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The absorption behavior of cyclosporin A lecithin vesicles in rat intestinal tissue

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Abstract

The purpose of the study was to investigate the absorption behavior of lecithin vesicles of cyclosporin A (CsA-VES), prepared by the rotary evaporation method and treated further with sonication. The everted gut sac technique and in situ circulation method were used to examine: (1) relationship between the CsA-VES absorption velocity and the CsA-VES content; (2) the influence of the intestinal mucus, blank vesicles, concentration of Na^+, energy inhibitor and P-gp inhibitor on the absorption of CsA-VES; and (3) the respective accumulated content of CsA in the incubating medium and the sacs after incubation with Sandimmum Neoral®(CsA-NEO) and CsA-VES. Our results showed there was a saturated absorption of CsA. Most CsA-VES accumulated in the mucus before it reached the intestinal tissue. There was no significant difference in the accumulated absorption content in the incubating medium and the sacs of CsA-NEO and CsA-VES. The addition of blank vesicles and concentration of Na^+ had no significant influence on the accumulated absorption of CsA ($P > 0.05$).The energy inhibitor and P-gp inhibitor influenced the accumulated absorption of CsA significantly ($P < 0.05$). CsA-VES may be transported by phagocytosis. Mucus was a barrier blocking the diffusion of CsA-VES. CsA-NEO and CsA-VES showed equal absorption levels in the intestine.

Keywords: Lecithin vesicles; Cyclosporin A; Everted gut sac technique; In situ circulation method; Absorption behavior

1. Introduction

Knowledge of lipid vesicles has become more diverse and deep after many years of research. However, the absorption mechanism of lipid vesicles from the gastrointestinal tract is still unclear (Chen and Langer, 1998).

We previously incorporated the model drug CsA, a lipophilic cyclic polypeptide with irregular oral absorption (Klompmaker et al., 1993), into lipid vesicles and compared its pharmacokinetic behavior in rabbits with Sandimmum Neoral®(CsA-NEO), a microemulsion formulation of CsA (Kovarik et al., 1994). The results demonstrated both preparations were bioequivalent (Guo et al., 2001). In the present study, in order to further investigate the absorption of CsA-VES in rats, the everted gut sac technique and in situ circulation method are used to test: (1) the relationship between CsA-VES absorption velocity and CsA-VES content, (2) the influence of the intestinal mucus, blank vesicles, concentration of Na^+, energy inhibitor and P-gp inhibitor on the absorption of CsA-VES, and (3) the respective accumulated content of CsA in the incubating medium and the everted-sacs after incubation with Sandimmum Neoral® (CsA-NEO) and CsA-VES. This research is

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based on the quantification of intestinal absorption of CsA-VES.

The everted-sac is a simple and useful in-vitro model to study the drug absorption (Barthe et al., 1999). The system provides information on drug absorption mechanisms through testing the drug content in the intestinal and transported through the intestinal tissue. It has been used to study the uptake of lipid vesicles (Rowland and Woodley, 1981), proteins and macromolecules with oral drug delivery potential, bioadhesive lecithins and synthetic nondegradable polymers. It reflects the uptake and absorption of tested drug quantitatively. However, in situ circulation method is the nearest to the in vivo system and is good for producing some kinetic data, particularly as the blood supply, innervations and clearance capabilities of the animal remain intact. Furthermore the input of the drug and the drug compound can be closely controlled in term of concentration, pH, osmolality, intestinal region, and flow rate (Barthe et al., 1999).

2. Materials and methods

2.1. Materials

Soybean lipid (purity >80%, lot number: 980607) was purchased from Shanghai Fuda Pharmaceutical Manufacturing Factory (China) and lecithin was freshly prepared by column chromatography on aluminum oxide according to the established methods. Only lecithin with purity >95% was used in this study. CsA powder (USP 23, lot number: 040396) was obtained from Galena (Czech Republic). Sandimmum Neoral® (lot number: 322MFD0598) was purchased from Novartis Pharma AG, Basle Switzerland. Verapamil was purchased from Henrui Pharmaceuticals, Lianyungang China. Methanol, chloroform, sodium chloride and other reagents, all p.a. were products of Nanjing Chemical Corporation (China).

2.2. Preparation of lecithin vesicles

Lecithin vesicles of CsA (CsA-VES) were prepared by the conventional evaporation-sonication method. Appropriate amounts of lecithin (4 wt.%) and CsA (0.375 wt.%) were dissolved in co-solvent of methanol and chloroform (1:1). The mixture was dried to a thin film under vacuum. This film was then hydrated with 0.9% NaCl solution to make a coarse lipid suspension. Sonication was carried out at 4 °C (JY 92-II ultrasonic processor, China) to obtain small vesicles.

2.3. Determination of CsA

Concentrations of CsA were determined by high-pressure liquid chromatography (HPLC). The HPLC system consisted of a pump (Model LC-5A, Shimadzu, Japan), a shim-pack CLC-ODS column (150 mm × 6 mm i.d., Shimadzu, Japan) maintained at 70 °C, an UV detector (Model SPD-6A, Shimadzu, Japan) at 210 nm and a data station (Model C-R6A, Shimadzu, Japan). The mobile phase was composed of 72% (v/v) methanol and 28% water, and delivered at a flow rate of 1.2 ml min^{-1}. The injection volume was 20 μl and the relative retention time was found to be 11 min.

Content determination was carried by HPLC by dissolving both preparations in absolute ethanol.

2.4. Intestinal everted-sac experiments

SD rats weighing (200–250 g, provided by Central Animal Laboratory of China Pharmaceutical University) were anesthetized with 20% ethyl carbamate solution by two 0.7 ml, i.p. injections 15 min apart. A 20 cm segment of jejunum was quickly removed, rinsed with Tyrode buffer and everted (Barthe et al., 1998). This segment was tied at one end with a cotton thread, filled with oxygenated Tyrode buffer and then tied at the other end. The resultant large sac was divided into four 3–4 cm sacs by tying at intervals. Each sac contained about 0.5 ml oxygenated Tyrode buffer. The rat was then sacrificed by injection of ethyl carbamate into the heart.

Each sac was individually placed in a 15-cm high glass tube containing 3 ml oxygenated Tyrode buffer kept in a 37 °C water bath. After equilibration at this temperature for 5 min, 1 ml of lecithin vesicle suspension was added to each tube to initiate the experiment. The whole samples of inside medium were taken at 15 min, 30 min, 1 h, 1.5 h, and 2 h, then fresh oxygenated Tyrode buffer was added to the sacs and incubation was continued.

At the end of the 2 h experiment, the everted-sacs were removed from the tube, rinsed with cold

Tyrode buffer three times, and blotted dry. The area of each sac was measured. Then each sac was homogenized with 2 ml double distilled water after the measurement. One hundred microlitre sample of inside medium or homogenized sac were extracted with 2.5 ml of ether and 2.5 ml of hexane. After shaking for 5 min and centrifugation for 5 min at 3000 rpm, the organic phase was transferred to a 5 ml conical tube for evaporation until dryness, under a stream of nitrogen at 35 °C. Residues were re-dissolved in 100 μl of the mobile phase and 150 μl of hexane, then shaken for 1 min and centrifuged for 5 min at 3000 rpm. An aliquot of 20 μl was injected into the HPLC.

2.5. In situ uptake experiment

The in situ circulation method (Chaing and Weiner, 1987; Fagerholm et al., 1996) was used in these experiments. SD rats weighing 200–250 g were anesthetized with 20% ethyl carbamate solution by two 0.7 ml i.p. injections 15 min apart. A midline abdominal incision was made and the small intestine was exposed. The proximal end of the jejunum was cannulated with a glass tube. A second cannula was then placed 20 cm distal to the first and the segment was rinsed with normal saline at 37 °C until the washing appeared clear. The cannulae were then connected to two plastic tubes that were connected to a constant-flow pump. A 10 ml measuring cylinder was used to contain the circulation fluid. CsA-VES (1 ml/200 g) was added to the measuring cylinder and diluted to 10 ml with normal saline. The experiment began when the circulation fluid flowed into the segment. The volume of the circulation fluid at 15 min, 30 min, 1 h, 1.5 h, and 2 h was recorded. A 50 μl sample was then taken and 50 μl blank circulation fluid was added to the measuring cylinder. The sample was diluted 100 times with absolute ethanol and measured by HPLC. The area of the segment was measured after the experiment.

2.6. Study design

2.6.1. The absorption of CsA-VES in rat intestinal tissue

CsA-VES of five different concentrations from low to high (2.05, 2.86, 6.07, 8.06, and 9.16 mg ml^{-1}) were prepared. The absorption velocity of CsA-VES at each concentration in the rat intestinal in 2 h were tested by the everted-sac technique. Then CsA-VES of other five different concentrations (1, 1.5, 2.4, 3.8, and 7.5 mg ml^{-1}) were prepared. The absorption velocity was tested by the in situ circulation method. Information on the absorption of CsA-VES in rat intestinal could be obtained from the relationship between the CsA-VES absorption velocity and the CsA-VES content.

2.6.2. The influence of mucus, blank vesicles, concentration of Na^+, energy inhibitor and P-gp inhibitor

Four everted-sacs were incubated with CsA-VES by the everted-sac technique. At the end of the 2 h experiment, four everted-sacs were removed from the tube, rinsed with cold Tyrode buffer three times. Then two were blotted dry and the mucus was peeled. The mucus of the other two was left as it was. CsA was extracted from the four sacs as described before, compared the CsA content of the sacs' intestinal walls with and without the mucus.

Blank lecithin vesicles whose lecithin concentration was 15 mg ml^{-1} were prepared. First, this suspension was circulated in the empty rat intestinal for half an hour, and then the CsA-VES was added into this solution and operated as the in situ circulation method, recording, taking samples and measuring.

The Na^+ requirement was examined by lowering the Na^+ concentration from 154 mM (control) to 20 mM (the osmolarity was compensated with K^+). The CsA-VES was diluted using solution with 20 mM Na^+ to 10 ml and it was circulated for 2 h using the above mentioned method, recording, taking samples and measuring.

One millimolar 2,4-dinitrophenol (DNP) and 1 mM verapamil was added to the circulation fluid separately and then the fluid was circulated for 2 h, recording, taking samples and measuring as the method mentioned above.

2.6.3. The comparison of CsA-NEO and CsA-VES

Eight everted-sacs were incubated with CsA-NEO and CsA-VES of same concentration (3.72 mg ml^{-1}). At the end of the 2 h experiment, the everted-sacs were removed out, drained of the inside medium, rinsed with cold Tyrode buffer three times and blotted dry.

The CsA content of CsA-NEO and CsA-VES in the inside medium and the sacs were determined and compared.

3. Results

3.1. Determination of CsA

The regression equation for CsA content ($\mu g\,ml^{-1}$) in ethanol solution ranging from 0.1 to 8 $\mu g\,ml^{-1}$ was $A = 7.52 \times 10^3 C - 74.66$ ($r^2 = 0.9999$). The mean recovery was $97.4 \pm 5.9\%$ ($n = 3$). Precision assay showed the average of the relative standard deviations (S.D.) within 1 day was 1.5% and between day was 2.0%.

The regression equation for CsA content ($\mu g\,ml^{-1}$) in the intestinal ranging from 10 to 100 $\mu g\,ml^{-1}$ was $A = 4.73 \times 10^3 C - 4382.8$ ($r = 0.9993$). The regression equation for CsA content ($\mu g\,ml^{-1}$) in the circulation fluid ranging from 0.5 to 10 $\mu g\,ml^{-1}$ was $A = 8.27 \times 10^3 C - 129.71$ ($r = 0.9999$).

3.2. The absorption behavior of CsA-VES in rat intestinal tissue

Fig. 1 shows the relationship of the CsA-VES absorption velocity and its concentration in rat sac. It can be seen that the absorption velocity increased with the increase of the concentration up to 5.77 mg/ml. However, the absorption velocity remained the same when the concentration increased from 5.77 to 9.16 mg/ml. Fig. 2 shows the relationship of the cumulative CsA-VES absorption and its concentration in situ. When the concentration of drug in CsA-VES is increased to 2.4 $mg\,ml^{-1}$, the cumulative absorption also increased. However, the absorption seemed saturated and no increase in cumulative absorption was observed when the concentration of drugs in CsA-VES increased from 2.4 to 7.5 $mg\,ml^{-1}$.

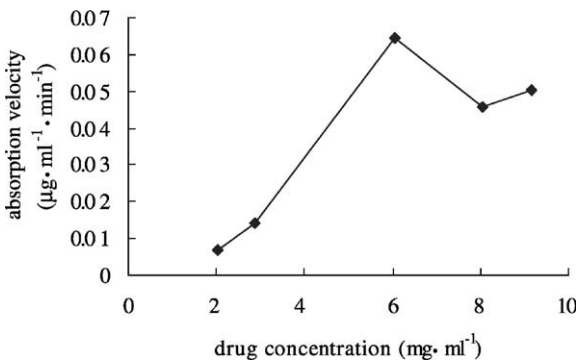

Fig. 1. The relation between the concentration of CsA and the absorption velocity in rat sac.

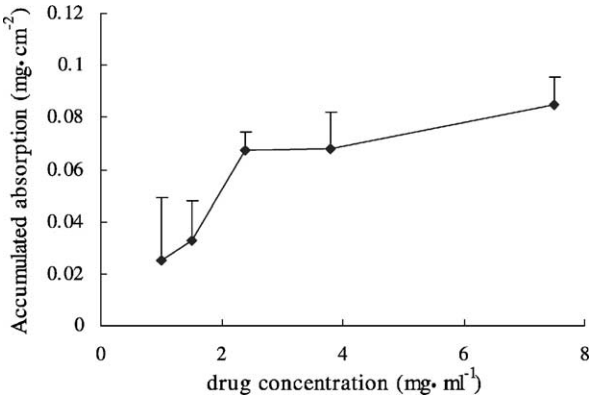

Fig. 2. The relation between the concentration of CsA and the accumulated absorption in situ.

Fig. 3 shows the change of the absorption of the CsA-VES after using blank lecithin vesicles, lowering the concentration of Na^+, adding DNP and Verapamil. Based on cross group *t*-test results, there were no significant effects of adding the blank lecithin vesicles and lowering Na^+ ($P > 0.05$). However, DNP and Verapamil had significant effects on the absorption ($P < 0.05$).

After the 2 h incubation, the CsA content in sacs with mucus and without mucus was $16.39 \pm 3.50\ \mu g\,cm^{-2}$ and $2.36 \pm 0.76\ \mu g\,cm^{-2}$, respectively. The former was seven times more than that the latter.

The accumulated CsA content of CsA-NEO and CsA-VES in inside medium were $0.40 \pm 0.17\ \mu g\,cm^{-2}$ and $0.50 \pm 0.09\ \mu g\,cm^{-2}$. Cross group *t*-tests showed there were no significant differences in the accumulated CsA content of inside medium between CsA-NEO and CsA-VES ($P > 0.05$). Similarly, cross group *t*-test showed there were no significant differences in the accumulated CsA content in sacs of both preparations ($P > 0.05$).

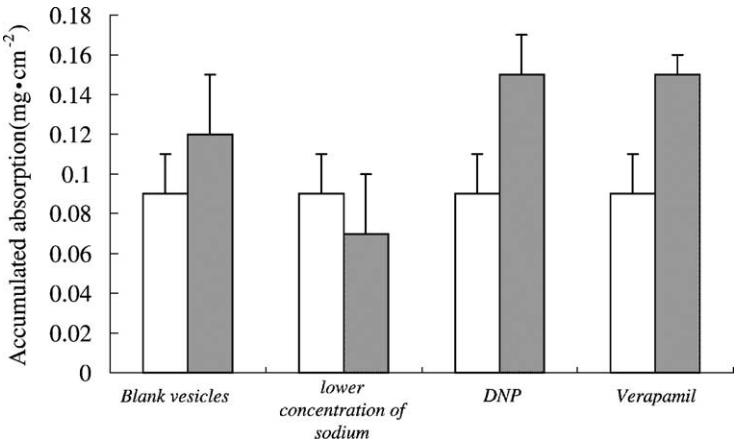

Fig. 3. The influence of blank vesicles, lower concentration of sodium, energy inhibitor DNP and P-gp inhibitor verapamil on the accumulated absorption of CsA in situ.

4. Discussion

According to this study, there are several conclusions which need to be evaluated further.

First, P-gp inhibitors can significantly increase the absoprtion of CsA-VES in the rat intestine. Therefore, the vesicles can not completely protect cyclosporin A from the efflux of P-gp. Second, the Na^+ concentration had no significant influence on the absorption of CsA-VES. Third, the absorption of CsA-VES is not energy-dependent. Otherwise, DNP would inhibit the absorption. The finding that DNP significantly increased the absorption of CsA-VES showed yet DNP inhibits the effect of P-gp. It is inferred that CsA-VES may be transported by phagocytosis because the absorption reached saturation.

Most CsA-VES accumulated in the mucus before it reached the intestinal wall. The main ingredient of mucus was mucoserous polysaccharides and proteins (Tu, 1998). CsA-VES may be adsorbed or combined with the mucoserous polysaccharides and proteins. So mucus proves to be the barrier blocking the diffusion of CsA-VES. The retention in the mucus or the adsorption to or combination with mucoserous proteins strengthened the reaction between the CsA-NEO and the absorption site. It prolongs the retention time of drug at the absorption site and promotes the transport.

There was no significant difference in the accumulated absorption content in the incubating medium and the sacs of CsA-NEO and CsA-VES. This is consistent with the results we have obtained from bioavailability experiments carried out in rabbits (Guo et al., 2001). The everted gut sac technique and in situ circulation method further demonstrated that the Sandimmum Neoral® (CsA-NEO) and CsA-VES are bioequivalent.

Acknowledgements

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