or ultrarapid metabolisers of CYP2D6 metabolised drugs (26). As an example, significant pharmacogenetic variation in the gene encoding of CYP2D15 resulted in variation in celecoxib metabolism (CYP2D15 substrate) in purebred Beagle dogs leading to elimination half-lifes of 1.5–2 h and 5 h for extensive and poor metabolisers, respectively (27). CYP2D15 and CYP2B11 are the CYP enzymes in dogs equivalent to the human CYP2D6 and CYPB6, respectively (28,29). The dogs included in the study were screened for CYP2D15 and CYP2B11 polymorphisms in order to assess whether genetic polymorphism of the metabolizing enzymes (CYP) could be the underlying reason for the dramatic differences in exposure observed between the dogs. However, there are only three single nucleotide polymorphisms that can lead to amino acid exchange (data in Supplementary Material). Dog 1, 2 and 3 had the same genetic polymorphism, one amino acid changing Single Nucleotide Polymorphism (SNP) in CYP2B11 and dog 4 was the only dog with an amino acid changing SNP in the CYP2D15 gene. The observed polymorphism patterns could therefore not explain the differences in exposure of cinnarizine administered from the aqueous suspension.

A highly variable bioavailability has been observed for cinnarizine in dogs in a previous study. The bioavailability of cinnarizine from a capsule containing powder and from an aqueous suspension formulation was $0.8 \pm 0.4\%$ and $8 \pm 4\%$ in dogs ($n=4$, mean $\pm$ SEM), respectively (30). The high variation between the individual dogs obtained after administration of the suspensions is most likely due to the normal variation that exists between individuals. Varying amounts of liquid available for dissolution, variation in intestinal transit time and variation in bile salt levels are just a few examples of the physiological variations that may exist. In the present study we used dogs with controlled neutral gastric pH, but the actual gastric pH was not measured during the study as this can also add some noise to the obtained bioavailability data. As a consequence, it cannot be ruled out that some dogs had a lower gastric pH, which could result in faster dissolution of cinnarizine from the suspensions. The SNEDDS formulation principle was able to markedly reduce the variation observed between individuals; exposure of cinnarizine well above the limit of quantification in plasma was obtained in all individuals, which was in strong contrast to the obtained result with the suspension formulations.

In Vitro In Vivo Relation

In vitro characterization using the lipolysis model does not take into account that prior to entering the duodenum and small intestine the formulations are exposed to the environment in the stomach. This includes exposure to a low pH environment and gastric lipase. It is known from a previous study (5) that cinnarizine distributes out of the nanoemulsion droplets at low pH. The fate of the cinnarizine/nanoemulsion upon transfer from a low gastric pH to a higher pH in the intestine is not known; cinnarizine may precipitate or redistribute into the nanoemulsion droplets. In the present study the pH shift was avoided by induction of neutral gastric pH administering esomeprazole to the dogs. Using this approach should improve the likelihood of the *in vitro* lipolysis model to predict the obtained *in vivo* data.

Three different cinnarizine loading levels in SNEDDS did not result in any significant differences in oral bioavailability, despite the fact that substantial amounts of cinnarizine precipitated during *in vitro* lipolysis of the SNEDDS with the highest loading. This is in accordance with our previous findings where SNEDDSs with substantial precipitation of cinnarizine during *in vitro* digestion could not be related to lower bioavailability, in fact a higher bioavailability was observed from the formulations with substantial precipitation (5). In contrast, studies using other drug compounds e.g. danazol and anethol trithione, found that a better *in vivo* performance of poorly soluble drug compounds from SNEDDS was related to higher amounts of drug compound in the aqueous phase during *in vitro* lipolysis (31,32). However, in these studies the

comparisons were made between SNEDDSs containing short, medium and long chain triglyceride. It is well known that significant differences in physiological response to medium and long chain lipids exist. Kossena *et al*. have shown that a 2 g dose of long chain lipid results in stimulated gall bladder contraction and increased levels of intestinal bile, phospholipid and cholesterol in man, whereas dosing the same amount of medium chain lipid only results in minor changes (33). In agreement with the present study, similar halofantrine bioavailability was obtained from long chain lipid containing SNEDDSs at 75% and 150% of saturation solubility, where one formulation had a higher drug content in the aqueous phase (10). In addition, SNEDDS loaded with 150% simvastatin resulted in a higher bioavailability compared with the same SNEDDS loaded with 75% and the latter had the highest simvastatin content in the aqueous lipolysis phase (11). In the cases involving halofantrine, simvastatin and cinnarizine, the drug substances have been proven to form an amorphous precipitate instead of a crystalline during *in vitro* lipolysis studies (10,11,34).

CONCLUSION

Large variations in cinnarizine bioavailability between dogs were obtained upon dosing aqueous suspensions; two dogs out of four did not have any measureable cinnarizine exposure. This was not caused by CYP polymorphism in CYP2B11 and CYP2D15 between the dogs, but could possibly be due to physiological variation between the dogs. Utilization of the SNEDDS concept reduced the variation between individuals as compared with the aqueous suspensions as all dogs had measureable cinnarizine exposure when dosed from SNEDDS. The cinnarizine loading level in SNEDDS, and thereby the amount of SNEDDS vehicle dosed, did not affect the bioavailability. However, decreasing the amount of vehicle leads to a significant increase in cinnarizine precipitation during *in vitro* dynamic lipolysis of the SNEDDS.

The present findings suggest that optimizing for a higher solubility of the drug compound than needed for dissolving the dose in the SNEDDS preconcentrate may be unnecessary in some cases. Furthermore, based on the obtained results, optimizing SNEDDSs towards keeping the drug compound solubilized during *in vitro* lipolysis may not be as important as previously suggested, at least not in cases where the drug precipitates in an amorphous state.

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