Water-In-Oil-In-Water Double Emulsions: An Excellent Delivery System for Improving the Oral Bioavailability of Pidotimod in Rats

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ABSTRACT: The aims of this study were to prepare fine pidotimod-containing water-in-oil-in-water (W/O/W) double emulsions and to investigate the possibility of those emulsions as a delivery system for promoting the oral bioavailability of pidotimod. A modified two-step emulsification procedure was applied to prepare the double emulsions using medium-chain triglyceride as the oil phase, Tween 80 as the hydrophilic emulsifier, and Span 80 alone or in combination with different amount of phospholipids as the lipophilic emulsifiers. A fine W/O/W emulsion, with the encapsulation efficiency of 82 ± 3.4%, mean oil-droplet diameter of 3.93 ± 0.25 μm, and viscosity of 36.4 ± 0.93 mPa·s at 25°C and 300 s^{-1}, was stable for 1 month at 4°C. In addition, the oral bioavailability of pidotimod in rats, after intragastric administration of W/O/W double emulsions, was significantly higher than that of pidotimod control solution. Moreover, the maximum uptake time was significantly prolonged, suggesting an extra absorption pathway for W/O/W emulsions: a lymphatic circulation pathway. Those results demonstrated that W/O/W emulsions could become a potential formulation for improving the oral bioavailability of poorly absorbable drugs and suggested an important technology platform for the oral administration of peptide and peptidomimetic drugs. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:2203–2211, 2011

Keywords: emulsion; stability; bioavailability; pharmacokinetics; oral drug delivery; lymphatic transport

INTRODUCTION

Oral delivery of peptide and peptidomimetic drugs has attracted wide attention over the past decade.[1] Generally, peptide drugs delivered orally are poorly absorbed because of extensive enzymatic degradation existing in the gastrointestinal tract, and insufficient membrane permeability caused by high molecular weight and low lipophilicity.[2] Therefore, some methods have been employed to conquer the peroral delivery problem, the additives such as absorption enhancer,[3,4] protease inhibitors,[5] pharmaceutical means such as changing the drug delivery route,[6,7] and some other methods such as chemical modification.[8,9] Among these studies, it was found that an emulsion formulation could promote the absorption of peptide drugs into rat intestines, especially for insulin.[10–12]

Double emulsions are compartmentalized liquid dispersions in which the droplets of the dispersed phase contain smaller dispersed droplets that are similar to the continuous phase.[13] The inner dispersed globule/droplet in the double emulsions is separated (compartmentalized) from the outer liquid phase by a layer of another phase.[14] Commonly, double emulsions are mainly classified into following two types: water-in-oil-in-water (W/O/W) emulsions and oil-in-water-in-oil (O/W/O) emulsions. Mainly, the W/O/W emulsions are utilized in pharmaceutical fields. The internal droplets encapsulated by the oil coating can be seen as a storage chamber, in which the hydrophilic bioactive substances are stored.[15] Because of the outer oil coating, the drug dissolved in the internal aqueous phase is protected, and some research showed that this structure could sustain drug release and increase its oral bioavailability to a certain extent.[16]

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However, the industrial application of double emulsions is limited due to their instability against heat, organic solvents, or pH changes. Double emulsions containing polydispersed droplets are thermodynamically unstable and have a strong tendency to coalescence, flocculation, and creaming.[17] Polymeric amphiphilic conjugates, such as sodium caseinate–maltodextrin conjugates,[14] caseinate–dextran conjugates,[18] complex of modified pectin and whey protein isolate (WPI),[19] and WPI–polysaccharide complexes,[15] could be employed as effective steric stabilizers to prepare a relatively stable emulsion, and in most cases, those stabilizers function on the oil–water interface. The stability of double emulsions has always been evaluated by the changes in appearance, viscosity, droplets size distribution, and encapsulation efficiency of the emulsions. The use of different indicators, electrolytes such as $MgSO_4$,[20] sugars such as glucose,[21] and dyes such as new coccine[22] enabled us to study the yield, release, and permeation of entrapped solutes in the emulsions. In addition, fluorescent probes such as 1,3,6,8, pyrenetetrasulfonic acid,[23] tetrasodium salt, some drugs such as diclofenac sodium[24] and dihydralazine[25] have also been used for formulation, stability, and release studies of W/O/W emulsions. Unfortunately, no pharmaceutical double emulsions have overcome the research phase and been marketed because powerful emulsifiers for these types of emulsion are only available in cosmetic grade and not in pharmaceutical grade. Therefore, fine W/O/W emulsions that contain conventional emulsifiers and have high encapsulation efficiency, high stability, and so on need more research.

Pidotimod, (R)-3-[(S)-(5-oxo-2-pyrrolidinyl) carbonyl]-thiazolidine-4-carboxylic acid, is a biological response modifier with a peptide-like structure, which can stimulate both the primary and acquired immune responses to virus and bacteria.[26–28] Studies have demonstrated that pidotimod itself does not have antibacterial activity, but when combined with antibacterial agents, pidotimod can be effective in improving clinical effects, promoting recovery, and shortening therapy time.[29,30] Until now, no research has observed significant side effects of pidotimod, and it is commonly used in clinical practice. However, the pharmacokinetics study of pidotimod in humans showed that the oral bioavailability was lower than 45%.[31]

No research has been performed to improve the oral bioavailability of pidotimod in past. The aim of this study was to investigate the potentiality of W/O/W emulsions delivery system for promoting the absorption of pidotimod. In this context, a modified two-step emulsification procedure[32] was applied to prepare the W/O/W double emulsions. Medium-chain triglycerides (MCTs) were used as the oil phase, the hydrophilic emulsifier Tween 80 as the external aqueous-phase emulsifier, and the lipophilic emulsifier Span 80 alone or in combination with different amount of phospholipids[33] as the emulsifier or stabilizer for primary emulsions. Then, the W/O/W emulsions were evaluated by the characteristics, stability, and absorption enhancement of pidotimod in rats.

MATERIALS AND METHODS

Materials

Pidotimod (99.6% purity) was supplied by the Wuzhong Medicine Co., Ltd. (Suzhou, China). Bean phospholipids were purchased from Taiwei Co., Ltd. (Shanghai, China). Tween 80, Span 80, and MCT were obtained from Well Chemical Co., Ltd. (Nanjing, China). Methanol of high-performance liquid chromatography (HPLC) grade was obtained from Yuwang Chemical Reagents Co. (Shandong, China). Perchloric acid was purchased from Linfeng (Shanghai, China). Isopropanol, sodium dihydrogen phosphate, alcohol, sodium hydroxide, glucose, and gelatin were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water was purified by redistillation and filtered through a 0.22-μm membrane filter (Zongxing Filter Co., Ltd., Nanjing, China) before use.

Preparation of W/O/W Emulsions

Double emulsions were prepared by a modified two-step emulsification procedure. The composition of W/O/W double emulsions is described in Table 1. First, the internal aqueous phase (W_1) was prepared by dispersing pidotimod in solution, when adjusting the pH to 6.0 ± 0.5 (PHS-25; Magnetic, Shanghai, China). Different amounts of phospholipids (0, 4, 8, and 16 wt %) were dispersed in an adequate alcohol solution and added to the internal aqueous phase. The oil phase (O) was MCT containing the lipophilic surfactants Span80. The W_1/O primary emulsions were formed by dropping the aqueous phase into the oil

Table 1. Typical Composition of W/O/W Double Emulsions Stabilized with Different Amount of Phospholipids (in wt %)

	EM0	EM1	EM2	EM3
w/o primary emulsion				
Pidotimod	8%	8%	8%	8%
Bean phospholipids	0%	4%	8%	16%
MCT	52%	48%	44%	36%
Span 80	17.5%	17.5%	17.5%	17.5%
0.5% gelatin solution	2.5%	2.5%	2.5%	2.5%
H_2O	20%	20%	20%	20%
w/o/w double emulsion				
w/o primary emulsion	50%	50%	50%	50%
Tween 80	5%	5%	5%	5%
Glucose	5%	5%	5%	5%
H_2O	40%	40%	40%	40%

W/O/W, water-in-oil-in-water; w/o, water-in-oil; MCT, medium-chain triglyceride.

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phase under moderate magnetic stirring (magnetic stirrer with hot plate). Then, the mixtures were homogenized with a rotor-stator system (9500 rpm for 3 min, CAT X620; Zipperer GmbH, Staufen, Germany). To improve the stability of the primary emulsions, the alcohol contained in the primary W_1/O emulsions was volatized during moderate stirring (magnetic stirrer with hot plate) at 60°C for 30 min. The speed of agitation was fixed at 2000 rpm. In the second step, the 50 wt % W_1/O primary emulsion was gradually added into the external aqueous phase (W_2, containing 40 wt % H_2O, 5 wt % Tween80, and 5 wt % glucose) under moderate stirring conditions (1500 rpm) at room temperature (25°C). Thus, the amount ratios of phospholipids in the final double emulsions were 0, 2, 4, and 8 wt %. The system was stirred for 15 min until the formation of the W/O/W double emulsions, which was checked by microscopy.

Measurement of Droplet Size

The droplet size distribution of double emulsions was measured by dynamic light scattering using a Malvern Particle Size Analyzer (Mastersizer 2000S; Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a He–Ne laser (623 nm), using deionized water as a dispersion medium. The refractive index of dispersed phase and continuous phase was 1.600 and 1.333, respectively, and the absorbance value of the emulsion droplets was 0.1. Droplet size measurements were carried out in duplicate on each emulsion, and the results are reported as the typical droplet size distribution (size range measured from 0.020 to 2000.00 μm) and the volume average diameter (d_{43}) was determined. Three replicate analyses were carried out for each formulation, and data presented as means ±SD. The SD was from three batches of emulsions, and each lot of emulsion was measured for three times.

Measurement of Viscosity

The rheological measurements of the W/O/W emulsions were performed with a programmable rheometer (DVIII; ULTRA Premium Oilfield Services, LTD., Brookfield, Ohio) operating with cone plate geometry (cone diameter 60 mm, angle 1°, and gap 0.058 mm). Portions of the samples (about 5 mL) without dilution were transferred to the instrument and allowed to equilibrate to 25 ± 1°C for 10 min before the measurement. The apparent viscosity was measured over a shear rate range of 0.1–300 s^{-1}. The mean constant shear viscosity (mPa·s) was determined from the data obtained at 300 s^{-1}. Three replicate analyses were carried out for each formulation, and data presented as means ±SD. The SD was from three batches of emulsions, and each lot of emulsion was measured for six times.

Microscopic Observation

The diluted double emulsions (one part emulsion and nine parts deionized water, 5 μL) were dropped on a slide, covered by a cover glass, and observed with a Nikon model Eclipse T*i* optical microscope equipped with Nikon Digital Camera model DS-Ri 1 and Nikon NIS-Elem F 3.0 (Nikon Corporation, Tokyo, Japan). The samples were observed at room temperature immediately after preparation.

Measurement of Encapsulation Efficiency

Measurements of encapsulation efficiency were carried out by centrifuging.[14,17] Briefly, 5 mL of the W/O/W emulsions was added to 5 mL of purified water in a centrifuge tube. Then, the mixture was gently inverted and centrifuged at 10,000 × *g* for 20 min at 4°C in a refrigerated centrifuge (Sigma 3K30, Harz, Germany). One milliliter of the clear part of the solution in the tube bottom was taken by a syringe and filtered using a 0.22-μm Whatman filter paper (Whatman-Xinhua Filter Pape Co., Ltd., Hangzhou, China) to eliminate the oil droplets. One hundred microliters of filtrate was diluted and mixed with 900 μL of purified water. Samples were analyzed using an HPLC assay.

The quantitative determination of pidotimod was performed on a Shimadzu LC-20AT (Shimadzu Cooperation, Tokyo, Japan) with a C18 column (phenomenex®ODS, 250 × 4.6 mm^2 i.d., 5 μm). The mobile phase was composed of 0.01 M sodium dihydrogen phosphate–methanol–isopropanol (97:2:1, v/v), with the flow rate of 1.0 mL/min. Detection was recorded at wavelength of 210 nm by a Shimadzu SPD ultraviolet detector, with a sample injection volume of 20 μL. The pidotimod stock solution was diluted to working solutions ranging from 0.1 to 20 μg/mL, and the standard calibration curves were constructed by plotting concentrations against peak areas. A good linearity was achieved with a correlation coefficient of 0.9999 over the concentration range of 0.1–20 μg/mL. The relative standard deviations of interday and intraday precision as well as accuracy were all less than 2% at low, medium, and high concentrations.

The encapsulation efficiency was defined by $(m_0 - m_{out})/m_0 \times 100$, where m_0 is the original theoretical amount of pidotimod encapsulated in the internal aqueous phase, m_{out} is the amount of pidotimod leaked into the external aqueous phase and was calculated using the initial volume of the external aqueous phase. The encapsulation efficiencies of double emulsions were determined immediately after manufacture and after 30 days of storage at 4°C. Three replicate analyses were carried out for each formulation, and data presented as means ±SD. The SD was from three batches of emulsions, and each lot of emulsion was measured for three times.

Oral Administration of W/O/W Emulsion Containing Pidotimod to Rats

The usage of animals was agreed by China Pharmaceutical University Animal Management and Ethics Committee. Male Wistar rats were obtained from Qinglong Mountain (Nanjing, China) and acclimatized for 7 days. Weights of the rats were 200–250 g. The rats were fasted overnight for 17 h before dosing. Before the intragastric administration, W/O/W emulsions containing pidotimod were diluted with purified water to a concentration of 8 mg/mL. At time zero, 80 mg/kg of sample emulsions were given to rats by gavage ($n = 6$). Phosphate-buffered saline (PBS) (pH 7.2) as a control solution, which contained the same concentration of pidotimod, was also given to rats by intragastric administration.

Half, 1, 1.5, 2, 3, 4, 6, 8, 10, 24 h after intragastric administration, the rats were anesthetized with ether. Blood samples (0.5 mL) were collected from the eye ground vein in heparinized plastic centrifuge tubes using 0.8–1.1-mm capillary glass tube. Individual plasma samples were prepared by centrifugation at 2600 × *g* for 15 min in an Nr.12154 rotor (Sigma 3K30). Two hundred microliters of plasma was taken by pipettor (Eppendorf, Hamburg, Germany) and mixed with equal volume of 10% perchloric acid solution to precipitate proteins. The mixture was stirred on a vortex mixer for 90 s and centrifuged at 7500 × *g* for 10 min. Then, 20 μL of supernatant was analyzed by HPLC. The column was an Aminex Ion Exclusion HPX 874 with a Polymeric Reversed Phase (PRP) precolumn, the mobile phase was 0.05% sulfuric acid–acetonitrile (88:12, v/v), the flow rate was 0.6 mL/min, and the detection wavelength was 210 nm.[34] The method showed good linearity ($R^2 > 0.999$) in the range of 0.500–50.0 μg/mL in rat plasma. The mean extraction recoveries of pidotimod at QC concentrations (1.00, 10.0, and 50.0 μg/mL) were 91.5 ± 3.8%, 84.9 ± 2.7%, and 80.9 ± 4.5%, respectively. The interday and intraday RSDs for pidotimod were less than 3.20% at the above concentrations. After storage for 72 h at −20°C and three freeze–thaw cycles, pidotimod remained stable in plasma.

Statistical Analysis

Statistical analysis was performed with Dunnett's test for multiple comparisons. A p value of 0.05 was used as the significant level for all tests. All data are presented as the mean ±SD unless otherwise noted.

RESULTS AND DISCUSSION

Droplet Size of W/O/W Double Emulsions

Immediately after preparation, the droplet size distribution of the oil globules in the W/O/W emulsions prepared with different ratios of phospholipids (EM0, EM1, EM2, and EM3) as measured in static light scattering is given in Figure 1. The results showed that when Span 80 alone or in combination with 2 wt % phospholipids was employed as lipophilic emulsifier, a unimodal distribution centered on approximately 3.9 μm was observed. However, the droplet size displayed a bimodal distribution as the phospholipids concentrations increased between 4 and 8 wt %. The proportion of the particle size distribution range below 10 μm declined and that from 10 to 100 μm increased, with the mean droplet size (d_{43}) typically increased to approximately 21.2 μm. This increased droplet size value could be explained by the change of curvature angle caused by the high amount of phospholipids that tend to form reverse micelles and lamellar structures, respectively.[35]

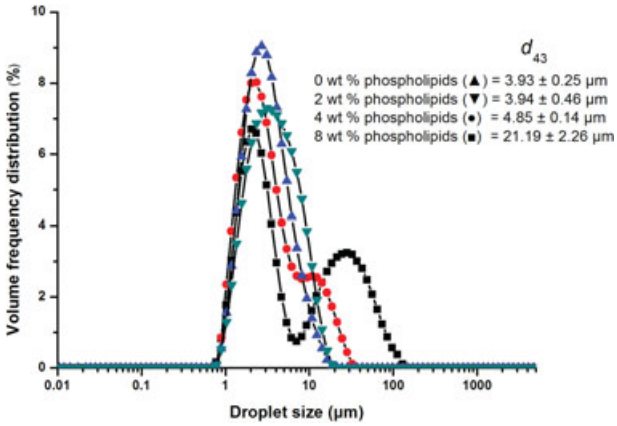

Figure 1. Typical droplet size distribution and mean droplet size (d_{43}) of water-in-oil-in-water double emulsions prepared with Span 80 alone (▲) or in combination with 2 (▼), 4 (●), 8 wt % (■) phospholipids as the internal emulsifier, and 5 wt % Tween 80 in the external water phase, immediately after manufacture.

Moreover, we observed some changes when the double emulsions were stored at room temperature, such as droplets coalescence and phase separation. For EM0 and EM1, with 2 wt % or less phospholipids, the aqueous phase was observed to separate after 7 days, and the phase separation of EM0 was comparatively more severe and easier to form. In this case, instability was attributed to film rupture around the oil globules.[14] Formulations EM2 and EM3 did not show phase separation after 7 days.

Figure 2 shows the droplet size distribution range of the EM2 and EM3 stored at 4°C. With 4 wt % of phospholipids, the droplet size of double emulsions was almost unchanged throughout the 30-day storage period, whereas with higher content of phospholipids (8 wt %), droplet size was decreased to 7.8 μm after 30 days, indicating that droplets flocculation and coalescence may occur during the storage period. It was noted that too high a content of phospholipids is not conducive to improve the stability of the emulsions.

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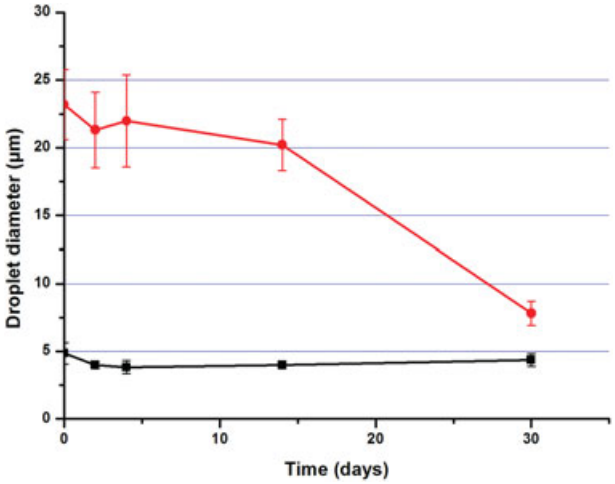

Figure 2. Stability at 4°C of water-in-oil-in-water multiple droplets stabilized with Span 80 in combination with phospholipids as the internal emulsifier. Double emulsions composition is described in Table 1. (■), 4 wt % phospholipids; (●), 8 wt % phospholipids. Data represent the means ±SD for three batches of samples; each lot of emulsion was measured for three times.

Viscosity of W/O/W Double Emulsions

Rheological measurements were performed immediately after preparation and after 30 days of storage at 4°C. Figure 3 shows the viscosity of the double emulsions stabilized by Span 80 in combination with 4 and 8 wt % of phospholipids. It was found that with higher phospholipids concentration (8 wt %), the emulsions had higher viscosity. The viscosity of double emulsions is derived from the entanglement of emulsifier tails on the interface among droplets. The entanglement is increased due to the clustering of the droplets.[19] Thus, higher viscosity of the double emulsions indicated clustering of larger droplets with higher phospholipids concentration, which was consistent with previous studies (as shown in Fig. 1).

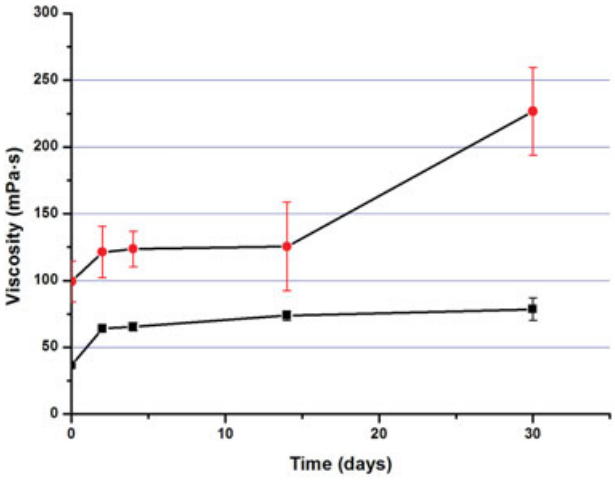

Figure 3. Influence of phospholipids concentration on the viscosity of the double emulsions stabilized with Span 80 in combination with (■) 4 and (●) 8 wt % phospholipids as the internal emulsifier, upon 30 days of storage at 4°C. Double emulsion composition is described in Table 1. Data represent the means ±SD for three batches of samples; each lot of emulsion was measured for six times.

After 30 days, the viscosity data of the double emulsions showed that significant increase happened to the viscosity of the double emulsions with the highest concentration of phospholipids (EM3, 8 wt %), from 99.3 ± 15.4 to 226.7 ± 32.9 mPa·s. However, the increase in viscosity was not as significant as EM3 when the concentration of phospholipids was 4 wt % (EM2). Viscosity increased from 36.4 ± 0.93 to 73.8 ± 0.34 mPa·s. This result led us to conclude that the appropriate amount of phospholipids is predominant in stabilizing the double emulsions. It seems that the main factor influencing double-emulsions viscosity is the amount of water in the continuous phase. As explained previously, in hypertonic double emulsions, water tended to penetrate from the high osmotic pressure phase into the low osmotic pressure phase to equalize the osmotic pressure. The osmotic pressure of the internal aqueous phase is higher than the osmotic pressure of external aqueous phase in the double emulsions that we made. So, aqueous phase tends to permeate from the external phase to the internal phase, and the viscosity value will increase with decreasing amount of water in the external phase. However, an appropriate amount of phospholipids existing on the internal water–oil interface could prevent water transference and reduce the rupture of the double emulsions film that can stabilize the emulsions.

Microscopy Images of W/O/W Double Emulsions

To verify the inclusion of the external aqueous phase solution into the oil droplet of the W/O/W double emulsions, we observed the microscopy images. All emulsions (EM0, EM1, EM2, and EM3) were macroscopically homogeneous, and the observed images are shown in Figure 4. The structure observed from optical microscopy is in accordance with the characteristics of double emulsions. The dispersed oil droplets contain smaller dispersed aqueous droplets.

Encapsulation Efficiency and Encapsulation Stability of W/O/W Double Emulsions

The encapsulation efficiencies of EM0–EM3 were measured just after preparation and after 30 days. The results are shown in Figure 5. The encapsulation efficiency of the emulsions on day 0 significantly increased with the increasing concentration of phospholipids ($p < 0.05$), and EM2 (4 wt % phospholipids) showed the highest encapsulation efficiency value, which was 82 ± 3.4%. However, it must be pointed out that the encapsulation efficiency was 76 ± 1.3%,

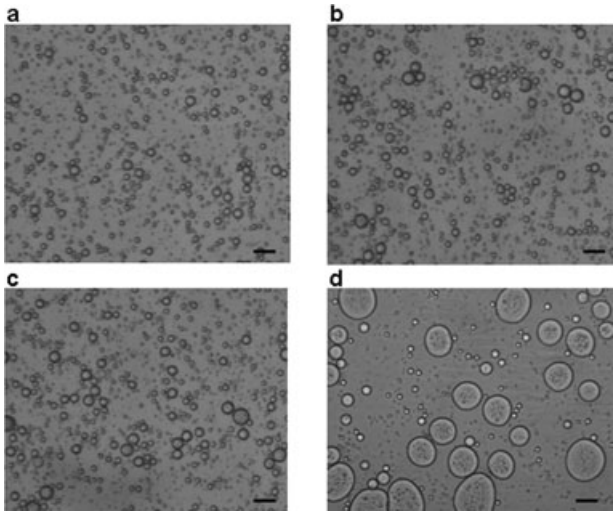

Figure 4. Photomicrographs of water-in-oil-in-water double emulsions stabilized with Span 80 alone (a) or in combination with (b) 2, (c) 4, and (d) 8 wt % phospholipids as the internal emulsifier, immediately after prepared by "two-step" method. Bar is 10 μm.

as the phospholipids concentration was increased to 8 wt %.

Polymeric amphipathic molecules are known to increase the interfacial coverage during emulsification, and that could improve the encapsulation efficiency and control release of the substance entrapped in the internal core of the double-emulsion droplets.[18] In this case, phospholipid molecules supplied powerful protection to the entrapped substance because of the amphiphilic structure and high molecular weight,

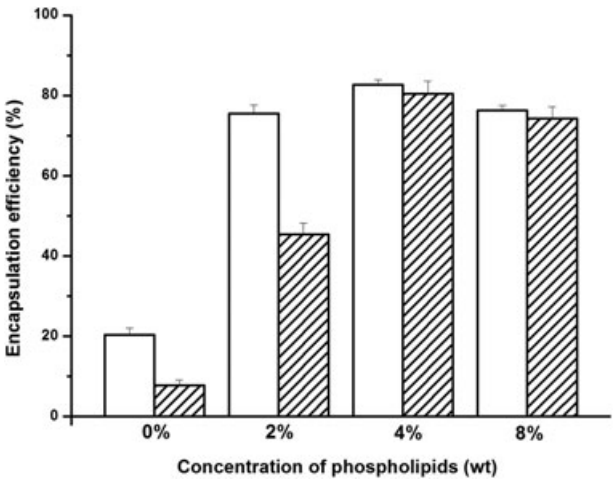

Figure 5. Effect of different phospholipids concentration on the encapsulation efficiencies of double emulsions stabilized with Span 80 in combination with different amount of phospholipids as the internal emulsifier. Blank bar, day 0; netted bar, 1 month at 4°C. Data represent the means ±SD for three batches of samples; each lot of emulsion was measured for three times.

and most of them existed on the water–oil interface to form a comparatively hard polymeric film with the oil phase and act as a barrier to control the water permeation. Thus, the encapsulation efficiency was increased while phospholipids were applied as the emulsifiers or stabilizers for primary water-in-oil emulsions. For the double emulsions (EM0) without phospholipids, the low encapsulation efficiency, 20 ± 1.7%, was attributed to the lack of full coverage that induced the weakness of the polymeric film.[15]

After 30 days storage at 4°C, the encapsulation efficiencies of the W/O/W emulsions (EM0 and EM1) prepared with 0 or 2 wt % of phospholipids significantly decreased ($p < 0.05$), whereas no significant change of encapsulation efficiency was detected during the storage period for those with 4 and 8 wt % phospholipids. The oil droplets of double emulsions (EM0 and EM1) were inclined to coalesce, and the leakage of pidotimod from the internal aqueous phase to the external aqueous phase could increase.

According to the previous study, data of the viscosity and volume mean droplet size (d_{43}) of EM3 (8 wt % phospholipids) made us conclude that droplet flocculation and coalescence had occurred during the storage period, whereas the encapsulation efficiency was kept at 76 ± 1.3% after 30 days storage. This result provided further evidence to prove that phospholipid molecules absorbed on the water–oil internal interface could prevent the entrapped substance from leaking out. It appears that although some changes were observed in the double emulsions at higher phospholipids concentration, the internal water droplets still remain intact and disperse well in the oil phase.[36]

Oral Administration of W/O/W Emulsion Containing Pidotimod to Rats

Owing to the general properties and stability, double emulsions (EM2, with 4 wt % phospholipids) were selected for the *in vivo* absorption experiments in rats. Meanwhile, pidotimod was dissolved in PBS (pH 7.2) as control.

Figure 6 shows the plasma pidotimod concentration versus time profile curves of the subject rats. The pharmacokinetic parameters are listed in Table 2. The maximum plasma pidotimod concentration (C_{max}) of control solution was 47.1 ± 19.2 μg/mL, which is higher than that of W/O/W double emulsions (EM2), 33.2 ± 13.6 μg/mL. On the contrary, the maximum uptake time (T_{max}) of control solution occurred at 1.20 ± 0.13 versus 3.00 ± 0.27 h of the emulsions. Plasma concentrations of pidotimod decreased with time at different rates. The plasma half-life of pidotimod following oral delivery in PBS or double emulsions was inversely correlated with the elimination constants calculated.[37] The plasma half-life of pidotimod given in PBS was determined to be 0.920 h with a k_{elim} of 0.753 h^{-1}, whereas that of pidotimod given

Table 2. Pharmacokinetic Parameters of Pidotimod Uptake in Rats Following Oral Delivery (by Gavage) of 80 mg/kg Pidotimod Reconstituted in PBS or W/O/W Double Emulsions ($n = 6$)

Parameter	PBS	W/O/W double emulsions
C_{max} (μg/mL)	47.1 ± 19.2	33.2 ± 13.6
T_{max} (h)	1.20 ± 0.13	3.00 ± 0.27
$AUC_{0-\infty}$ [(μg/(h mL)]	83.2 ± 18.1	181.1 ± 37.7*
k_{elim} (h^{-1})	0.753	0.171
$t_{1/2}$ (h)	0.920	4.05

*$p < 0.05$ versus control group; Tukey test.
W/O/W, water-in-oil-in-water.

in double emulsions was 4.05 h with a k_{elim} of 0.171 h^{-1}. The pidotimod control solution showed a lower absorbability, and area under the curve $(AUC)_{0-\infty}$ was 83.2 ± 18.1 μg/(h mL). An increase in $AUC_{0-\infty}$ [(181.1 ± 37.7 μg/(h mL)] was observed when pidotimod was encapsulated in double emulsions. Statistically, there was a significant difference between $AUC_{0-\infty}$ obtained from pidotimod given in PBS and that from W/O/W double emulsions treatment ($p < 0.05$). The enhancing ratio (2.2-fold) indicated that the use of double emulsions enhanced pidotimod intestinal absorption.

As we know, there is a great deal of interest recently in understanding the role of multiple emulsions in enhancing drug intestinal absorption. However, mechanisms guiding the intestinal absorption of drugs through W/O/W emulsions are not well understood. The absorption-enhancing effect has always been evaluated using an *in vitro* assay with Caco-2 monolayer and *in vivo* assay with rats. Recent reports suggested that oil droplets encapsulating drug permeated into the intestinal mucosa via a transcellular pathway in rats.[16] Because the double emulsions can easily incorporate absorption enhancers in each phase, in accordance with their solubility, it is expected that an increase in oral bioavailability of drugs could be achieved with formulation improvement. It is well known that phospholipids can act as a penetration enhancer for topically applied substances and can facilitate the transport of molecules into cells.[38] On the contrary, there are reports that medium-chain fatty acids, such as caprylate, caprate, and laurate, enhanced the intestinal membrane permeability of hydrophilic compounds via the paracellular route.[39] Thus, the absorption-enhancing effect of W/O/W double emulsions on the intestinal absorption of pidotimod may be partly ascribed to the phospholipids and medium-chain fatty acids contained in the emulsions.

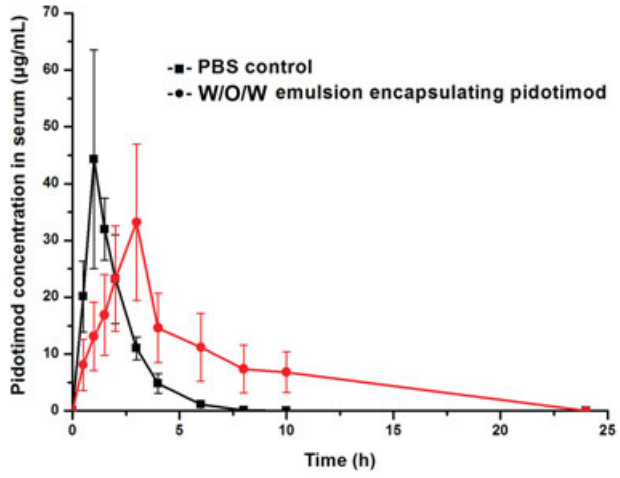

Figure 6. Serum pidotimod concentration versus time profiles after oral administration into rats. The dose of pidotimod was 80 mg/kg. Control formulation was 8.0 mg/mL pidotimod solution, and other was w/o/w emulsions encapsulating pidotimod. Pidotimod concentrations in w/o/w emulsions were all adjusted to 8.0 mg/mL. Each data point with error bar represents the mean ±SD of six experiments for same preparation.

In contrast to recent articles, we did not find the C_{max} of W/O/W emulsions to be higher than the C_{max} of the control. However, we found that the plasma concentration peaking time was prolonged, and the absorption progress of pidotimod in the emulsions was slower than in the control. Moreover, the plasma half-life of pidotimod was prolonged and the elimination was slowed, showing a sustained-release effect *in vivo*. These findings may implicate a different absorption mechanism of W/O/W emulsions containing pidotimod in contrast to the control solution. We suggest that the drug dispersed in the inner aqueous droplets of the emulsions, with the package of oil layer, may be absorbed into the lymphatic circulation in the duodenum through the chyle role of bile, and then enter the blood circulation, thus achieving sustained absorption and slow elimination. This idea is consistent with the findings that drug must be encapsulated into the oil droplets to enhance the intestinal absorption,[16] but needs further research. Further investigations are required to support this concept, and we will throw more emphasis on this problem in the next experimental theme.

CONCLUSION

The present study investigated the potential of improving the oral bioavailability of pidotimod by the promising W/O/W emulsions drug delivery system. To optimize the formulation, the influence of the phospholipids concentrations on the general properties and stability of W/O/W double emulsions was investigated. The fine W/O/W emulsions, containing 4 wt % of phospholipids, were stable for a whole month at 4°C. The change of volume mean droplet size (d_{43}) of oil droplets, the viscosity, and the encapsulation efficiency value with time were analyzed, and no obvious changes were observed. Moreover, we performed *in vivo* rat bioavailability studies and analyzed the

resulting plasma pidotimod concentration versus time profiles. The results of these analyses showed that the $AUC_{0-\infty}$ was 2.2-fold that of the control, indicating that the pidotimod bioavailability after oral administration of those emulsions was significantly higher than that of the control. The effect of the sustained absorption and slow elimination revealed a probable lymphatic circulation pathway for W/O/W emulsions and suggested a potential formulation for improving the oral bioavailability of poor absorbable hydrophilic drugs.

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