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Research paper

Enhanced bioavailability of silymarin by self-microemulsifying drug delivery system

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Abstract

The main purpose of this study was to prepare lipid-based self-microemulsifying drug delivery system (SMEDDS) to improve peroral bioavailability of silymarin. SMEDDS was a system consisting of silymarin, Tween 80, ethyl alcohol, and ethyl linoleate. Particle size change of the microemulsion was evaluated upon dilution with aqueous media and loading with incremental amount of silymarin. In vitro release was investigated by a dialysis or an ultrafiltration method. Results showed that release of silymarin from SMEDDS was limited, incomplete, and typical of sustained characteristics. Pharmacokinetics and bioavailability of silymarin suspension, solution, and SMEDDS were evaluated and compared in rabbits. Plasma silybin, which was treated as the representing component of silymarin, was determined by high-performance liquid chromatography. After gavage administration of silymarin suspension, plasma silybin level was very low and fell below limit of detection 4 h after. As for silymarin solution and SMEDDS, double peak of maximum concentrations were observed, which was characteristic of enterohepatic circulation. Relative bioavailability of SMEDDS was dramatically enhanced in an average of 1.88- and 48.82-fold that of silymarin PEG 400 solution and suspension, respectively. It was concluded that bioavailability of silymarin was enhanced greatly by SMEDDS. Alternative mechanisms, such as improved lymphatic transport pathway, other than improved release may contribute to enhancement of bioavailability of silymarin.

Keywords: Self-microemulsifying drug delivery system; Silymarin; Silybin; Bioavailability; Pharmacokinetics; In vitro release

1. Introduction

Silymarin, isolated from *Silybum marianum* L. Gaertn (milk thistle), is a mixture of mainly three flavonolignans, i.e., silybin, silydianin, and silychristin, among which silybin is the most active component [1]. Historically, milk thistle was used medicinally to treat disorders of the liver, spleen, and gallbladder. Well known as a hepatoprotector, silymarin was found effective clinically to treat a variety of liver disorders, including acute and chronic viral hepatitis, toxin- and drug-induced hepatitis and cirrhosis, and alcoholic liver disease [1–4]. It was also found effective in treating certain cancers, such as breast, prostate, and skin cancers [5–7]. Mechanisms of silymarin action include: stabilization of hepatocytes by inhibition of hepatotoxin binding to receptor sites on hepatocyte membranes; reduction of glutathione oxidation to increase glutathione levels in the liver and intestines; antioxidant activity; stimulation of ribosomal RNA polymerase and subsequent protein synthesis, leading to enhanced hepatocyte regeneration [1–3].

However, the effectiveness of silymarin as liver disease remedy was discounted by its poor water solubility and low bioavailability after oral administration [1]. Orally administered silymarin is absorbed rapidly with a t_{max} of about 2–4 h and a $t_{1/2}$ of 6 h [1]. Totally only 20–50% of oral silymarin is absorbed from the gastrointestinal tract after oral administration and undergoes extensive enterohepatic circulation. Eighty percent is excreted via bile as glucuronide and sulfate conjugates [8], and only 3–8% is excreted in the urine. Bile-associated silybin concentration

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is 60 times higher than that found in the serum [9]. In order to improve the dissolution and bioavailabilty of silymarin or silybin, several approaches have been employed, such as forming silybin–phosphatidylcholine complex [10,11] and incorporating into solid dispersion [12]. One of the silymarin preparations, bearing a brand name Legalon®, showed two- to three-fold increase in bioavailability, compared with other preparations [13,14].

Recently, self-emulsifying and self-microemulsifying drug delivery system (SMEDDS) [15–20] has drawn has great attention in pharmaceutical industry, benefiting from the success of cyclosporine formulations-Sandimmune® and Neoral®. SMEDDS are mixtures of drugs (usually water insoluble ones), lipids, surfactants, and cosurfactants, which form fine oil-in-water (O/W) microemulsion with a particle size of less than 100 nm when exposed to aqueous media under conditions of gentle agitation or digestive motility that would be encountered in the gastrointestinal (GI) tract. The spontaneous formation of microemulsion advantageously presents the drug in a dissolved form, and the resultant small droplet size provides a large interfacial surface area for drug release and absorption. In addition, the specific components of SMEDDS promote the intestinal lymphatic transport of drugs. Main mechanisms include increasing membrane fluidity to facilitate transcellular absorption, opening tight junction to allow paracellular transport, inhibiting P-gp and/or CYP450 to increase intracellular concentration and residence time by surfactants, and stimulating lipoprotein/chylomicron production by lipid [16,21–23]. Oral absorption of several drugs has been enhanced by SMEDDS employing single or combined mechanism [24–28].

In the previous study [29], we have studied the phase behavior of silymarin SMEDDS, through which optimal SMEDDS formulations composed of simply silymarin/ethyl linoleate/Tween 80/ethyl alcohol were selected. In this study, bioavailability of orally administered silymarin SMEDDS was evaluated in rabbits, and implication of the effect of microemulsion particle size and release characteristics on absorption was discussed.

2. Materials and methods

2.1. Materials

Silymarin was purchased from Panjinhuacheng Pharmaceuticals (Liaoning, China) with a purity of 80.6% as total flavonolignans determined by HPLC (silybin 40.93%, isosilybin 11.65%, silychrystin 17.08%, and silydianin 4.51%). Silybin standard was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with a purity of 99.8%. Tween 80 was obtained from Dazhong Pharmaceuticals (Shanghai, China). Ethyl linoleate was supplied by Aoqi Biopharmaceuticals (Wuxi, China). Deionized water was prepared by a Milli-Q purification system (Millipore, USA). HPLC-grade methanol was supplied by Merck (Darmstadt, Germany). All other chemicals used in the study were of analytical grade.

2.2. Preparation of silymarin SMEDDS

SMEDDS formulations should keep clear when enough drug was added and when exposed to aqueous phase. In a previous work [29], we studied the solubility of silymarin in a variety of oils, surfactants, and short-chain alcohols, and best solubilization and self-microemulsifying effect were found for ethyl linoleate/Tween 80/ethyl alcohol combinations. Through pseudo-ternary phase diagram investigation, optimal formulation (Table 1) was selected incorporating different amount of silymarin for in vitro and in vivo evaluation.

Preparation of silymarin SMEDDS was simply through mixing the components. Since silymarin was difficult to dissolve, it was better to dissolve silymarin first by ethyl alcohol. Then ethyl linoleate and Tween 80 were added slowly with gentle stirring until homogeneous mixture formed. The mixture was sealed in glass vial and stored under ambient temperature.

2.3. Determination of particle size

Silymarin SMEDDS was diluted with 0.1 M hydrochloride solution, or otherwise specified, to a definite volume in a flask. The flask was inverted and shaken gently to mix thoroughly. The particle size of so-formed microemulsion was determined by Nicomp™ 380 ZLS laser diffraction sizer (PSS Nicomp, Santa Barbara, CA, USA). The measurement conditions were: He–Ne laser; angle, 90°; temperature, 23 °C; reflection index, 1.333; wavelength, 632.8 nm; or with adjustment if needed.

2.4. In vitro release

First, in vitro release of silymarin SMEDDS was tested using a dialysis method [25] in 900 ml of 0.1 M hydrochloride solution containing 0.5% of Tween 80, based on ChP release test method II. The temperature was set to 37 °C and paddle revolution speed to 100 r/min. Silymarin SMEDDS was directly instilled with or without dilution using release medium into dialysis bag (MWCO 10,000, Spectrum Medical Industries Inc., USA). At definite time intervals, 3 ml of release sample was withdrawn and concentrations of silybin were analyzed by Agilent 1100 series

Table 1
Relative composition of the optimal silymarin SMEDDS formulation

Drug/excipient	Composition
Silymarin	55.5[a]
Ethyl alcohol	59.4
Tween 80	356.4
Ethyl linoleate	178.2

[a] Silybin equivalent: 22.7.

HPLC system (Agilent, USA). Release percentages were calculated as the ratio of silybin released/total silybin.

In order to obviate the effect of dialysis bag on release, an ultrafiltration method was also studied under similar experimental conditions. Silymarin SMEDDS was fitted into hard gelatin capsules before in vitro release test. At definite time intervals, 3 ml of release medium was withdrawn and centrifuged at 5000*g* for 10 min in an Amicon Ultra-4 ultrafiltration tube (MWCO 10,000, Millipore, USA). Concentration of silybin in filtrate was analyzed by Agilent 1100 series HPLC system.

2.5. Determination of silybin in rabbit plasma by RP-HPLC

As silybin is the sole component of silymarin, pharmacokinetic and bioavailability study of silymarin is always based on the determination of silybin in plasma [30,31]. In this study, a modified HPLC/UV method was employed to determine silybin in rabbit plasma.

The Agilent 1100 series HPLC system (Agilent, USA) was composed of a quaternary pump, a degasser, an autosampler, a column heater, and a tunable ultraviolet detector. Silybin isomers were separated by C18 column (Diamonsil, 5 μm, 4.6 mm × 250 mm, Dikma, China) guarded with a refillable precolumn (C18, 2.0 mm × 20 mm, Alltech, USA) and detected at 288 nm. The mobile phase was composed of methanol and 0.05 M KH_2PO_4 (adjusted to, pH 2, with 10% phosphoric acid) in a volume ratio of 49/51. The mobile phase was pumped at a flow rate of 1.0 ml/min. The column temperature was set to 40 °C.

Liquid–liquid plasma extraction procedure was as follows: in a 15 ml polypropylene screw-capped conical tube was added 1 ml of plasma followed by 25 μl of an internal standard (1-naphthol) solution and 0.5 ml of 0.1 M Na_2HPO_4. After vortex mixing for 30 s, 5 ml of ether anhydrous was added and vortexed for 15 min. After centrifuging at 3000*g* for 5 min, the organic layer was transferred to another tube and evaporated under a light stream of nitrogen at 40 °C. The residue was dissolved by 125 μl of mobile phase and 100 μl was injected for HPLC analysis. Quantification was based on area ratio ($A_{\text{silybin}}/A_{\text{IS}}$) and the area of silybin isomers was calculated as a whole.

Silybin isomers were separated well from impurities in plasma extracts, with retention times of 18.7 and 20.7 min, respectively. In the concentration range of 0.014–1.4 μg/ml, peak area ratio (R) of silybin isomers as a whole to internal standard correlated well to spiked plasma concentration: $R = 5.51229C - 0.01499$, ($r = 0.9999$, $n = 6$). Limit of quantification and limit of detection (S/N > 3) were 0.014 and 0.007 μg/ml, respectively. At concentrations of 0.07, 0.35, and 0.7 μg/ml, spiked recoveries of silybin from rabbit plasma were 98.65%, 101.0%, and 103.9%; intra-day precision was 3.41%, 3.02%, and 3.12%; inter-day precision was 3.63%, 4.50%, and 3.74%. After storage for 1 month at −18 °C and freeze–thawing for three times, silybin was stable in plasma.

2.6. Bioavailability studies

Bioavailability of silymarin SMEDDS was compared with silymarin suspension and solution. Silymarin suspension was prepared by milling silymarin powder with a small amount of 2.5% (v/v) hydroxymethylcellulose (15 cP, Dow Chemicals, USA) solution and diluted to a definite volume using the same vehicle afterwards. Since silymarin was practically insoluble in water, its solution was prepared by dissolving silymarin in polyethylene glycol (PEG) 400/dimethylformamide (20/1, v/v) mixture solvent.

Male rabbits weighing (2.5 ± 0.3) kg were fasted for 12 h prior to the experiment and water was available ad lib. After gavage administration of a dose of silymarin (300 mg/kg, expressed as silybin equivalents), about 2 ml of blood sample was collected through peripheral ear vein into heparinized tubes at 0, 0.33, 0.67, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 16 h. Blood samples were centrifuged at 5000*g* for 10 min using a high-speed centrifuging machine (TGL-16, Shanghai, China) and plasma samples were withdrawn and stored at −18 °C.

Since preliminary evaluation of silymarin suspension in six rabbits resulted in very low levels of plasma silybin, silymarin solution was treated as a reference when studying silymarin SMEDDS bioavailability. Another six rabbits were allocated at random to two treatment groups and administered SMEDDS and silymarin PEG 400 solution in a crossover design. The washout period between the two treatments was 7 days.

3. Results and discussion

3.1. SMEDDS formulation

In a previous pseudo-ternary phase diagram study [29], systems consisting of ethyl acetate, ethyl linoleate, and ethyl oleate as oil phase, Tween 80 as emulsifier (E), and low-chain alcohol or PEG 400 as co-emulsifier (CoE) were titrated with water, and self-emulsifying formulations were selected observing regions of infinite dilution. In the diagrams consisting of ethyl acetate as oil phase, the area of self-microemulsifying region was much bigger than that of ethyl linoleate and ethyl oleate. With the decreasing of emulsifier/co-emulsifier ratio (K_m), the area decreased slightly. Self-microemulsifying formulations could be obtained under the condition of K_m from 6/1 to 1/6, and oil/(E/CoE) equal to 1/9, 2/8, 3/7, and 4/6 even with those co-emulsifiers of high viscosity such as PEG 400, 1,2-propanediol, and propanetriol. Compared with other co-surfactants, the biggest area of self-microemulsifying region appeared in the diagrams with ethanol anhydrous as co-emulsifier. Ethyl linoleate was introduced to the system as the lipid for its relatively good self-microemulsifying efficiency and its potential as promoter of lymphatic transport. The optimal formulation of silymarin SMEDDS was selected regarding self-microemulsifying ability, solubilization ability, and reduced use of emulsifiers, as described in Table 1.

3.2. Particle size analysis

Particle size after microemulsification was the most important property of SMEDDS. Mechanisms of particle size effect on drug absorption may include improved release and facilitated lymphatic transport [19,21,25,32]. So, we studied the effect of several variables on particle size. Typical size distribution is shown in Fig. 1.

The effect of drug loading on particle size in different media is presented in Fig. 2. For this evaluation, totally 1 g of blank SMEDDS was added in 20 mg increments of silymarin and diluted to 100 ml with aqueous media to perform self-microemulsifying activity before particle size measurement. The mean size increased slightly with increased drug loading up to 100 mg. When drug loading further increased, particle size increased dramatically, even to as high as 100 nm or over, which was beyond the range of colloidal system and no more presented the properties of microemulsion.

In order to simulate in vivo dilution behavior of SMEDDS, effect of dilution volume by 0.1 M hydrochloride solution and other aqueous media on particle size was evaluated. It seemed that dilution volume within the investigated range had little effect on particle size (Fig. 3) and self-microemulsifying behavior.

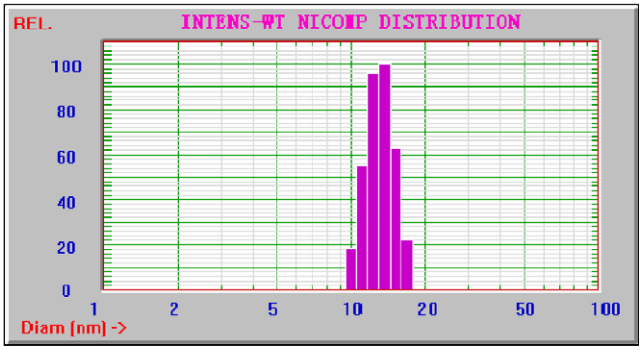

Fig. 1. Size distribution of silymarin self-microemulsifying drug delivery system determined by laser diffraction sizer after dilution to 100 ml with 0.1 mol/L hydrochloride solution.

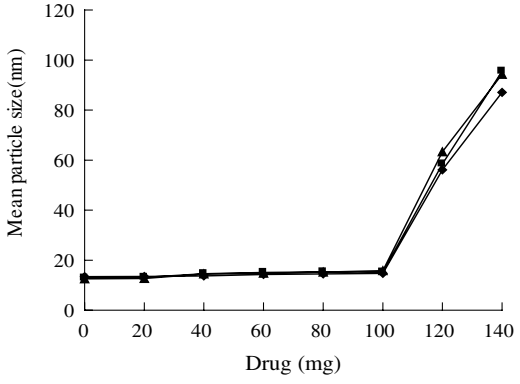

Fig. 2. Effect of drug loading on the particle size of self-microemulsifying drug delivery system in different media. -♦-, distilled water; -■-, phosphate-buffered saline (pH 6.8); -▲-, 0.1 mol/L hydrochloride solution.

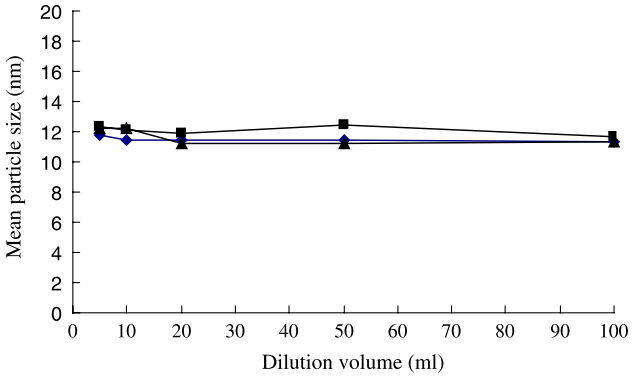

Fig. 3. The effect of dilution volume on particle size of silymarin self-microemulsifying drug delivery system. -♦-, distilled water; -■-, phosphate-buffered saline (pH 6.8); -▲-, 0.1 mol/L hydrochloride solution.

Self-microemulsifying behavior was also studied after SMEDDS had been sealed in glass vial and stored at −4, 25, and 37 °C for 90 days. Particle size measurement showed that no distinguishable difference was observed after storage (Fig. 4).

3.3. In vitro release study

Release of drugs from SMEDDS cannot be evaluated using a conventional release protocol, because dissolved and microemulsion-associated drugs must be separated before determination. For this purpose, methods must be developed to cut off microemulsion-associated drugs during sampling. Kang et al. [25] employed a dialysis bag with molecular weight cut-off of 10,000 to circumscribe escape of microemulsion into release medium. However, in our preliminary study, silymarin SMEDDS, sticky in nature, cannot be easily dispersed to form fine microemulsion droplets during release test process and only limited silymarin was released. So, we developed two modified methods for SMEDDS release evaluation: (1) dialysis method similar to that reported by Kang et al. [25]. But water was added to make silymarin SMEDDS self-microemulsify before

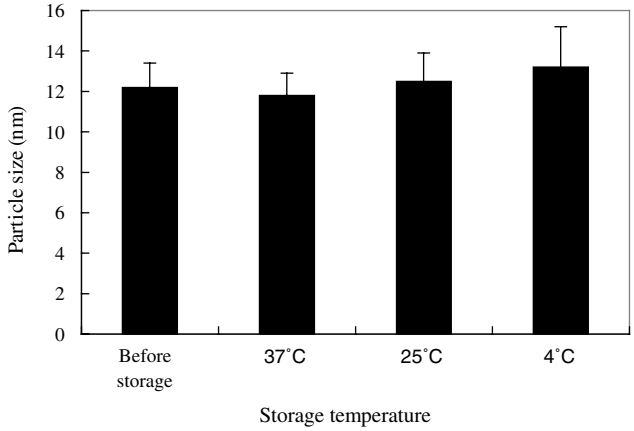

Fig. 4. Effect of storage temperature on particle size of silymarin self-microemulsifying drug delivery system.

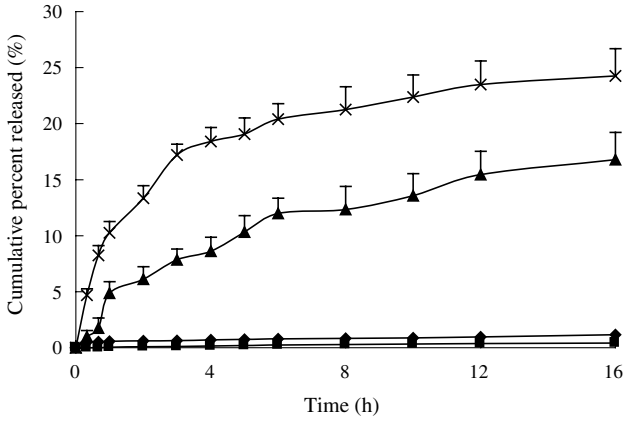

Fig. 5. Release profile of silymarin from several vehicles determined by a dialysis method (-♦-, SMEDDS without previous dilution; -■-, silymarin crude drug powder; -▲-, SMEDDS with previous dilution to 100 ml with 0.1 mol/L hydrochloride solution) and an ultrafiltration method (-×-, SMEDDS).

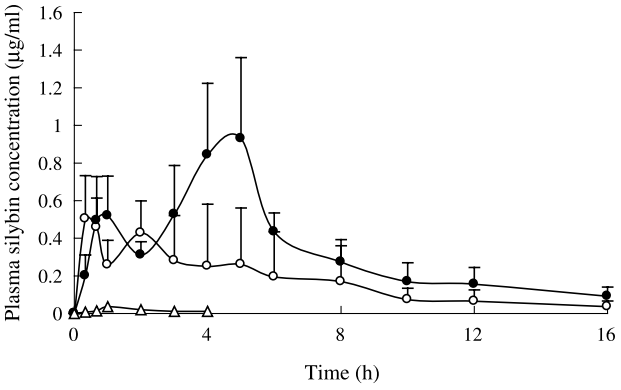

Fig. 6. Plasma silybin concentration–time plot after a single oral dose of silymarin in three formulations. -●-, SMEDDS; -○-, PEG 400 solution; -△-, suspension.

being instilled into dialysis bag; (2) ultrafiltration method. Release test is performed a conventional way, but ultrafiltrate release samples before assay.

Release of silymarin crude drug powder and SMEDDS was compared. Results indicated that release of silymarin crude drug powder was limited with not more than 0.5% released at 16 h. Undiluted SMEDDS in dialysis bag showed little release (smaller than 2%) after 16 h. It was important to dilute SMEDDS with water before being instilled into dialysis bag to guarantee correct measurement of release rate. Release profiles of SMEDDS determined by an ultrafiltration method or dialysis method with previous dilution displayed sustained characteristics, releasing about 25% and 15% of total dose (Fig. 5).

The results indicated that silymarin SMEDDS might be diluted previously with aqueous solutions before performing in vitro release test in a dialysis bag. Otherwise, SMEDDS will stick to the dialysis bag and circumscribe the inflow of release medium. After prior dilution with release medium, SMEDDS can be easily dispersed, and dissolved molecules can permeate out of the dialysis bag easily. Release of silymarin was greatly enhanced by SMEDDS and followed first-order kinetics, but far from completeness. Release profile was typical of sustained characteristics.

In vitro release determined by ultrafiltration also displayed sustained characteristics and first-order kinetics, while release rate was much higher than that determined by dialysis. Since an ultrafiltration method did not interfere with the release process, it served as a potential method for in vitro evaluation of SMEDDS release profiles.

3.4. Bioavailability study

Pharmacokinetic parameters of silymarin SMEDDS, solution, and suspension were compared in rabbits. Mean plasma silybin concentration was plotted as a function of time and shown in Fig. 6. After gavage administration of silymarin suspension, plasma level of silybin was very low, most of which (after 4 h) was under limit of detection, with AUC_{0-16h} of only 0.11 μg h/ml. As for silymarin solution and SMEDDS, double peak of maximum concentrations were obvious, characteristic of enterohepatic circulation [8].

Pharmacokinetic parameters of SMEDDS and solution of six rabbits are shown in Tables 2 and 3. Mean pharmacokinetic parameters for silymarin solution and SMEDDS were as follows – C_{max}: (1.01 ± 0.21) and (0.71 ± 0.18) μg/ml; t_{max}: (4.33 ± 0.82) and (2.39 ± 1.54) h; $AUC_{0-\infty}$: (6.23 ± 1.75) and (3.17 ± 1.63) μg h/ml; AUC_{0-16h}: (5.37 ± 1.45) and (2.85 ± 1.46) μg h/ml, respectively. Calculated on AUC_{0-16h}, the mean relative bioavailability of silymarin SMEDDS was 1.88- and 48.82-fold that of silymarin PEG 400 solution and suspension.

Relative bioavailability of silymarin solution and SMEDDS was dramatically enhanced compared to silymarin suspension. What was interesting was that the bioavailability of SMEDDS was about two times that of silymarin solution. It was rational to deduce that alternative mechanisms other than improved release may contribute to enhancement of bioavailability of silymarin. Moreover, release of silymarin SMEDDS was not big enough either.

High bioavailability of SMEDDS may attribute to its promotion of lymphatic transport through transcellular pathway [19]. It was reported that the long-chain oils promote lipoprotein synthesis and subsequent lymphatic absorption [33]. Yuan et al. [32] had studied in situ absorption of silymarin microemulsion, and good absorption was observed in the middle and distal parts of intestine. The authors proposed a mechanism of M cell-mediated transport in that part of the intestine that was rich in Peyer's patch.

The main rate-limiting barrier for drug absorption/diffusion is the single layer of intestinal epithelial cell. High content of surfactants in SMEDDS could increase the permeability by disturbing the cell membrane [22]. Surfactant monomers are capable of partitioning into the cell membrane where they can form polar defects in the lipid

Table 2
Pharmacokinetic parameters of silymarin self-microemulsifying delivery system

No.	t_{max} (h)	C_{max} (μg/ml)	$t_{1/2}$ (h)	AUC_{0-16h} (μg h/ml)	$AUC_{0-\infty}$ (μg h/ml)	MRT (h)
1	4	1.07	6.58	5.02	5.29	5.95
2	4	0.82	7.01	3.88	4.51	1.77
3	4	1.39	4.96	7.91	8.84	1.52
4	4	0.96	7.49	5.63	7.31	2.92
5	4	0.86	5.68	4.12	4.51	1.19
6	6	0.93	7.42	5.66	6.96	2.51
Mean ± SD	4.33 ± 0.82	1.01 ± 0.21	6.53 ± 1.02	5.37 ± 1.45	6.23 ± 1.75	2.64 ± 1.74

Table 3
Pharmacokinetic parameters of silymarin PEG 400 solution

No.	t_{max} (h)	C_{max} (μg/ml)	$t_{1/2}$ (h)	AUC_{0-16h} (μg h/ml)	$AUC_{0-\infty}$ (μg h/ml)	MRT (h)
1	2	0.47	5.91	1.42	1.76	1.24
2	0.33	0.96	4.55	5.03	5.56	1.07
3	2	0.60	4.30	2.15	2.24	0.55
4	5	0.82	7.81	4.34	4.90	1.78
5	2	0.61	6.58	1.97	2.32	1.62
6	3	0.75	5.87	2.19	2.33	0.80
Mean ± SD	2.39 ± 1.54	0.70 ± 0.18	5.81 ± 1.3	2.85 ± 1.46	3.17 ± 1.63	1.47 ± 0.88

bilayer. At high surfactant concentrations in the cell membrane, surfactant–surfactant contact occurs, and the membrane can be dissolved into surfactant–membrane mixed micelles [22]. It should be noted that the surfactant with best enhancement ability requires both hydrophilic and lipophilic domains reaching a balance with intermediate values of HLB such as Tween 80 used in our study; consist of a polyoxyethylene and intermediate hydrocarbon chain. Its structural characteristics impart both lipophilic and hydrophilic properties to the surfactant, allowing it to partition between lipid and protein domains [22]. Surfactant also demonstrated a reversible effect on the opening of tight junction; it may interact with the polar head groups of the lipid bilayers, modifying hydrogen bonding and ionic forces between these groups. It may also insert itself between the lipophilic tails of the bilayers, resulting in a disruption of the lipid-packing arrangement.

For drugs of low bioavailability, lymphatic pathway may play an important role in improving bioavailability by lipid-based delivery system, as reported [23] that very low levels, <1% dose, of lymphatic transport using CI-976, a lipophilic lipid regulator, could significantly increase the AUC. Although the in vitro release rate of silymarin SMEDDS was limited in our study, several factors, such as formation of microemulsion in nanometer range, high content of surfactant with proper HLB, and use of long-chain fatty acid, may contribute to increased bioavailability of silymarin after oral administration.

4. Conclusion

Drug loading up to 100 mg/1 g SMEDDS had little effect on particle size, while higher drug loading led to particle size increase dramatically. Dilution volume had no significant effect on particle size and self-microemulsifying behavior. In vitro release of silymarin from SMEDDS determined by a dialysis method and an ultrafiltration method showed first-order kinetics and typical of sustained characteristics. However, cumulative percent released was far from completeness. Relative bioavailability of silymarin SMEDDS was dramatically enhanced, approximately 1.88- and 48.82-fold that of silymarin PEG 400 solution and suspension, respectively. It was concluded that alternative mechanisms other than improved release contribute to enhancement of bioavailability of silymarin.

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