

Preparation of nobiletin in self-microemulsifying systems and its intestinal permeability in rats

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ABSTRACT - PURPOSE. The objective of this study was to prepare nobiletin self-microemulsifying drug delivery systems (SMEDDS) and investigate its intestinal transport behavior using the single-pass intestinal perfusion (SPIP) method in rat. **METHODS.** The characterizations of nobiletin SMEDDS were investigated. SPIP was performed in each isolated region of the small intestine (i.e. duodenum, jejunum, ileum and colon) over three concentrations of nobiletin (15, 30 and 60 $\mu\text{g/mL}$) at a flow rate of 0.2 ml/min. The concentrations of the samples were determined by HPLC and the effective permeability coefficients (P_{eff}) in rats were calculated. Considering the high correlation of rat P_{eff} values with those of human, the human intestinal permeability was predicted using the Lawrence compartment absorption and transit model. The intestinal permeability of nobiletin in SMEDDS, sub-microemulsions and micelles was compared. **RESULTS.** The particle size and zeta potential of nobiletin SMEDDS were (28.6 \pm 0.3) nm and (-22.6 \pm 3.5) mV, respectively. The P_{eff} in jejunum at 15 $\mu\text{g/mL}$ was significantly higher than that at 60 $\mu\text{g/mL}$ ($p < 0.01$). The P_{eff} in colon was higher at the same concentration comparing to the other intestinal segments. Moreover, there was no statistical difference in P_{eff} at each same concentration in jejunum, duodenum and ileum. The estimated human absorption of nobiletin for the SMEDDS dilutions was higher than that for sub-microemulsions ($p < 0.01$) and similar to that of the micelles ($p > 0.05$). **CONCLUSIONS.** Bases on the above results, the SMEDDS could enhance the intestinal permeability of the nobiletin, and may be presented as potential candidates for improving the oral absorption of the noblietin.

INTRODUCTION

In recent years, much attention has been focused on oral dosage forms using a self-microemulsifying drug delivery system (SMEDDS) for the purpose of improving the solubility and absorption of poorly water-soluble drugs. SMEDDS consists of a mixture of drugs, oils, surfactants and/or other additives. Gentle mixing of these ingredients in aqueous media generates microemulsions with a droplet size in a range of 10-100 nm. SMEDDS has been shown to improve absorption of drugs by rapid self-microemulsification in the stomach, with the microemulsion droplets subsequently dispersing in the gastrointestinal tract to reach sites of absorption [1]. The resultant small droplet size from SMEDDS provides a large interfacial surface area for drug release and absorption, and the specific components of SMEDDS promote the intestinal lymphatic transport of drugs [2]. Oral absorption of several drugs has been enhanced by SMEDDS [2-5]. Sandimmu Neoral[®] (CsA-NEO), a self-microemulsifying capsule of cyclosporine A, had been approved by FDA.

Nobiletin (5, 6, 7, 8, 3', 4'-hexamethoxy flavone), Figure 1, a citrus polymethoxy flavonoid, is a candidate of the anti-inflammatory drug [6, 7]. A number of studies have shown it to be effective in the treatment and precaution of stomach cancer [8-10]. It may also be effective as a sunscreen reagent for protection against photoinflammation and photoaging [11]. It is known that intestinal permeability is necessary for oral administration [12]. Because the nobiletin is a poor water-soluble drug, it has a very low absolute bioavailability (about 4%).

In this study, we developed an optimized formulation using SMEDDS to improve the solubility and oral absorption of nobiletin.

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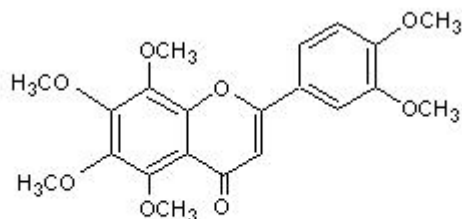


Figure 1. Structure of nobiletin (5, 6, 7, 8, 3', 4' – hexamethoxyflavone)

The 1:1 mixture of polyoxyethylene 35 castor oil and polysorbate 80 was used as the surfactant, polyethylene glycol 400 (PEG 400) as the co-surfactant and the 3:1 mixture of medium-chain triglyceride/tea oil as the oil phase in the formulations.

The intestinal permeability of the nobiletin was determined using the single pass intestinal perfusion (SPIP) in rats. The SPIP method provides the unique advantages of experimental control (e.g. compound concentration and intestinal perfusion rate), ability to study regional differences and an intact intestinal blood supply and a functional intestinal barrier [12-14].

MATERIALS AND METHODS

Materials

Nobiletin was obtained from Chinabest Drugs Research Ltd. (Nanjing, China). Polyoxyethylene 35 castor oil USP24/NF19 (PEG-35 castor oil, Cremophor EL 35) was purchased from BASF (Ludwigshafen, Germany). Medium-chain triglyceride (Caprylic/capric triglyceride, Cradamol GTCC) was a kind gift from CRODA (Singapore). Polysorbate 80 (Tween 80) was purchased from Jiangsu Chenpai Pharmaceutical Co., Ltd. (Haimen, China). Polyethylene glycol 400 (PEG 400) was purchased from Beijing Haidian Huiyou Refined Chemical Plant (Beijing, China). Other chemicals were of HPLC or analytic grade.

Animals

Sprague-Dawley male rats (180-220g) from Experimental Animal Center of China Pharmaceutical University were used in this paper. The protocol was approved by Local Animal Ethics Committee.

Preparation of nobiletin self-microemulsifying system

A mixture of the surfactants, co-surfactant and oils were prepared at a fixed ratio of 7:2:1 (g/g). Nobiletin was then dissolved in the resulting mixture by stirring magnetically at 40° C water bath. Before the SPIP experiment, sufficient Krebs-Ringer's buffer solution [15] was added to adjust the formulations to a designed concentration.

The intestinal permeability experiments of the SMEDDS solutions with different concentrations of the nobiletin were investigated and compared to both the micelle solution of equivalent surfactant and co-surfactant concentration and the sub-microemulsion of the 0.78:0.22:1 surfactants/co-surfactant/oils.

Particle size and zeta potential measurement

The formulation (0.5mL) of SMEDDS was diluted with double-distilled water to 10mL. Photo correlation spectrometer (Zetasizer 3000HS, Malvern Instruments Corp., U.K.) was employed to measure particle sizes and zeta potential of the SMEDDS dilutions, sub-microemulsions and micelles.

Binding and stability studies

Preliminary studies are necessary before commencing the SPIP studies to ensure that the loss of drug from perfusion is due to absorption and not by other losses (e.g. the binding of the intestinal wall or degradation).

Rat intestinal segments of approximately 10 cm size were clipped. The intestinal sac were turned over and put into 50 mL Krebs-Ringer's buffer solution, in which nobiletin (30µg/mL) was incubated at 37° C for 3 h. Samples (50µL) were

removed at 3 h, and were diluted with absolute methanol, and assayed by HPLC.

Nobiletin was also incubated in 10 mL perfused Krebs-Ringer's buffer for the intestinal segment at 37° C for 3 h. Samples (50µL) were removed at 60, 120 and 180 min and diluted with an appropriate volume of absolute methanol, and assayed by HPLC.

Single-pass perfusion of the rat intestinal segments

SPIP studies were performed according to previously described methods [12,16]. After an overnight fast, male SD rats weighing approximately 180-220 g (Central Animal Laboratory of China Pharmaceutical University, China) were anesthetized with an i.p. injection of 20% (w/v) urethane solution (urethane: 1g/kg). To maintain normal body temperature, rats were placed on a warming pad under a surgical lamp. The abdomen was opened with a midline incision, an interest intestinal segment of approximately 10 cm was chosen and cannulated with plastic tubing. Initially, the intestinal segment was rinsed with isotonic saline (37° C) until the outlet solution was clear. Using an infusion pump (FB-1, Kingdom Biochemical Instrument Co., Ltd., Shanghai, China), the intestinal segments were perfused at a flow rate of 0.2 ml/min with the bland Krebs-Ringer's buffer solution. The preparation time took approximately 30 min. Then the pump was connected to the reservoir containing the nobiletin-loaded SMEDDS dilutions with the marker (Phenol Red).

Setting $t=0$ when the buffer solution has been completely pushed out. Each perfusion experiment lasted for 180 min. and the perfusate was quantitatively collected in the following intervals: 0-60, 60-75, 75-90, 90-105, 105-120, 120-135, 135-150, 150-165 and 165-180 min. The steady state reached in 60 min.

Samples were stored in a refrigerator at 4° C until analysis by HPLC.

HPLC analysis of samples

All samples were diluted to 5 ml with absolute methanol and measured by HPLC. Aliquots of 20 µL were injected onto the HPLC system, equipped with a Shim-pack CLC-ODS column (5 µm, 150*4 mm, Kyoto, Japan). The HPLC system (LC-2010C,

SHIMADZU Corp., Kyoto, Japan) consisted of an auto-sampler and an ultraviolet detector. The detection wavelength was set at 332 nm. The mobile phase was a methanol-water (80:20, v/v) mixture with the flow rate of 1.0 ml/min.

Data Analysis

Water absorption or secretion (flux) was measured by methods: gravimetric, phenol red, and ¹⁴C PEG-3500 [17]. The net water flux (NWF) per cm of each segment was calculated using Equation (1) [18]:

$$\text{NWF } (\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-1}) = (1 - \text{CPR}_{\text{out}}/\text{CPR}_{\text{in}}) * \text{Q}_{\text{in}} / \text{L} * 1000 \quad (1)$$

Where CPR_{in} and CPR_{out} were the inlet and outlet concentration of phenol red, respectively. Q_{in} was the flow rate of the perfusion solution entering the intestinal segment, L was the length of the intestinal segment (cm).

Under most conditions, NWF is negative value, which indicates the intestinal lumen would absorb the water. Only when the high osmotic solution is administered, the intestinal lumen secretes the water and UWF is positive value [17, 19, 20].

The effective permeability, P_{eff} was calculated based on the inlet and outlet concentration of nobiletin (C_{in} and C_{out} , respectively). Steady state was considered to have been achieved when the concentration of phenol red and nobiletin were constant. The permeability values are calculated only from experiments where steady state was achieved.

The permeability was calculated using Equation (2) [12,17]:

$$P_{\text{eff}} = \frac{Q \ln(C_{\text{in}}/C_{\text{out}})'}{2\pi rL} \quad (2)$$

Where Q is the flow rate in ml/min, r the radius of the intestine (0.18 cm in rat and 1.75 cm in man), L is the length of the perfused intestinal segment (cm), and $(C_{\text{in}}/C_{\text{out}})'$ is the concentration ratio corrected for fluid flux.

The statistical significance of the differences among group means were assessed using the one-way unweighted means analysis of variance (ANOVA) with the least significant difference (LSD) test and a value of $p < 0.05$ was considered statistically significance.

RESULTS

SMEDDS formulations

SMEDDS formulations should be clear, monophasic liquid at ambient temperature, and should have suitable solvent properties to allow presentation of drug in solution [3]. Polyoxyethylene 35 castor oil and polysorbate 80 were used as the surfactants because of the higher solubility of nobiletin in polyoxyethylene 35 castor oil and polysorbate 80 observed. Caprylic/capric triglyceride (GTCC), medium-chain triglyceride, was selected as the oil phase for formulation development because it provided higher solubility than other oils and might influence the tight junctions of the epithelial cells [21]. Moreover, to obtain the more stable microemulsions with smaller particle size after self-microemulsification, the tea oil was selected as the other oil. The tea oil, the mixture consisting long chain fatty acids, stimulates the lipoprotein synthesis and subsequent lymphatic absorption [22]. According to the results of the pseudo-ternary phase diagram in the previous study, the 3:1 mixture of medium-chain triglyceride/tea oil as the oil phase was selected. PEG400 was introduced to the system as the co-surfactant for its higher solubilization ability of nobiletin and its low toxicity. The optimal formulation of SMEDDS was selected regarding solubilization ability, self-microemulsifying ability (including the size and stability of microemulsions after self-microemulsification) and reduced use of emulsifiers. The nobiletin SMEDDS were stable for at least 6 months at ambient temperature.

Particle sizes and zeta potential of three preparations

The particle sizes and zeta potential of the SMEDDS dilutions, sub-microemulsions and micelles are shown in Table 1. The

sub-microemulsions had a larger particle size and wider size distribution compared to the SMEDDS dilutions and micelles. According to the zeta potential values, three preparations had negative charge.

Validation of HPLC assay

The regression equation for the concentration of nobiletin (ng/mL) vs response in the perfusion fluid ranging from 80 ng/mL to 800 ng/mL was $A=95.88C+820.3$ ($r = 0.9999$). The mean recovery of nobiletin was $101.3 \pm 1.36\%$. The within-day and between-day RSD's were less than 3%.

Binding and stability studies

Nobiletin did not exhibit markedly binding to the intestinal wall. The remaining amount of nobiletin after incubated in the Krebs-Ringer's buffer with the intestinal sac for 3 h was $99.43 \pm 3.34\%$, which was not significantly decreased.

The stability of nobiletin was based on the decrease drug concentration as quantitated by HPLC. After 3 h incubation, at least 98.2% nobiletin remained in the perfused Krebs-Ringer's buffer for each intestinal segment. Neither of the potential metabolites was observed in the chromatograms.

Water absorption or secretion of intestinal lumens

The NWF of each segment is listed in Table 2. The NWF for all intestinal segments were negative value, which suggested that the water was mainly absorbed in the intestine tract. The NWF for the duodenum was significantly higher than that for the jejunum, ileum and colon ($P < 0.05$), but variance in NWF for the jejunum, ileum and colon were not significant ($P > 0.05$).

Table 1. Characterizations of the SMEDDS, sub-microemulsions and micelles (n=3).

Parameters	SMEDDS	Sub-microemulsions	Micelles
Particle size (nm)	28.6±0.3	184.9±3.5	10.1 ±1.5
Polydispersity index	0.068±0.026	0.547±0.010	0.086±0.021
Zeta potential (mV)	-22.6±3.5	-28.0±6.4	-20.7 ±4.2

Table 2. The net water flux (NWF) in each intestinal segment (n=4)

Time (min)	Duodenum	Jejunum	Ileum	Colon
75	-0.584±0.439	-0.144±0.082	-0.009±0.228	-0.146±0.127
90	-0.555±0.564	-0.117±0.121	-0.238±0.553	-0.197±0.103
105	-0.714±0.241	-0.341±0.199	-0.129±0.372	-0.237±0.037
120	-0.795±0.185	-0.209±0.454	-0.237±0.262	-0.426±0.176
135	-0.666±0.414	-0.206±0.119	-0.340±0.444	-0.298±0.110

SPIP studies

The intestinal permeability of nobiletin was studied as a function of concentration in each segment of the intestine. The upper boundary (60 µg/mL) of the concentration range was chosen because of the limited aqueous solubility of nobiletin.

Steady state is confirmed by plotting the concentration of nobiletin and phenol red versus time in Figure 2. Steady state, which was assessed by the constant concentrations of both phenol red and nobiletin, was reached about 60 min after the beginning of the perfusion. The permeability values are calculated only from experiments where steady state was achieved.

The permeability values of SMEDDS dilutions for each intestinal segment and concentrations are listed in Table 3. There appears to be a trend of decreasing permeability with increasing concentration. P_{eff} in the jejunum at 15 µg/mL

(0.445±0.026 cm/s) was significantly higher than P_{eff} at 60 µg/mL (0.377±0.057 cm/s, $p<0.01$). Moreover, there was no statistical difference in P_{eff} at three investigated concentrations in other segments (including duodenum, ileum and colon).

Comparisons across each segment revealed that the permeability at each concentration in colon was higher than all other segments ($p<0.01$). Moreover, the permeability values in duodenum, jejunum and ileum were not statistically different ($p>0.05$).

Figure 3 shows the permeability values of nobiletin in the formulation of SMEDDS dilutions, micelle and sub-microemulsion for each segment at 30 µg/mL. P_{eff} of SMEDDS dilutions and micelle in each segment was significantly higher than that of sub-microemulsions ($p<0.01$). Except the duodenum ($p<0.05$), P_{eff} of SMEDDS dilutions and micelle in the jejunum, ileum and colon did not all reach statistical difference ($p>0.05$)

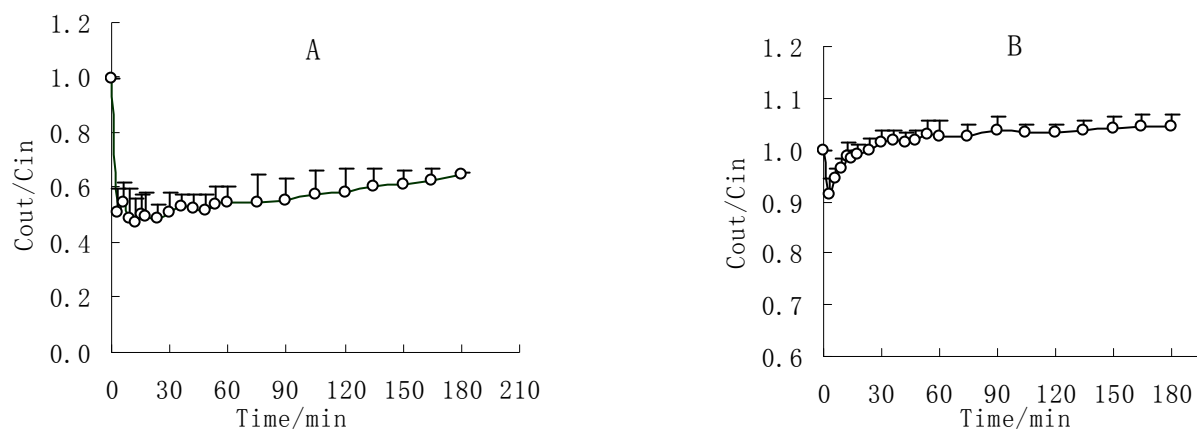
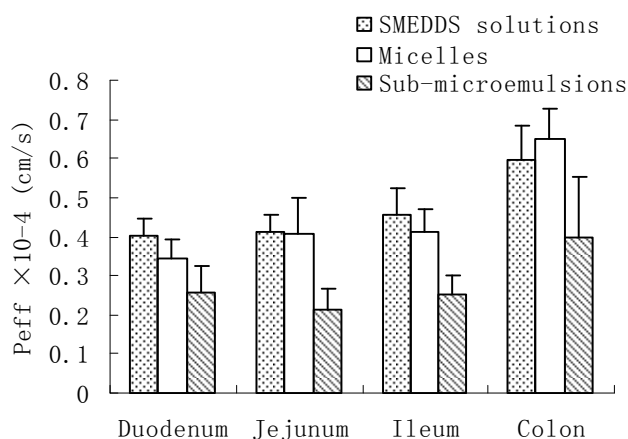


Figure 2. Representative plot of the concentration ratio of the outlet and inlet concentrations ($C_{\text{out}}/C_{\text{in}}$) vs. time. Phenol red in perfusion solution (B); Drug apparent absorption fraction curve (A).

Table 3. Comparison of effective permeation coefficients ($P_{\text{eff}} \times 10^{-4}$) of different intestinal segments with three concentrations. Results are given as mean \pm s.d. (n=4).

Conc. of nobiletin ($\mu\text{g}/\text{mL}$)	P_{eff} (cm/s)			
	Duodenum	Jejunum	Ileum	Colon
15	0.435 \pm 0.049	0.445 \pm 0.026	0.475 \pm 0.052	0.625 \pm 0.096
30	0.402 \pm 0.045	0.414 \pm 0.042	0.456 \pm 0.067	0.598 \pm 0.085
60	0.401 \pm 0.068	0.377 \pm 0.057	0.437 \pm 0.116	0.532 \pm 0.068

**Figure 3.** Comparison of effective permeation coefficient (P_{eff}) of the SMEDDS solutions, sub-microemulsions and micelles in different intestinal segments (n=4). The concentration of the nobiletin in the perfusion solutions was 30 $\mu\text{g}/\text{mL}$.

DISCUSSIONS

According to P_{eff} values in each intestinal segment (shown in Table 3), the P_{eff} at each concentration in the colon was significantly higher than those in other segments, which is consistent with previous studies [23-25]. As a multi-drug resistance-reversing agent [26], the intestinal absorption of nobiletin was limited due to the presence of the P-gp throughout the intestinal tract. There are various reports showing the regional difference in the expression and activity of P-gp throughout the gastric intestinal tract [27]. The order of the expression and activity of P-gp is ileum > duodenum and jejunum > proximal and distal colon [27]. The lower expression activity of P-gp conducted to be increasing the permeability of nobiletin in colon as compared to other sections (Table 3). Moreover, the SMEDDS dilutions, sub-microemulsion and micelle had native charge. It has been shown that there is a selectivity of permeation depending of the gut sections. The small intestine has more cationic selectivity, whereas, the

colon has more anionic selectivity [29]. Another possible mechanism for higher P_{eff} values in colon was that the higher crypt surface area in the colonic mucosa compared with the small intestine could account for its high paracellular permeability [29].

Linear correlation of intestinal permeability and the fraction absorbed between human and rats has been reported for various drugs [30, 31]. The extent of drug absorption in human can be predicted from rat intestinal permeability experiments. Human intestinal permeability values may be estimated using the Lawrence compartmental absorption and transit (CAT) model [18, 32]:

$$F_a = 1 - [1 + P_{\text{eff, man}} \times 0.54]^{-7} \quad (3)$$

$$P_{\text{eff, man}} = 3.6 \times P_{\text{eff, rat}} + 0.03 \times 10^{-4} \quad (4)$$

According to this equation (3) and (4), the estimated F_a for the nobiletin in the SMEDDS dilutions, sub-microemulsions and micelles were 83.5 \pm 2.8%, 63.0 \pm 7.1% and 82.5 \pm 4.9%, respectively. It suggested that the estimated oral absorption in

human for SMEDDS dilutions would be higher than that for sub-microemulsions ($p < 0.01$), and similar with that for the micelles ($p > 0.05$). The lower oral absorption for the sub-microemulsions may be related to its larger particle size with wider size distribution (Polydispersity index in table 1: 0.547 ± 0.010), as suggested in the previous studies, where a better absorption profile was observed after oral administration of the microemulsions containing cyclosporine [33, 34]. SMEDDS are known to improve the oral absorption of lipophilic drugs. The main mechanism reported include increasing membrane fluidity, opening tight junction, inhibiting P-gp and/or CYP450 by surfactants and stimulating lipoprotein/chylomicron production by lipid [2,35]. Similar oral absorption of the SMEDDS and micelles showed that the surfactant-induced membrane fluidity and inhibition of P-gp might play an important role in the permeability change and the increase of drug

absorption in the gastrointestinal tract [3, 36]. Moreover, the nobiletin SMEDDS and its dilutions were more stable than the nobiletin micelles (the data is not listed).

CONCLUSIONS

According to these results, several conclusions can be drawn. The nobiletin SMEDDS had a small particle size (28.6 ± 0.3 nm) and a native charge. The P_{eff} in jejunum at $15 \mu\text{g/mL}$ were significantly higher than that at $60 \mu\text{g/mL}$ ($p < 0.01$). Compared to the jejunum, duodenum and ileum, the higher P_{eff} in colon were observed at the same concentration. The estimated absorption of nobiletin in human for the SMEDDS dilutions was higher than that for sub-microemulsions ($p < 0.01$) and similar with that for the micelles ($p > 0.05$). Moreover, the nobiletin SMEDDS and its dilutions were more stable than the nobiletin micelles.

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