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Spray-dried oil-in-water emulsion to improve the intestinal absorption and oral bioavailability of ZLR-8, a nitric oxide-releasing derivative of diclofenac

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Abstract

Objectives Spray-dried emulsion (SDE) was prepared and characterized to improve the intestinal absorption and oral bioavailability of ZLR-8, a nitric oxide-releasing derivative of diclofenac, currently under preclinical development.
Methods The intestinal absorption of ZLR-8 was characterized by single pass intestinal perfusion technique to obtain its absorption and permeability parameters. SDE of ZLR-8 was prepared and characterized by particle size measurements and in-vitro release study. Accurate and precise RP-HPLC methods for the detection of ZLR-8 and its metabolite diclofenac were constructed to perform the bioavailability study.
Key findings It was demonstrated that ZLR-8 was absorbed in the whole intestine, of which the duodenum segment exhibited the largest absorption ability. ZLR-8 can be classified into BCS Class 2. SDE significantly enhanced the intestinal absorption rate of ZLR-8 in duodenum and jejunum but had indistinctive effect on permeability. All concentrations of ZLR-8 in rat plasma was lower than the limit of detection. A bicompartment model gave the best fit to the plasma diclofenac concentration–time curves. Calculated on $AUC_{0–12h}$, the mean relative bioavailability of SDE was 105.4-fold that of ZLR-8 suspension.
Conclusions SDE significantly improved the intestinal absorption of ZLR-8 and resulted in a dramatic improvement in its bioavailability.
Keywords bioavailability; intestinal absorption; spray-dried emulsion; ZLR-8

Introduction

ZLR-8 (2-(2,6-dichlorophenylamino)-benzeneacetic acid 4-(4-pheny-1,2,5-oxadiazole-2-oxide) -methoxylphenyl ester), the nitric oxide (NO)-releasing derivative of diclofenac (Figure 1), is a novel therapeutic agent currently under preclinical development for the treatment of fever, pain and inflammation. As an NO-releasing compound, ZLR-8 has been synthesized with the aim of achieving lower gastrointestinal toxicity in comparison with diclofenac while maintaining its anti-inflammatory action. In previous pharmacological studies, it has been proved that ZLR-8 has greater anti-inflammatory and analgesic activity and lower gastrointestinal side effects than diclofenac and other NSAIDs.[1,2] However, ZLR-8 was demonstrated to be insoluble in water (<0.1 μg/ml in water and aqueous buffer, pH 1.5–9.6) in a previous preformulation study. Traces of water will result in the rapid precipitation of ZLR-8 from a solution in ethanol–PEG 400 (1 : 1). The extreme hydrophobicity of ZLR-8 may lead to poor absorption and low bioavailability.[3] Jiao *et al*. developed an HPLC method to determine ZLR-8 and its metabolite diclofenac after single oral administration of ZLR-8 crude material in beagle dogs.[4] However, no absorption, bioavailability or formulation was studied. So far, the formulation of ZLR-8 has only been reported by our research team based on the preliminary findings of the low bioavailability. ZLR-8 spray-dried emulsion was prepared by spray-drying its liquid oil-in-water (o/w) emulsions in a laboratory Büchi B-290 spray dryer, following the experimental screening and formulation optimization.[5]

Recently, spray-dried emulsion (SDE) had been used as an effective carrier for some insoluble drugs to improve their solubility and hence the absorption and bioavailability.[6] Compared with the conventional emulsion, SDE displayed enhanced stability due to the absence of water. Besides, the toxicity was reduced as a result of the lower level of

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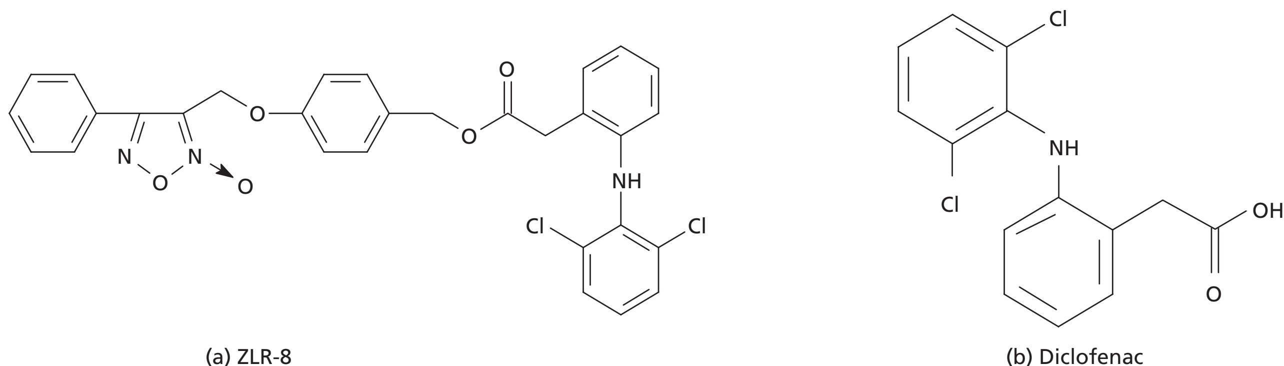

Figure 1 Structure of ZLR-8 (a) and diclofenac (b).

surfactants. Once introduced to the digestive fluid in the gastrointestinal tract, the SDE may be rapidly reconstituted to form an o/w emulsion.[7] So far as we know, the effect of SDE on the intestinal absorption profiles of ZLR-8 and its oral bioavailability have not ever been reported.

The main objective of this study was to characterize the intestinal absorption and permeability features of ZLR-8 and to investigate the effect of SDE on them. In addition, an in-vivo bioavailability study of SDE was performed, in comparison with ZLR-8 suspension, to demonstrate its bioavailability enhancement.

Materials and Methods

Materials

ZLR-8 was synthesized by the Center of Drug Discovery, China Pharmaceutical University. Its purity was determined to be 99.80%. Labrafac CC (medium-chain triglyceride, caprylic/capric triglyceride) was kindly supplied by Gattefosse (Saint-Priest, France). PEG 400 was obtained from Nanjing Well Chemical Co., Ltd (Nanjing, China). Hydroxylpropylmethylcellulose (HPMC, Methocel E3-LV, 3 mPa·s) was provided by Shanghai Colorcon Coating Technology Co., Ltd (Shanghai, China). Maltodextrin was provided by DMV International Pharma (Veghel, The Netherlands). Solutol HS15 was obtained from BASF (Ludwigshafen, Germany). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade.

Animals

The rat has been used widely as an animal model and is particularly suited for the study of intestinal absorption and pharmacokinetics. All rats used in the study were in good health. The intestinal absorption and bioavailability studies were realized using conscious adult male Sprague–Dawley rats, weighing 201–232 g at the beginning of the experiments. The studies were approved by the Experimental Animal Ethical Committee of China Pharmaceutical University. The rats were housed for at least two weeks before experiments with free access to food and water at 25 ± 3°C and 50 ± 20% relative humidity under a 12-h light–dark cycle. All rats used for experiments were 8–12 weeks old. In the intestinal absorption experiments all rats were fasted for 15–18 h and anaesthetized by intraperitoneal injection of urethane (1.2 g/kg). In the pharmacokinetics experiments, before surgery, rats were anaesthetized by intraperitoneal injection of pentobarbital, then the right jugular vein was cannulated with a cannula made up of polyethylene tubing (PE50, Intramedic Polyethylene Tubing; Becton–Dickinson, Baltimore, USA) to allow blood sampling. After the surgery, rats were transferred to restraining cages and were fasted for 24 h before the experiment. However, free access to water was allowed throughout the experiment.

Preparation of spray-dried emulsion of ZLR-8

In previous study, the optimized spray-dried redispersible emulsion was prepared after the solubility test of ZLR-8 in various media and the screening of HPMC and maltodextrin.[5] In brief, ZLR-8 (500 mg) was dissolved in the mixture of Labrafac CC (2 g) and PEG 400 (3 g). One-hundred millilitres of 9% HPMC (3 mPa·s) containing 6 g of maltodextrin was added and vortexed for 2 min. The resultant coarse emulsion was then homogenized (Avestin Emulsiflex05; Avestin Inc., Ottawa, Canada) for five cycles at a pressure of 1×10^5 kPa. To obtain a physically stable solid redispersible dry emulsion, homogenized liquid emulsion was subsequently spray dried using a mini spray-dryer equipped with a high-performance cyclone (Büchi B-290; Büchi Labortechnik AG, Flawil, Switzerland) with a 0.7-mm nozzle, using the following standard operating conditions: inlet temperature, 110°C; aspirator setting, 100% (40 m3/h); spray flow rate, 600 l/h; pump setting, 2.72 ml/min; These conditions resulted in an outlet temperature of 58–60°C.

Particle size measurement

In a 20-ml tube, 1 g of the SDE powders was redispersed with 5 ml distilled water by hand-shaking (10 s), then the emulsion was vortexed for 1 min at 20 rpm and samples were withdrawn for droplet size determination. The size distribution of the droplets from the coarse primary emulsion (PE), the homogenized emulsion (HE) and SDE was determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). This instrument uses an approximation of Mie-scattering theory, which uses the

refractive index of the dispersed phase and its absorption. Each sample was measured at 25°C in triplicate. PCS yields the volume weighted mean particle size and the polydispersity index (PI) of the liquid emulsion. All results were recorded as volume distributions expressed as mean, d(4,3) or median, d(v,0.5) volumetric diameters. The width of the droplet size distribution was expressed by the SPAN value obtained by the following relation: SPAN = [d(v,0.9)-d(v,0.1)] /d(v,0.5).

In-vitro dissolution study

A modified two-phase stirred transfer device was used for in-vitro dissolution comparison;[7] it consisted of a beaker at 37°C with thermostat water bath, one glass bladed impeller in the centre of the upper phase (125 ml hexane) with a rotation speed of 50 rpm, a magnetized agitator in the bottom phase (250 ml 40% (v/v) ethanol) giving an opposite equal rotation to reduce the vortex effect at the phase interface. Before two-phase stirring, samples (all equivalent to 2 mg drug), including ethanolic solution, PE, HE and SDE, were transferred into the bottom phase with a glass funnel. The transfer of ZLR-8 into hexane was investigated dynamically. Samples (5 ml) were collected from the upper phase at scheduled times during 2 h and ZLR-8 concentration was analysed by UV spectrophotometry ($\lambda = 273$ nm).

Intestinal absorption

The composition of the blank perfusion buffer (in mg/ml) was: NaCl 7.8, KCl 0.35, $CaCl_2$ 0.37, $NaHCO_3$ 1.37, NaH_2PO_4 0.32), $MgCl_2$ 0.02 and glucose 1.4. The pH was maintained at 7.4. To avoid the precipitation of ZLR-8, 3% Solutol HS15 was added. Stock solution of ZLR-8 (1.0 mg/ml) was prepared in ethanol. Perfusion solution of ZLR-8 was prepared by diluting this stock solution into a certain concentration with perfusion buffer. ZLR-8 SDE was weighed and redispersed as described in the section on particle size measurement, above.

ZLR-8 in samples was analysed by a reversed phase HPLC (Shimadzu Corp., Kyoto, Japan) comprised of a LC-10ATvp pump and SPD-10Avp UV detector. A C18 column (Waters, 150 mm × 4.6 mm; 5 μm) was employed for the separation of analytes. Mobile phase composed of methanol–acetonitrile–0.025mol/l ammonium dihydrogen phosphate solution (13 : 82 : 5; v/v/v). Flow rate was maintained at 1 ml/min and detection wavelength was set at 270 nm. Elution time for ZLR-8 was about 3.64 min. The calibration curve of ZLR-8 was linear over the range 0.5 μg/ml~100 μg/ml ($r = 0.9998$, $n = 6$). At 20, 40 and 80 μg/ml, mean recovery of the method was 96.16%, 97.04% and 95.37% ($n = 3$), respectively, and relative standard deviation (RSD) was lower than 2.0%. Additives from SDE and intestinal secretion had no interference on chromatogram of ZLR-8.

ZLR-8 solution (40 μg/ml in 3% Solutol HS15 aqueous solution) was incubated at 37°C for 2 h. Samples were collected at appropriate time intervals and analysed by HPLC. No drug adsorption to the tube and glass surface was noted. A stability study of ZLR-8 (40 μg/ml) in intestinal perfusion buffer was also conducted by incubating for 3 h at 37°C. Samples were collected at predetermined time points and analysed by HPLC to monitor any drug degradation.

Single-pass intestinal perfusion (SPIP) is a well established technique employed to study the intestinal absorption of drugs. It provides the advantages of experimental control (e.g. permeant concentration, intestinal perfusion rate), intact intestinal blood supply and ability to determine regional absorption rates of drugs in intestinal segments. The surgery for SPIP of the rat small intestine was performed as described.[8,9] Briefly, rats were anaesthetized and the abdomen was opened with a midline longitudinal incision. The corresponding intestinal segment of approximately 10 cm was measured and cannulated with plastic tubing (4 mm o.d., inlet tube 40 cm, outlet tube 25 cm). Care was taken to avoid injury to the local circulatory system. Each intestinal segment was rinsed with intestinal perfusion buffer maintained at 37°C for approximately 30 min until the outlet solution was visually clear. A bolus dose of 3–5 ml of drug solution was allowed to equilibrate with the intestinal segment. Thereafter the intestinal segment was perfused at a constant flow rate (Q_{in}) of 0.2 ml/min with a peristaltic pump (HL-2; Qingpu Huxi Instrument Co. Ltd, Shanghai, China). Each perfusion experiment lasted for 120 min and samples were collected at 15-min intervals in pre-weighed glass tubes. All the perfusion solutions collected were weighed and stored at −18°C until analysis. At the end of the experiment, the rat was euthanized with a cardiac injection of saturated KCl solution. Finally, the intestine was removed and the length and radius of intestine were measured.[10–12]

In-vivo bioavailability study

Before gavage administration, jet-milled ZLR-8 powder (Jetpharma; MCOne, Balerna, Switzerland) was dispersed in sodium carboxylmethylcellulose solution (0.5%) to produce the suspension (25 mg/ml). ZLR-8 SDE was reconstituted in distilled water to obtain an o/w emulsion at a concentration of 25 mg/ml. The rats were allocated randomly into three groups, five rats in each group. The first group received a suspension of ZLR-8 (25 mg/ml). The second received the reconstituted ZLR-8 SDE (25 mg/ml). The third received an aqueous solution of diclofenac sodium (20 mg/ml). Oral formulations were delivered by an oral intubation cannula and followed by 2 ml of distilled water to ensure the delivery of the whole dose. Rats received ZLR-8 at the dose of 50 mg/kg (body weight) and received diclofenac sodium at the dose of 40 mg/kg. During each experiment, about 0.4 ml of blood was collected from the catheter and put into heparinized tubes at 0, 0.083, 0.25, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h post-dose. Plasma was separated by centrifugation (10°C, 10 000*g*, 15 min) using a refrigerated table-top centrifuge (Sigma 1–15 K; Sigma, Osterode am Harz, Germany) and kept frozen at –20°C until analysis.

Determination of ZLR-8 and diclofenac in plasma

In this study, an HPLC/UV method was employed to determine the concentration of ZLR-8 and diclofenac in rat's plasma using reversed phase HPLC (Shimadzu LC 10AD; Shimadzu Corporation, Kyoto, Japan).

ZLR-8 was detected by a C18 column (Shimadzu VP-ODS column, 150 mm × 4.6 mm) guarded with a precolumn (Shimadzu) and the wavelength was set at 270 nm. The mobile phase for ZLR-8 consisted of acetonitrile–0.025 mol/l ammonium dihydrogen phosphate (80 : 20, v/v), pumped at a

flow rate of 1.0 ml/min. For the detection of diclofenac, the active metabolite of ZLR-8, was detected at 280 nm using the ternary mixture of acetonitrile–methanol–0.025mol/l ammonium dihydrogen phosphate (25 : 35 : 40, v/v/v) as the mobile phase, pumped at a flow rate of 1.0 ml/min.

The degree of interference to ZLR-8 or diclofenac determination by endogenous co-eluent components was evaluated through comparison of chromatograms derived from blank plasma samples, spiked plasma samples and plasma taken from drug-administered rats. Stock solutions of ZLR-8 (500 μg/ml), diclofenac (1080 μg/ml), gemfibrozil (250 μg/ml) and indometacin (50 μg/ml) were prepared in methanol and stored in brown glass bottles. Two calibration curves were prepared for ZLR-8 and diclofenac, respectively.

Quantification of ZLR-8 was based on peak area ratio (Y_1 = ZLR-8/gemfibrozil). ZLR-8 and gemfibrozil were separated well from impurities in plasma extracts, with retention times of 13.098 min and 4.032 min, respectively. In the concentration range of 0.25–37.5 μg/ml, peak area ratio correlated well to spiked plasma concentration (X_1): $Y_1 = 0.02649X_1 + 0.06321$ ($r = 0.9998$, $n = 6$). After storage for 15 days at −20°C and freeze–thawing three times, ZLR-8 was stable in plasma. The limit of quantification and limit of detection for ZLR-8 were 0.0625 μg/ml and 0.01875 μg/ml, respectively; at concentrations of 0.25, 6.25 and 37.5 μg/ml, spiked recoveries of ZLR-8 from rat plasma were 96.72 ± 3.01%, 103.3 ± 8.65% and 97.53 ± 6.49% ($n = 5$), respectively; intra-day precision was 1.82%, 2.10% and 2.54%; inter-day precision was 6.06%, 6.85% and 5.18%.

Quantification of diclofenac was based on peak area ratio (Y_2 = diclofenac/indometacin). Diclofenac and indometacin were separated well from impurities in plasma extracts, with retention times of 8.798 min and 11.432 min, respectively. In the concentration range of 0.45–54.0 μg/ml, peak area ratio correlated well to spiked plasma concentration (X_2): $Y_2 = 0.1096X_2$-0.0005 ($r = 0.9997$, $n = 6$). After storage for 15 days at −20°C and freeze–thawing three times, diclofenac was stable in plasma. The limit of quantification and limit of detection for diclofenac were 0.135 μg/ml and 0.0405 μg/ml, respectively; At concentrations of 0.45, 9.0 and 54.0 μg/ml, spiked recoveries of diclofenac from rat plasma were 100.9 ± 2.70%, 102.6 ± 0.57% and 101.8 ± 1.90% ($n = 5$), respectively; intra-day precision was 2.03%, 0.24% and 1.81%; inter-day precision was 7.38%, 3.92% and 6.41%.

Frozen plasma samples were allowed to thaw and were warmed to room temperature followed by vortex mixing. For the determination of ZLR-8, 100 μl of rat plasma was supplemented with 25 μl of gemfibrozil solution as an internal standard (50 μg/ml in methanol) and vortexed for 20 s. Acetonitrile–methanol (95 : 5, 0.25 ml) was added. The mixture was vortexed for 5 min and then centrifuged for 10 min (10°C, 10 000*g*). Supernatant (20 μl) was injected into an HPLC system for ZLR-8 analysis. For the determination of diclofenac, 100 μl of rat plasma was mixed with 25 μl of indometacin solution as an internal standard (50 μg/ml in methanol) and vortexed for 20 s. Acetonitrile–methanol (95 : 5, 0.25 ml) was added. The mixture was vortexed for 5 min and then centrifuged for 10 min (10°C, 10 000*g*). Supernatant (20 μl) was injected into an HPLC system for diclofenac analysis.

Data analysis

Intestinal absorption analysis

Drug concentrations in the perfusion samples were corrected for changes in the water flux during each time interval. Density corrected gravimetric method was utilized for the calculation of net water flux across the incubated intestinal segment. The advantage of this method over the usage of non-absorbable markers (like phenol red and ^{14}C polyethylene glycols) is that it does not interfere with analytical method and has no radiation safety issues. The density of collected samples was determined by weighing the contents using an electronic weighing balance of a known volume of perfusate.[13]

Net water flux (NWF) was calculated by using the following equation:

$$\mathrm{NWF} = \left[1 - \left(\frac{Q_{out}}{Q_{in}}\right)\right]\frac{Q_{in}}{l} \tag{1}$$

Where Q_{in} is the measured flow rate (ml/min) of entering intestinal perfusate, Q_{out} is the measured flow (ml/min) of exiting intestinal perfusate for the specified time interval calculated from the actual intestinal perfusate density (g/ml) and l is the length (cm) of intestinal segment perfused.

Absorption rate constant K_a and $C_{out(corr)}$ were calculated from the following equations:

$$K_a = \left(1 - \frac{C_{out(corr)}}{C_{in}}\right)\frac{Q}{V} \tag{2}$$

and

$$C_{out(corr)} = C_{out}\frac{Q_{out}}{Q_{in}} \tag{3}$$

Where $C_{out(corr)}$ is the water flux corrected concentration of the compound measured in the exiting perfusate at the specified time interval (45, 60, 75, 90, 105 and 120 min), C_{in} denotes concentration of the drug measured in entering perfusate, Q is the perfusion rate (0.2 ml/min) and V is the volume of perfused segment.

The permeability in SPIP was estimated at a steady state of absorption. Steady state was confirmed by plotting the ratio of the outlet to inlet concentrations (corrected for water transport) versus time.[14] Permeability calculations across rat small intestine (P_{eff}) were performed from intestinal perfusion samples collected over 45–120 min (steady state). P_{eff} of ZLR-8 was calculated from the following equation:

$$P_{eff} = \frac{[-Q_{in}\ln(C_{out(corr)}/C_{in})]}{A} \tag{4}$$

Where Q_{in} is the flow rate (ml/min) of entering perfusate, $C_{out(corr)}$ the water flux corrected concentration of the permeant in the exiting perfusate, C_{in} the concentration in entering perfusate and A is the surface area (cm^2) of the intestinal segment perfused.

Pharmacokinetics analysis

Pharmacokinetic analysis was performed by means of a model independent method using the DAS 2.0 computer program (issued by the State Food and Drug Administration of China for pharmacokinetic study). The area under the plasma concentration–time curve from zero to 12 h (AUC_{0-12h}) was calculated using the trapezoidal rule. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from plasma data. All results were expressed as mean ± SD.

Statistical analysis

Kruskal–Wallis one-way analysis of variance was used to evaluate the difference of absorption/permeability parameters in four intestinal sections or three ZLR-8 concentrations between crude material and SDE. Nemenyi's test was used to evaluate the effect of intestinal section or ZLR-8 concentration in crude material or SDE. In the dissolution test, repeated measures analysis of variance was performed to compare the dissolution curves of four samples. The dissolution amount at 120 min was statistically compared using a paired-samples *t*-test. Mann–Whitney *U*-test was used to compare the pharmacokinetic properties between crude material and SDE.

Results

Particle size and distribution

The particle size of PE was 63.88 ± 0.076 μm ($n = 3$) with a wide Span of 1.352 and a large PI of 0.633. To decrease the side effect the gut, a semi-synthetic polymer, HPMC, was used, which has relative poor emulsifying efficacy. Without strong mechanical power in the process of preparation, the resultant coarse emulsion showed a large particle size and multi-peak distribution (Figure 2). After homogenization, the particle size of HE was decreased sharply to 0.211 ± 0.001 μm ($n = 3$) with a PI of 0.092 and a Span of 0.460 due to the high pressure of the homogenizer. After the reconstitution of SDE, the particle size was determined to be 1.282 ± 0.004 μm ($n = 3$) with a PI of 0.105 and Span of 0.775. The increase in particle size may be due to the adhesion and amalgamation of small homogenized emulsion drops to form large emulsion drops during the spray-drying process because of the instantaneous drying.

The particle size of the reconstituted emulsion was close to that of the conventional emulsion (1–100 μm). It was reported that the mean particle size of emulsion is a function of the type and content of the oil used in the formulation and generally larger than the liquid emulsion before drying (1.5–3 μm).[15,16] Christensen *et al*. demonstrated, in the preparation of a blank dry emulsion, that the particle sizes of homogenized emulsion and reconstituted liquid emulsion containing 30–80% oil were 0.82–3.15 μm and 0.79–7.73 μm, respectively, and increase with the increase in percentage of oil in the composition.[17] The particle size of reconstituted liquid emulsion containing 50% of oil (1.282 μm) was slightly lower than the particle size value in the reference. In the references, the reconstituted liquid emulsion was prepared by vortexing for 1 h using a Heto Mastermix rotator 20 rpm[18] or Heidolph Reax 2 rotam at 20 rpm for 2 h.[19] In the study, the reconstituted liquid emulsion was prepared just by vortexing for 1 min. If it was vortexed at 20 rpm for 30 min, the particle size may decrease to be 0.545 μm, a value close to HE.

In-vitro release study

A biphasic stirred transfer device provided a discriminating way to compare the dissolution profiles of the ethanolic solution, PE, HE and SDE according to their droplet sizes. The ethanolic solution underwent rapid dissolution, with 83% ZLR-8 released after approximately 30 min (Figure 3). Although transferred at a relative slower rate than the solution, the homogenized emulsion and the reconstituted SDE achieved a similar complete dissolution amount ($P > 0.05$), with about 80% released at 2 h. Before the transfer of ZLR-8 in SDE into hexane, a dispersion process of SDE in the bottom liquid phase (40% ethanol) and subsequent dissolution were necessary. This caused the relative slower release of ZLR-8 from SDE. As a comparison, the ethanolic solution of ZLR-8 can immediately begin the transfer process once the rotation began. In the earlier release period, the relative lower release of SDE compared with HE demonstrated the effect of the dispersion process. It can also be concluded that the spray-drying process had no significant effect on the final dissolution amount of HE. SDE containing ZLR-8 could recover the

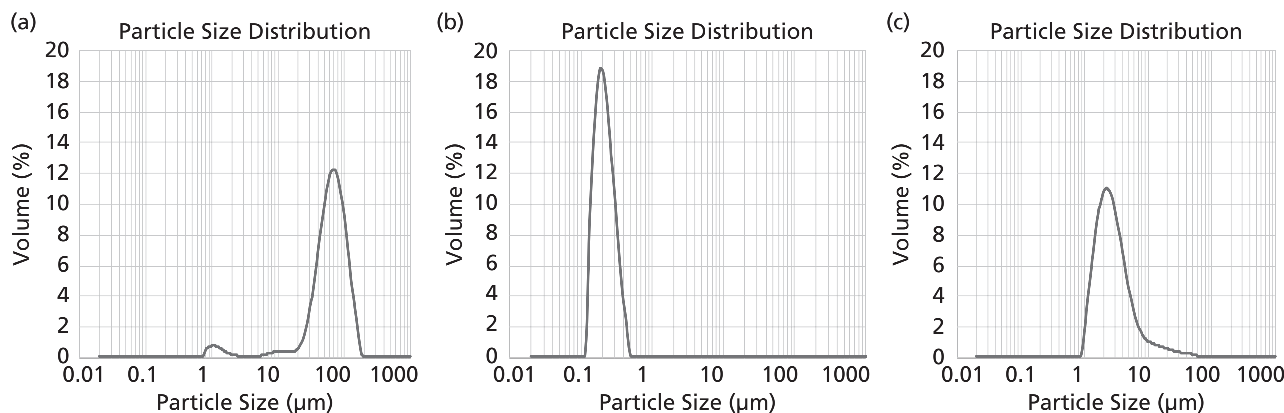

Figure 2 Droplet size distribution of coarse primary emulsion (a), homogenized emulsion (b) and reconstituted spray-dried emulsion (c).

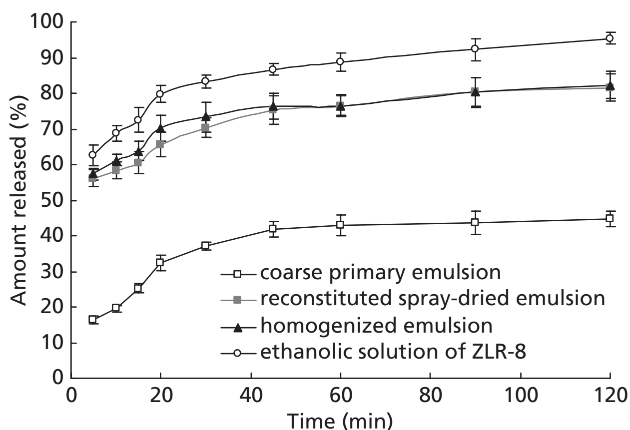

Figure 3 Percentage of ZLR-8 transferred into hexane versus time plots from different solutions. Bars represent the standard deviation obtained from the individual data ($n = 6$).

initial HE upon reconstitution without altering the drug dissolution. Compared with them, the primary emulsion with the particle size of 63.88 μm had a diminished drug release, with only 44.70% released at 2 h.

Intestinal absorption

SPIP experiments in rat's small intestine were undertaken to determine absorption rate constant and intestinal permeability of ZLR-8. Solutions of ZLR-8 with various drug concentrations were prepared for perfusion studies. To facilitate the rapid HPLC determination of ZLR-8 in perfusion buffer (less than 6 min for a sample), the chromatographic condition was different from that used for plasma determination, which requires good separation between ZLR-8 and the internal standard. ZLR-8 was found to be stable enough during the whole experiment and determination.

For each segment, absorption and permeability were determined at 40 μg/ml. As shown in Table 1, ZLR-8 was absorbed throughout the whole intestine, of which the duodenum segment exhibited the largest absorption ability. No significant difference in the two absorption parameters was found between jejunum and ileum ($P > 0.05$). Rank order of absorption rate constants and intestinal permeability of different intestine segments for ZLR-8 was duodenum > jejunum ≈ ileum > colon. The absorption and permeability of the duodenum segment were significantly higher than those in the jejunum, ileum and colon ($P < 0.01$).

It can also seen from Table 1 that SDE of equivalent ZLR-8 concentration significantly enhanced the intestinal absorption rate of ZLR-8 in the duodenum and jejunum ($P < 0.01$), which are the main absorption sites in rats.[19,20] However, SDE exhibited an indistinctive effect on the absorption in ileum and colon. It is suggested that the upper section of the small intestine is the main absorption site of ZLR-8. Meanwhile, no significant change in the permeability was observed in all intestinal sections of rats ($P > 0.05$).

The duodenum segment was selected for the perfusion at different drug concentrations. It was shown that the drug concentration from 20 μg/ml to 80 μg/ml had no significant effect on the absorption rate constant and intestinal permeability of ZLR-8 ($P > 0.05$) (Table 2).

Enhancement of bioavailability

To confirm the usefulness of SDE in improving the bioavailability of ZLR-8, an in-vivo test was carried out in rats and pharmacokinetic parameters of ZLR-8 solution, ZLR-8 suspension and ZLR-8 SDE were compared.

After gavage administration of ZLR-8 suspension, the plasma levels of diclofenac were very low, all being below the limit of detection, with AUC_{0-12h} of only about 0.32 (μg·h)/ml. The main pharmacokinetic parameters of diclofenac in rats after oral administration of diclofenac sodium solution and SDE are summarized in Table 3. A two-compartment model gave the best fit to the plasma diclofenac concentration–time curves. Mean plasma diclofenac concentration was plotted as a function of time and shown in Figure 4. It was found that the relative bioavailability of SDE was dramatically enhanced compared with ZLR-8 suspension. Calculated on AUC_{0-12h} of diclofenac, the mean relative bioavailability of SDE was 105.4-fold that of ZLR-8 suspension. Compared with diclofenac sodium solution, ZLR-8 SDE produced a significantly decreased C_{max} and a significantly increased T_{max}.

Discussion

Intestinal absorption

As a scientific framework, the Biopharmaceutics Classification System (BCS) takes into account three major factors that govern the rate and extent of drug absorption from solid dosage forms (dissolution, solubility and intestinal permeability) and classifies the drugs into four categories: Class 1(high solubility/high permeability), Class 2 (low solubility/high permeability), Class 3 (high solubility/low permeability) and Class 4 (low solubility/low permeability).[21] A drug can be considered highly permeable when the extent of absorption in humans is determined to be greater than 90%. Apart from direct measurements across human intestinal membrane, human intestinal permeability values may be predicted from rat SPIP experiments. According to the intestinal permeability correlation between humans and rats, the human permeability ($P_{eff,\ Human}$) and the dose absorbed in humans (fa_{Human}) can be estimated by the following equation:[22]

$$fa_{Human} = 1 - e^{-\left[2\times\left(3.6\times P_{eff,rat} + 0.03\times10^{-4}\right)\times t_{trans}\right]/(2.8\times r)} \quad (5)$$

where t_{trans} is the average small intestine transit time (3 h) and r is the average human small intestine radius (1.75 cm).

It was indicated that the dose absorbed in humans increased with increase in the permeability and the absorption will be greater than 90% when $P_{eff,Human} \times t_{res}/(2.8 \times r)$ is not less than 1.15 or $P_{eff,rat}$ is greater than 8.69×10^{-3} cm/min. Applying this equation to ZLR-8 for the lowest observed $P_{eff,rat}$ (1.59 cm/s, duodenum at 40 μg/ml) in this study gives an estimated $P_{eff,Human}$ for ZLR-8 of 92%. Taking into account the extremely low aqueous solubility and high permeability, ZLR-8 can be classified into BCS Class 2.

It is essential that we spare no effort to improve the bioavailability of this BCS Class 2 drug. Although solution

Table 1 Absorption rate constant and permeability of ZLR-8 crude material and spray-dried emulsion in different intestinal sections

Intestinal section	ZLR-8 crude material		ZLR-8 Spray-dried emulsion	
	$K_a \times 10^{-2}$(1/min)	$P_{eff} \times 10^{-4}$(cm/s)	$K_a \times 10^{-2}$(1/min)	$P_{eff} \times 10^{-4}$(cm/s)
Duodenum	16.81 ± 2.23	1.59 ± 0.18	28.16 ± 2.06[a]	1.62 ± 0.12[b]
Jejunum	11.43 ± 0.76	0.99 ± 0.13	18.73 ± 1.97[a]	1.07 ± 0.13[b]
Ileum	12.17 ± 0.06	0.11 ± 0.04	14.02 ± 0.64[b]	0.098 ± 0.019[b]
Colon	6.59 ± 0.88	0.074 ± 0.0011	7.21 ± 1.03[b]	0.076 ± 0.017[b]

Data represent means ± SD, $n = 6$. [a]$P < 0.05$, compared with the values of ZLR-8 crude material; [b]$P > 0.05$, compared with the values of ZLR-8 crude material.

Table 2 Absorption rate constant and permeability of ZLR-8 crude material and spray-dried emulsion from duodenum at different concentration

Concentration (μg/ml)	ZLR-8 crude material		ZLR-8 Spray-dried emulsion	
	$K_a \times 10^{-2}$(1/min)	$P_{eff} \times 10^{-4}$(cm/s)	$K_a \times 10^{-2}$(1/min)	$P_{eff} \times 10^{-4}$(cm/s)
20	16.49 ± 2.61	1.69 ± 0.37	25.21 ± 1.75[a]	1.76 ± 0.22[b]
40	16.81 ± 2.23	1.59 ± 0.18	28.16 ± 2.06[a]	1.62 ± 0.12[b]
80	15.29 ± 2.69	1.64 ± 0.32	21.43 ± 3.45[a]	1.68 ± 0.19[b]

Data represent means ± SD, $n = 6$. [a]$P < 0.05$, compared with the values of ZLR-8 crude material; [b]$P > 0.05$, compared with the values of ZLR-8 crude material.

Table 3 Pharmacokinetic parameters of diclofenac in rats following oral administration of diclofenac sodium solution and ZLR-8 spray-dried emulsion

Parameter	Diclofenac sodium solution	ZLR-8 spray-dried emulsion
A (μg/ml)	107.4 ± 54	34.42 ± 16
A (1/h)	1.921 ± 0.64	0.6000 ± 0.043
B (μg/ml)	6.094 ± 0.48	1.067 ± 0.29
B (1/h)	0.1168 ± 0.038	0.01000 ± 0.00
K_a (1/h)	6.885 ± 1.3	0.8660 ± 0.15
K_{10} (1/h)	0.8894 ± 0.38	0.3030 ± 0.072
K_{12} (1/h)	0.9900 ± 0.35	0.2530 ± 0.082
K_{21} (1/h)	0.2669 ± 0.037	0.07300 ± 0.015
$t1/2_{Ka}$ (h)	0.1033 ± 0.018	0.8190 ± 0.14
$t1/2_{\alpha}$ (h)	0.3963 ± 0.14	1.160 ± 0.082
$t1/2_{\beta}$ (h)	6.425 ± 2.0	60.63 ± 19
$AUC_{(0-12)}$ ((μg·h)/ml)	75.39 ± 8.8	25.19 ± 6.9
$AUC_{(0-\infty)}$ ((μg·h)/ml)	88.98 ± 13	33.73 ± 6.7
T_{max} (h)	0.3 ± 0.11	1.6 ± 0.22
C_{max} (μg/ml)	47.88 ± 4.1	5.642 ± 1.4
V_c (ml/g)	0.7474 ± 0.40	6.451 ± 2.3
CL ((g·h)/ml)	0.4732 ± 0.069	1.851 ± 0.46

A, distribution phase; B, elimination phase; α, distribution rate constant; β, elimination rate constant; k_a, absorption rate constant; $t1/2_{\alpha}$, distribution half-life time; $t1/2_{\beta}$, elimination half-life time; $t1/2_{Ka}$, absorption half-life time; k_{12} and k_{21}, transportation constants; k_{10}, elimination constant; AUC, area under curve; V_c, apparent volumes of distribution of the central compartments; CL, total body clearance; C_{max}, maximum concentration; T_{max}, time to reach C_{max}. Data represent means ± S.D, $n = 5$.

may enhance its solubility, ZLR-8 may precipitate immediately once in contact with gastrointestinal fluid to form a suspension. So the preparation of SDE meets this goal just in time by keeping ZLR-8 in a solubilized state even in aqueous fluid.

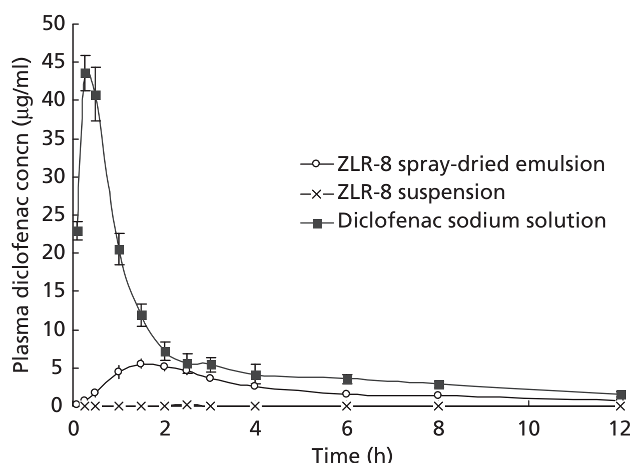

Figure 4 Mean plasma diclofenac concentration–time curves after intragastric administration in rats. Bars represent the standard deviation obtained from the individual data ($n = 5$).

Enhancement of bioavailability

As a NO-releasing derivative of diclofenac, ZLR-8 may be metabolized into diclofenac along with the release of NO; the therapeutic action of ZLR-8 may be the concurrent effect of diclofenac and NO.[23] Thus, although the bioavailability of ZLR-8 SDE was 37.91% that of diclofenac sodium solution, ZLR-8 has evident anti-inflammatory and analgesic activity and has lower gastrointestinal side effects than diclofenac.[1,2] It was previously demonstrated in an in-vitro study that NO can hardly be released from ZLR-8 suspension. The slow release of diclofenac from ZLR-8 SDE might be due to the NO release behaviour of ZLR-8, which may be beneficial for achieving lower gastrointestinal toxicity.

In addition, we constructed an accurate and precise RP-HPLC analytical method for ZLR-8 detection and tried to

perform a bioavailability study of ZLR-8 given to rats. However, the concentration of ZLR-8 in rat plasma was lower than the limit of detection. Jiao *et al*. determined the concentration of ZLR-8 in beagle dogs to be below 0.6 μg/ml.[4] The limit of quantification for ZLR-8 of their gradient HPLC method was 0.05 μg/ml, similar to that in our report (0.0625 μg/ml). This indicates the difference in absorption of ZLR-8 in different animals and ZLR-8 might have a poorer absorption from the gastrointestinal tract in rats. In in-vitro studies, the solubility of ZLR-8 in aqueous media was found to be lower than 0.1 μg/ml and the ZLR-8 will precipitate immediately from its PEG 400 solution even if a small amount of aqueous media was present. It was difficult to determine its *logP*, which is estimated by ACD/*logP* (Advanced Chemistry Development Inc. Toronto, Ontario, Canada) to be as high as 7.7 ± 0.54.

Conclusions

In summary, we prepared a spray-dried emulsion of ZLR-8, the nitric oxide-releasing derivative of diclofenac, which was demonstrated to be BCS Class 2 drug. SDE significantly improved its intestinal absorption by maintaining its solubilized state in aqueous fluid and resulted in a dramatic improvement in its bioavailability. Considering the significant increase in bioavailability compared with ZLR-8 crude material and the thermodynamically stable solid drug delivery system, SDE can be an effective formulation strategy for other drugs with low oral absorption.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Yu XL *et al*. Pharmacological activity screening of furoxan-coupled diclofenac compounds. *J Chin Pharm Univ* 2001; 32: 301–305.
2. Li RW *et al*. Synthesis and anti-inflammatory analgesic activities of phenylfuroxan-coupled diclofenac. *Acta Pharm Sin* 2002; 37: 27–32.
3. Kagan L *et al*. Selection of drug candidates for gastroretentive dosage forms: pharmacokinetics following continuous intragastric mode of administration in a rat model. *Eur J Pharm Biopharm* 2008; 69: 238–246.
4. Jiao H *et al*. Simultaneous determination of ZLR-8 and its active metabolite diclofenac in dog plasma by high-performance liquid chromatography. *Biomed Chromatogr* 2007; 21: 382–388.
5. Zheng ZJ *et al*. Formulation study of spray-dried emulsion of ZLR-8: a nitric oxide releasing anti-inflammatory drug. *J Chin Pharm Univ* 2006; 37: 33–36.
6. Dollo G *et al*. Spray-dried redispersible oil-in-water emulsion to improve oral bioavailability of poorly soluble drugs. *Eur J Pharm Sci* 2003; 19: 273–280.
7. Hansen T *et al*. Process characteristics and compaction of spray-dried emulsions containing a drug dissolved in lipid. *Int J Pharm* 2004; 287: 55–66.
8. Cummins CL *et al*. In vivo modulation of intestinal CYP3A metabolism by P-glycoprotein: studies using the rat single pass intestinal perfusion model. *J Pharmacol Exp Ther* 2003; 305: 306–314.
9. Berggren S *et al*. Characterization of jejunal absorption and apical efflux of ropivacaine, lidocaine and bupivacaine in the rat using in situ and in vitro absorption models. *Eur J Pharm Sci* 2004; 21: 553–560.
10. Issa C *et al*. Implications of density correction in gravimetric method for water flux determination using rat single-pass intestinal perfusion technique: a technical note. *AAPS Pharm Sci Tech* 2003; 4: E16.
11. del Amo EM *et al*. In vitro–in vivo correlation in P-glycoprotein mediated transport in intestinal absorption. *Eur J Pharm Sci* 2009; 36: 200–211.
12. Dahan A *et al*. Segmental-dependent membrane permeability along the intestine following oral drug administration: evaluation of a triple single-pass intestinal perfusion (TSPIP) approach in the rat. *Eur J Pharm Sci* 2009; 36: 320–329.
13. Szakács G *et al*. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). *Drug Discov Today* 2008; 13: 379–393.
14. Rathore R *et al*. Simultaneous determination of hydrazinocurcumin and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation. *J Pharm Biomed Anal* 2008; 46: 374–380.
15. Christensen KL *et al*. Technical optimization of redispersible dry emulsions. *Int J Pharm* 2001; 212: 195–202.
16. Shively ML. Characterization of oil-in-water emulsions prepared from solid-state emulsions: effect of matrix and oil phase. *Pharm Res* 1993; 10: 1153–1156.
17. Christensen KL *et al*. Preparation of redispersible spray-dried emulsions by spray drying. *Int J Pharm* 2001; 212: 187–194.
18. Christensen KL *et al*. Physical stability of redispersible spray-dried emulsions containing amorphous sucrose. *Eur J Pharm Biopharm* 2002; 53: 147–153.
19. Jannin V *et al*. Approaches for the development of solid and semi-solid lipid-based formulations. *Adv Drug Deliv Rev* 2008; 60: 734–746.
20. Zhang L *et al*. Preclinical characterization of intestinal absorption and metabolism of promising anti-Alzheimer's dimer bis(7)-tacrine. *Int J Pharm* 2008; 357: 85–94.
21. US Food and Drug Administration. *Guidance for Industry: Waiver of in Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*. Rockville, MD: US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, 2000.
22. Fagerholm U *et al*. Comparison between permeability coefficients in rat and human jejunum. *Pharm Res* 1996; 13: 1336–1342.
23. Feng XC *et al*. Effect of ZLR-8 on the healing of gastric ulcer and NO releasing in vitro. *J Chin Pharm Univ* 2004; 35: 357–360.