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Original article

The Passive Diffusion Improvement of Vitamin B₁₂ Intestinal Absorption by Gelucire that fit for Commercialized Production

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Original Article

Title: The Passive Diffusion Improvement of Vitamin B₁₂ Intestinal

Absorption by Gelucire that fit for Commercialized Production

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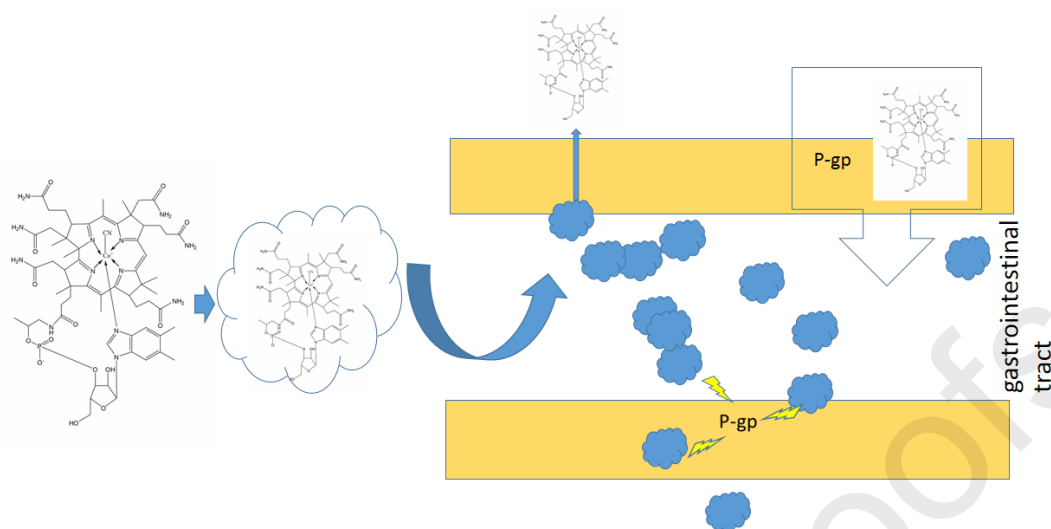
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Abstract:

Vitamin B₁₂ (VB₁₂) is a vital micronutrient to maintain the normal state of the hematopoietic system. It must be obtained from the diet since the human body cannot synthesize it. Moreover, the absorption of VB₁₂ needs to be mediated by intrinsic factor on the gastrointestinal (GI) track. The abnormalities in the stomach or lack of such intrinsic factors may result in poor oral absorption of VB₁₂. However, the very advanced formulation strategies were generally very costly and still in the development stage. Thus, the objectives of the present study were to increase the VB₁₂ intestinal absorption by conventional excipients of Gelucire 44/14 (G44/14) or Labrasol, which could be potentially formulated as a cost effect balanced product.

The *in vitro* Caco-2 cell model was applied for the absorption study. A novel VB₁₂ solid dispersion was subsequently prepared and further characterized by Differential scanning calorimetry, Fourier transform infrared spectroscopy, and Scanning electron microscopy, respectively. The membrane permeability of the VB₁₂ solid dispersion was finally evaluated using *ex vivo* rat everted gut sac method. The results suggested that G44/14 could significantly enhance the intestinal absorption of VB₁₂ via P-glycoprotein inhibition *in vitro* ($P < 0.01$). The membrane permeability of VB₁₂ could be significantly ($P < 0.01$) improved by G44/14-VB₁₂ solid dispersion at a proportion of carrier: drug ratio of 20:1. The liquidified solid dispersion was finally directly filled in the hard gelatin capsules. In conclusion, the cheap and simplified process of VB₁₂ complex prepared by G44/14 could potentially increase VB₁₂ intestinal absorption, which may be liable to commercial manufacturing.

Graphical abstract.



The chemical structure of vitamin B₁₂.

Keywords:

Vitamin B₁₂ (VB₁₂), P-glycoprotein (P-gp), Gelucire 44/14 (G44/14), Intestinal absorption, Caco-2 cells, Everted gut sac.

1 Introduction

It is well-known that Vitamin B₁₂ (VB₁₂) is an essential and vital micronutrient for the body to maintain normal physiological functions. As a cofactor of methyltransferase, VB₁₂ is involved in the *in vivo* synthesis of methionine and thymine, and subsequently promoting the biosynthesis of proteins and nucleic acids.

However, the human body cannot synthesize VB₁₂, and it must be obtained from the diet. The natural food sources of vitamin B₁₂ are limited to animal foods (Pawlak R, 2016). In general, vegetarians and seniors possess a high risk of VB₁₂ deficiency, but it also occurs in young people occasionally (Ekabe et al., 2017). The daily Recommended Dietary Allowance (RDA) of VB₁₂ for adults is 2.4 µg/day (Bailey, 2004). The deficiency in VB₁₂ may lead to anemia, fatigue, emotional disorders, and other neurological complications, as well as increase the risk of myocardial infarction and stroke (Butler et al., 2006). VB₁₂ is predominantly absorbed in the terminal ileum. It is the only vitamin that needs to be absorbed with the help of an intrinsic factor, which is a glycoprotein secret by gastric parietal cells (Dror and Allen, 2008).

However, some people may still have pernicious anemia even though the dietary VB₁₂ source is sufficient, due primarily to the abnormalities in the stomach or lack of such intrinsic factors. In some countries, the prevalence of VB₁₂ deficiency is as high as 50% (McLean et al., 2008). In such a case, improving the intestinal absorption by the mechanism of passive diffusion may be an effective solution, although only 1 - 2% of VB₁₂ is generally absorbed in this way, due to the unique characters of it (Brito et al., 2018).

VB₁₂ possess high solubility and low intestinal permeability in character (Jain et al., 2015), which is not easily absorbed by the GI track. Sarti et al. have proved that VB₁₂ is the substrate of P-glycoprotein (P-gp), which also contributes to the poor membrane transportation of VB₁₂ in the GI track (Sarti et al., 2013). Although the alternative formulation of injections is also available on the market, it is not compliance to the patients for daily administration. Several other studies have summarized and compared various advanced preparations for improving the bioavailability of VB₁₂ in recent years, including nano-particles, microspheres, buccoadhesive films, etc. (Brito et al., 2018; Tiwari et al., 1999; Zhang et al., 2015). However, these methods were generally in the stage of research and development. Moreover, the strategies of these approaches are costly and are not easy for pharmaceutical or nutraceutical manufacturing scale-up. After all, the benefits groups who need to improve the oral absorption of VB₁₂ via passive diffusion are in the minority. The relatively small production batches are not conducive to the cost control for manufacturers, due to the high cost of additives and equipment that are necessary to produce those advanced formulations. Thus, a relatively simple and cost-effective formulation design that can assist the VB₁₂ absorption via passive diffusion may necessary.

The verapamil, quinidine, and cyclosporin A are well-known P-gp inhibitors (Hunter and Hirst, 1997) that may potentially be applied to improve the absorption of P-gp substrate, such as VB₁₂. However, the pharmacological active properties and unwanted side effects of these drugs limit this application (Constantinides and Wasan, 2002). Thus, adding the conventional P-gp inhibitors of conventional pharmaceutical excipients or additives to the formulation may be an effective solution. Some non-ionic surfactants were proved to possessing P-gp inhibitory effect that may potentially be used as absorption enhancers to improve the absorption of VB₁₂ (Dubray et al., 2016).

As far as we know, Okuda et al. (1960) had identified the effects of several non-ionic surfactants (Span 85, Tween 20, Tween 80, Tween 85, G-1096, G-672, Arlacel A, and Myrij52) on VB₁₂ absorption in rats. The results suggest that only Tween 80,

Tween 85 and G-1096 were found to be effectively improving the absorption of VB₁₂ when they were fed orally before the VB₁₂ administrated per oral. However, the dose exceeded the oral LD₅₀ of these surfactants (Okuda et al., 1960). Therefore, other higher safety commercially available lipid-based excipients, such as G44/14 and Labrasolare in line with our scope (Chambin and Jannin, 2005; He et al., 2005; Lukovac et al., 2010; Mori et al., 2004), as they were applied to enhance the absorption of water-soluble drugs by both increasing the passive diffusion and inhibiting the P-gp efflux (Dubray et al., 2016). Thus, there is a great opportunity to improve the oral absorption of VB₁₂ following these ways. Moreover, both G44/14 and Labrasol consist of mono-, di and triglycerides and of mono-, and di-fatty acid esters of polyethylene glycol. These excipients are mixtures of nonionic surfactants and form self-emulsifying micro-emulsion, when in contact with the water phase in the GI track. It could thus be applied for orally administrative drug formulations (Koga et al., 2002).

The aim of the current study was to identify whether G44/14 and Labrasol were able to improve the absorption of VB₁₂ applying *in vitro* Caco-2 cells transport assay method (Netsomboon et al., 2016). The appropriate formulation was subsequently designed, characterized and evaluated accordingly (Ma et al., 2016).

2 Materials and Methods

2.1 Materials

VB₁₂ standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Quinidine and cyclosporin A were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). Verapamil was from Heowns Biochemical Technology Co., Ltd. (Tianjin, China). Fluorescein sodium standard was purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICBP, China). Labrasol and G44/14 were generous gifts from Gattefossé (Saint-Priest, France). Krebs's Ringer Buffer (KRB) was purchased from Yuanye Biochemical Technology Co., Ltd. (Shanghai, China). Caco-2 cells were obtained from Key GENBioTECH Corp., Ltd. (Nanjing, China). Dulbecco's Modified Eagle's Medium (DMEM), were obtained from Gibco™ (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Ex Cell Biology, Inc. (Shanghai, China). MTT was from Sangon Biotech Co., Ltd. (Shanghai, China). Hank's balanced salt solution (HBSS), phosphate buffer saline (PBS) powder, trypsin, penicillin-streptomycin solution (100X) and dimethyl sulfoxide (DMSO, cell culture grade) were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). Transwell® Permeable Supports were from Costar® (Corning Inc., USA). The solvents were of the High Performance Liquid Chromatography (HPLC) grade. Other reagents were of the Analytical Reagent (AR) grade.

2.2 Animals

The Healthy male Sprague-Dawley (SD) rats (180-210 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. The experiment was approved by the ethics committee of Tianjin International Joint Academy of Biomedicine (Laboratory animal use approval number: SYXK (JIN) 2017-0003). The study procedures were performed in accordance with the standards set forth in the eighth edition of the Guide to the Care and Use of Laboratory Animals (published by the National Academy of Sciences, US). The animals fasted for 24 hs under a controlled ventilation room, but free access to water.

2.3 Assay of VB₁₂ and fluorescein sodium

The VB₁₂ and fluorescein sodium quantification methods are established in the current study, respectively. The fluorescein sodium, as the marker, was applied for the evaluation of paracellular leakage of the Caco-2 absorption studies (Khan et al., 2003). Both VB₁₂ and fluorescein sodium were quantified using a reversed-phase HPLC-UV system, which consisted of a L-2130 pump, and a L-2400 UV detector (Hitachi L-2000, Japan). The samples were injected into the column (250 mm × 4.6 mm, 5 μm) (Venusil XBP C18(L) at room temperature, which pre-connected with a guard column (Zorbax SB-C18, 4.6 mm i.d. × 12.5 mm, 5 μm, Agilent Technologies, USA) and eluted at the flow-rate of 1 mL/min. The mobile phase used for VB₁₂ elution consisted of the mixture of methanol and water (30:70, v/v), while in the case of fluorescein sodium assay, a mixture of methanol, water and acetic acid (70:30:0.075, v/v/v) was applied. The chromatogram was monitored at 361 nm and at 493 nm for VB₁₂ and fluorescein sodium, respectively. The limit of detection (LOD), the limit of quantitation (LOQ), the linearity, the within-day and between-day analyses precision and accuracy, and recoveries were evaluated, accordingly. The data were shown in Supplementary Material Table S1.

2.4 *In vitro* Caco-2 cells transport study

2.4.1 Cell Culture

Caco-2 cells were grown routinely on 100 mm cell culture dishes (Jet Bio-Filtration Co., Ltd., Guangzhou, China). The cell culture media composed of DMEM basic(1X) containing 4.5 g/L D-Glucose, L-Glutamine and 110 mg/L Sodium Pyruvate, supplemented with 10% of FBS and 1% penicillin-streptomycin solution (100X) was incubated at 37°C, 5% CO₂. The cells were split when reaching 70~80% confluence using 0.25% trypsin-EDTA.

2.4.2 Cell viability

Cells were plated at a density of 1.0×10^4 cells/well in 96-well microplates (Thermo Fisher Scientific, USA), incubated overnight, and were then exposed to VB₁₂ (200, 250, 500 µg/mL), G44/14 (50, 100, 200, 500, 1000, 2000, 2500, 5000 µg/mL), Labrasol (50, 100, 200, 500, 1000, 2000, 2500, 5000 µg/mL), triton X-100 (positive control, 0.2%) and the culture medium (negative control) for 24 h, respectively. Then, a 100-µL aliquot of MTT (1 mg/mL) was replaced and returned to the incubator for 4 hs at 37°C, 5% CO₂. Finally, the absorbance was measured with Multiskan™ FC microplate reader (Thermo Fisher Scientific, USA) at 570 nm.

2.4.3 Transport study of VB₁₂ in Caco-2 cells

Caco-2 cells were seeded at a density of 3.75×10^4 cells/cm² on Transwell® permeable supports (0.4 µm mean pore size, 1.12 cm² polyester membrane, Corning, USA) and grown for 21 days (Netsomboon et al., 2016). Cell monolayer integrity and viability were assessed by monitoring the transepithelial electrical resistance (TEER) using a RE1600 epithelial cell voltohmmeter (JingongHongtai Technology Co., Ltd., Beijing, China) and the leakage of fluorescein sodium. Prior to each experiment, cell monolayers grown on Transwell® inserts were rinsed twice with pre-warmed (37°C) PBS solution. The inserts were then immersed in HBSS solution for 1.5 mL and 0.5 mL on the basolateral side and apical side, respectively. After equilibration in an incubator for 30 mins, the VB₁₂ (500 µg/mL), the combinations of G44/14 (50 µg/mL, 200 µg/mL) and VB₁₂ (500 µg/mL), Labrasol (50 µg/mL, 200 µg/mL) and VB₁₂ (500 µg/mL), cyclosporin A (50 µg/mL) and VB₁₂ (500 µg/mL), verapamil (50 µg/mL) and VB₁₂ (500 µg/mL) in HBSS were added in the apical chamber, respectively, while the basolateral chamber received HBSS. Samples (200 µL) were taken from the basolateral chamber at 30, 60, 90, 120, 150, and 180 min accordingly followed by an immediate replacement of the same volume of pre-warmed fresh HBSS. The sample was subsequently treated and quantified by HPLC-UV. The apparent permeability coefficient (P_{app}) reflecting intestinal absorption was calculated as follows [20].

$$P_{app} = \frac{Q}{Act}(2)$$

Where the unit of P_{app} is cm/s. Q is the total amount of test solution permeation throughout the incubation period (µg). A is the intestinal area or Transwell® inserts membrane area (cm²). c is the initial concentration of the test solution (µg/cm³). t is the time of the transportation study (s).

Statistical data analyses were performed using one-way ANOVA (GraphPad Prism 6, GraphPad Software, USA). The values of $P < 0.05$ and $P < 0.01$ were considered significant, and very significant, respectively. All values were expressed as mean \pm SD.

2.5 Preparation of VB₁₂ solid dispersions

VB₁₂ solid dispersions were prepared by the solvent evaporation method. Briefly, VB₁₂ was completely dissolved in ethanol (20 mL) and added dropwise to the molten G44/14 at 60°C following by a thoroughly mixing for 15 mins. The ethanol was subsequently evaporated under vacuum at 45-55°C and dried in a vacuumed oven (Taisite Instrument Co., Ltd., Tianjin, China.) (under-0.1 MPa) at room temperature for 48 hours to remove the residual solvent. The solid dispersion of VB₁₂ was obtained (The mass ratio of VB₁₂: G44/14 = 1:20). It was stored at 4°C and protected from the light.

2.6 Characterization of solid dispersions

2.6.1 Differential scanning calorimetry (DSC)

The samples of VB₁₂ (a), G44/14 (b), VB₁₂ and G44/14 physical mixtures (c), VB₁₂, and G44/14 solid dispersions (d) were prepared for the present study. Prior to the test, the temperature and heat flow rate of the DSC were calibrated using the standard Indium. The samples were dried at 25°C under -0.1 MPa in the vacuum desiccators with silica gel to remove residual water. The DSC chromatogram was recorded using an EXSTAR DSC 6200 (Seiko Instruments Inc., Japan). Newly prepared samples (5 mg) were weighed and sealed in an aluminum pan prior to the test executed. A continuously heating of 10°C/min was applied to cover the range of 25-350°C.

2.6.2 Fourier transform infrared spectroscopy (FT-IR)

Samples of VB₁₂ (a) and G44/14 solid dispersions (b) in equal amounts were prepared by the potassium bromide (KBr) pellet technique and were measured using an FT-IR spectrum 65 (PerkinElmer Inc., USA). In general, dry sample powder (1.5 mg) was blended with 200 mg dry KBr. Then, the blend was ground into a fine powder using an agate mortar before being compressed into a KBr disc. Nevertheless, both the G44/14 and the physical mixtures couldn't be compressed into the KBr discs in the present study, due to the high viscosity of G44/14. Thus, only VB₁₂ and VB₁₂ dispersion samples were prepared for the FT-IR assessment. The wave number accuracy and wave number repeatability were calibrated using the standard

polystyrene film before the evolution of the sample carried out. The characteristic peaks were obtained over a wave number region of 400-4000 cm^{-1} at a resolution of 4 cm^{-1} with 4 scans.

2.6.3 Scanning electron microscopy (SEM)

The surface morphology of the dispersion was examined by a scanning electron microscopy (JCM-6000 Plus, JEOL, Japan) at an excitation voltage of 10 kV. Samples were previously mounted on a column holder using conductive tapes and coated with a thin layer of gold in a vacuum to make sure that the samples are electrically conductive. The coated films were scanned and micrographs were taken by JCM-6000 Plus.

2.7 Ex vivo rat everted gut sac transport study of solid dispersion

Although the G44/14-VB₁₂ solid dispersion was successfully prepared, the *in vivo* absorption improvement of it needs to be evaluated. However, the amount of animal's consumption may be comparatively high for carrying out an *in vivo* study. Thus, the *ex vivo* rat everted gut sac transport study method was applied in the present study, as it closely similar to the *in vivo* method but few animals used (Alam et al., 2012).

2.7.1 Preparation of everted gut sac

The test of drug absorption using an everted gut sac model was performed as mentioned previously (Li et al., 2011). Briefly, male Sprague-Dawley rats (body weight 180-210 g) were selected and fasted for 24 hours (free access to water) before the experiment. The rats were euthanized using carbon dioxide and the ileum was isolated. The contents of the intestine were gently washed with oxygenated physiological saline, and divided into 4 segments of 5.0 to 5.5 cm in length. The gut segments were inverted with a glass tube, and an intestinal sac was formed by ligaturing one end with a cotton thread, while the other end was fixed to a soft hollow plastic tube with the cotton thread. The Krebs's-Ringer buffer (500 μL) (37°C) that applied for the gut sac incubation solution was injected into the sac.

To verify the integrity and viability of the intestinal sac under the incubation conditions, the glucose transport experiments in the everted gut sacs were performed using a D-glucose assay kit (Sigma, Glucose (GO) Assay Kit, USA) to measure the D-glucose concentration both on the serosal and mucosal side (Ma et al., 2016; Li et

al., 2011).

2.7.2 Absorption study of G44/14-VB₁₂ solid dispersion using the everted gut sac method

The sacs were kept and incubated in 34 mL 37°C oxygenated Krebs's-Ringer buffers containing VB₁₂ (500 µg/mL) in the absence (control group) or presence of verapamil (50 µg/mL) or quinidine (422.5 µg/mL) (Ma et al., 2016). Different mass ratio of vitamin B₁₂ to G44/14 in the G44/14-VB₁₂ solid dispersion (1:1, 1:2, 1:5, 1:10, 1:20, the corresponding G44/14 experimental concentrations are 500, 1000, 2500, 5000, 10000 µg/mL) was also evaluated, intended to identify the starting concentration of G44/14 that would improve the permeability of VB₁₂ significantly. A gas mixture of O₂/CO₂ (95:5) was continuously supplied to the buffer. The total amount of VB₁₂ transported from the mucosa side to the serosa side in each group was measured by sampling 100 µL of the serosal medium after 90 mins and quantified using the HPLC-UV method.

2.8 Statistical data analyses

Statistical data analyses were performed using one-way ANOVA (GraphPad Prism 6, GraphPad Software, USA). The values of $P < 0.05$ and $P < 0.01$ were considered significant, and very significant, respectively. All values were expressed as mean \pm SD.

3 Results

3.1 In vitro Caco-2 cells transport study

3.1.1 Cell viability

Caco-2 cells were exposed to G44/14, Labrasol, Triton X-100 (positive control), and the culture medium (negative control) for 24 h. The cell viability was assessed with the MTT colorimetric assay (Fig. 1). The cell viability of three different concentrations of VB₁₂ was shown in Fig. 1a. These results showed that when the concentration of VB₁₂ was in the range of 200~500 µg/mL, the cell viability was greater than 90%, which was considered that the Caco-2 cells had high viability, and VB₁₂ was not toxic to the Caco-2 cells in this concentration range during the 24 hs of incubation (Zhou et al., 1995). Thus, 500 µg/mL of VB₁₂ was used for the transport study in Caco-2 Cells in the current study. The result also suggests that when the concentration of G44/14 (Fig. 1b) and Labrasol (Fig. 1c) were above 500 µg/mL and 1000 µg/mL, respectively, the cell viability significantly decreased (less than 90%). Thus, the relatively lower concentrations of G44/14 and Labrasol were applied in the following CaCO-2 transport studies that were within the safe concentration range.

Moreover, the concentrations of G44/14 and Labrasol were prepared at the same levels for the purpose of comparing the P-gp inhibition effects of the two excipients in the following studies. A high concentration (200 $\mu\text{g}/\text{mL}$) and a low concentration (50 $\mu\text{g}/\text{mL}$) were evaluated for the extent of P-gp inhibition by each excipient.

3.1.2 Transport study in Caco-2 Cells

The integrity and viability study of Caco-2 cells monolayer was firstly carried out. The permeability of fluorescein sodium tested for our study was 3.62 $\mu\text{g}/(\text{h}\cdot\text{cm}^2)$. While the TEER values of the monolayers were all above $350\Omega\cdot\text{cm}^2$ during the transport studies, which indicated that the integrity and viability of the Caco-2 Cells monolayers was suitable for the following transport study in the Transwell® Permeable Supports.

The P_{app} value of the VB_{12} (500 $\mu\text{g}/\text{mL}$) transported from the apical side to the basolateral side of the cell monolayer was measured (Fig. 2), which was the same as the previous report (Sarti et al., 2013). When the transport experiment was performed on the Transwell® chamber without seeding Caco-2 cells, the P_{app} value of VB_{12} was significantly increased compared with the control group ($P<0.01$). It further demonstrates the successful establishment of the Caco-2 cell monolayers. The P-gp inhibitors of both cyclosporin A (50 $\mu\text{g}/\text{mL}$) and verapamil (50 $\mu\text{g}/\text{mL}$) significantly promote the transport of VB_{12} by 1.88 and 2.29 times, respectively ($P<0.01$). These results indicate that the well-known P-gp inhibitors of cyclosporin A and verapamil reduced the efflux of VB_{12} by P-gp. It confirmed that VB_{12} is the substrate of the P-gp.

The results also suggest that the lower (50 $\mu\text{g}/\text{mL}$) and a higher (200 $\mu\text{g}/\text{mL}$) concentration of G44/14 could both significantly promote the transport of VB_{12} by 1.57 and 2.48 times ($P<0.01$), respectively, due to the P-gp inhibition of G44/14. Another study also suggested the same inference, in which G44/14 allowed a significant reduction in the secretory transport of a well-known P-gp substrate rhodamine 123 in Caco-2 cells in the same manner as verapamil (Dubray et al., 2016).

In contrast, the Labrasol, which was measured at the same concentrations, did not significantly promote transmembrane absorption of VB_{12} ($P>0.05$), although, it was recognized as the P-gp inhibitor that could potentially enhance the membrane permeability and the intestinal absorption of cephalexin in a previous study (Koga et

al., 2006). It may be due to the difference in polydispersity between the nonionic surfactants that may contribute to the difference in membrane lipid fluidity, which subsequently changes the membrane permeability (Koga et al., 2006).

3.2 Formulation and the characterization of VB₁₂ solid dispersions

The mass ratio of VB₁₂:G44/14 = 1:20 was applied to prepare the solid dispersion in the present study using the melt–fusion method. The characterization studies were also carried out since such a study is the part of the quality control.

3.2.1 Differential scanning calorimetry (DSC)

The DSC results of VB₁₂, G44/14, VB₁₂/G44/14 physical mixture, and VB₁₂dispersion are shown in Fig. 3. The spectrums of VB₁₂ (Fig. 3a) and G44/14 (Fig. 3b) are consistent with other studies (Ma et al., 2017; Mohamad et al., 2017). A phase transition point of VB₁₂ was recorded at 232.7 °C. Two sharp endothermic peaks were identified at 235.8 °C and 289.4 °C, respectively. These two endothermic peaks are related to the decomposition of ligands coordinated to the cobalt atoms²⁸, while G44/14 showed a sharp endothermic peak at 42.9 °C.

A sharp endothermic peak with a peak-to-valley of 42.5 °C appeared in the thermogram of VB₁₂ and G44/14 physical mixture (Fig. 3c), which is not much different from 42.9 °C of G44/14 endothermic peak (Fig. 3b). The temperature of 42.5 °C is also the characteristic peak of G44/14. There is an endothermic zone between 227.9 °C and 350 °C, which belongs to the endothermic peak group of the VB₁₂. The phase change point of 227.9 °C is close to 232.7 °C of the VB₁₂ endothermic peak (Fig. 3a). This may be due to the formation of a small amount of complex during the melting process (Knyazev et al., 2014), but in general, they are still a physical mixing.

The endothermic peaks of VB₁₂ and G44/14 solid dispersions appeared around 39.9 °C and 146.8 °C (Fig. 3d), and the phase change endothermic peak of VB₁₂ near 232.7 °C - 350 °C disappeared. The endothermic peak of the G44/14 also decreased from 42.9 °C to 39.9 °C. These results collectively suggest that VB₁₂ has been highly dispersed in G44/14. A solid dispersion and/or a solid solution may form.

3.2.2 Fourier Transform Infrared (FT-IR)

The FT-IR spectra of VB₁₂ reference standard (Chinese Pharmacopeia, 1995), VB₁₂ sample and VB₁₂dispersion are shown in Fig. 4. The spectrum of VB₁₂ sample was (Fig. 4b) in accordance with the VB₁₂ reference standard (Fig. 4a), which showed a small peak between 2700 cm⁻¹ and 3000 cm⁻¹ corresponding to the typical C-H stretch vibrations (Mohamad et al., 2017). The O-H and N-H groups of VB₁₂ showed broad peaks at 3388 cm⁻¹. (Mohamad et al., 2017) While the figure also showed a sharp peak at 2134 cm⁻¹ corresponding to the C≡N stretching vibration. Other peaks at 1668, 1571 and 1500 cm⁻¹ were observed referring to C=O, C=C, C=N respectively (Mohamad et al., 2017). The FT-IR spectrum of the VB₁₂ solid dispersion (Fig. 4c) showed that the C≡N peak of VB₁₂ changed from 2134 cm⁻¹ to 1971 cm⁻¹, suggesting that a strong intermolecular interaction of G44/14 and C≡N of VB₁₂. However, no new chemical bonds were formed, because no new characteristic absorption peaks were detected. Moreover, the C=O and C=C stretching vibration peaks were changed from 1668 cm⁻¹ and 1571 cm⁻¹ to 1671 cm⁻¹ and 1581 cm⁻¹, respectively. Meanwhile, the peaks of C-H, O-H, and N-H also changed slightly according to the FT-IR spectrum of the VB₁₂ solid dispersion in Fig. 4.

The above results collectively indicated that the wave number of the corresponding functional groups in the spectrum of the solid dispersion has different degrees of migration, compared with the FT-IR spectrum of VB₁₂.

The absorption peaks will be shifted and the peak shape will be asymmetrically widened if an intermolecular force generated between the compounds. In the current study, the wave number migration occurs. However, the wave number after the migration is still within the wave number range of the functional groups. The migration may be due to the forces between the molecules or other physicochemical interactions occurred. Therefore, it is suggested that VB₁₂ does not form a simple physical mixture with G44/14 they may combine into a new complex.

3.2.3 Scanning electron microscopy (SEM)

SEM has been used as a tool for the characterization of formulations containing G44/14 in previous studies (Kallakunta et al., 2013). Fig. 5 shows the surface morphological characteristics of VB₁₂ and the G44/14-VB₁₂ solid dispersion. VB₁₂ showed a clear crystal structure, while the G44/14-VB₁₂ solid dispersion showed the amorphous structure without crystal appearing (Fig. 5b). The amorphous structure may have formed during the preparation process in which the VB₁₂ was coated with Gelucire 44/14 before the concentration of the VB₁₂ gradually increased until saturation. In such a way, the growth of VB₁₂ crystals was inhibited (Ping et al., 2012).

3.3 *Ex vivo* rat everted gut sac transport study of the solid dispersion

Glucose is a necessary nutrient for cell survival, which can be transported through the cell membrane actively. It could thus be used to verify the integrity and viability of the everted gut sac before the experiment carried out (Fig. 6a). The glucose at the mucosal side was actively transported into the serosal side of the intestinal wall. During 90 minutes of incubation, the glucose concentration on the serosal side was significantly increased, while the level on the mucosa side decreased, indicating that the glucose was active transport of through the small intestine (Li et al., 2011). However, the concentration of glucose at the serosa side was significantly reduced after 90 mins, due to the decreased viability of the everted intestinal sac (Ma et al., 2017). Therefore, 90 mins was chosen as the incubation time for the following transport study of VB₁₂.

The results for the transport of VB₁₂ by the solid dispersion from the mucosa to the serosa side using the *ex vivo* rat everted gut sac method are shown in Fig. 6b. The transport of VB₁₂ by the combination of verapamil (50 µg/mL) or quinidine (422.5 µg/mL) was significantly higher than VB₁₂ ($P < 0.01$), respectively. It is further confirmed that VB₁₂ is the substrate of P-gp. Moreover, the combination of VB₁₂ with Gelucire 44/14 also showed a very significant transport improvement at the concentration of 10mg/mL suggesting the effectiveness of Gelucire on the permeability of VB₁₂. However, no significant improvement was detected in the case of combination of labrasol.

4 Discussion

To determine the increase of the VB₁₂ in intestinal absorption, the *in vitro* Caco-2 cell model and the *ex vivo* rat everted gut sac method were applied for absorption study due to the high reliability and reproducibility of these methods. These methods had been reported applied to quantify paracellular transport of hydrophilic molecules and to evaluate the role of absorption enhancers (Dixit and Dumbwani, 2012; Sarti et al., 2012). Thus, these methods were applied to seek for the appropriate excipients and determine the optimized drug-carrier proportion of VB₁₂ solid dispersion in the present study, respectively.

The current *in vitro* Caco-2 cells transport study result suggests that the P-gp inhibition effect of different inhibitors may not all valid for the same drug substrate (Koga et al., 2002). Significant inhibition was identified only for G44/14 rather than

Labrasol in the present study.

A novel Vitamin B₁₂ solid dispersion using G44/14 was subsequently prepared. The G44/14 has been widely used as the carrier for commercialized pharmaceutical products, such as LIPOFEN®, due to relatively easy for manufacturing scale-up, as the liquefied carrier of G44/14 could be filled in the hard gelatin capsules at very lower temperature. In such a way, the pharmaceutical process only consists of two steps, mixing and filling. Meanwhile, the compositions of the formulation were only VB₁₂ and G44/14. Thus, this simplified composition makes it easier to further scale up commercially.

This formulation was subsequently characterized using Differential Scanning Calorimetry, Fourier Transform Infrared, and Scanning Electron Microscopy. These evaluations all suggest that a VB₁₂-G44/14 complex was formed rather than a simply physical mixture. Although the physical mixture may also contribute to the a certain level of dispersing of VB₁₂ in the G44/14, the partially important reason that using G44/14 is that after orally administrated the G44/14 based solid dispersion capsules, the self-emulsifying were being triggered when contacting with liquid in the stomach, which subsequently improving the membrane permeability. Of course, the P-gp inhibition of G44/14 is another important reason for its application of it in the present study.

VB₁₂ is known possesses high solubility and low intestinal permeability in character⁷. The high HLB value of G44/14 are mostly applied to the lipophilic active compounds. However, the amphiphilic property of G44/14 makes it also applicable for the improvement of polar compounds such as VB₁₂. In practice, the G44/14 was liquefied at 44-45°C and filled in the hard gelatin capsules. It's facilitate the scale up process. Moreover, this process may also be further modified by using the hot-melt extrusion process, as this continuous production mode can further reduce equipment procurement costs, and the production process is more easily controlled. It could thus become a cheap and effective supplementary therapy for VB₁₂ deficiency associated diseases.

This formulation was ultimately evaluated by the *ex vivo* transport study. The result suggest that the concentration of G44/14 used in the present study may significantly affect VB₁₂ absorption. For the G44/14-VB₁₂ solid dispersion, following the concentration increases, the P_{app} value of VB₁₂ transported from the mucosal side to the serosal side was gradually increased, indicating that the G44/14 had a

concentration-dependent effect on the intestinal absorption of VB₁₂. For the present study, the absorption of VB₁₂ in the everted gut sac was significantly increased by 1.52 times ($P < 0.01$) when the higher concentration of 10 mg/mL of G44/14 was combined with the VB₁₂ (G44/14=1:20). The US National Institutes of Health indicated that the average daily recommended amount for adults is 2.4 μ g (Bailey, 2004). The oral administration dose of VB₁₂ tablets in the Chinese market is 100 μ g/day (4 tablets). There is 1-2 μ g of VB₁₂ absorbed (1-2 % oral absorption), which is almost equivalent to the recommended dose. However, Gelucire based VB₁₂ could significantly improve the bioavailability. It may reduce the daily dose and further improve patient compliance.

5 Conclusion

In conclusion, the Caco-2 cells transport assay method and the rat everted gut sac method were proved to be appropriate models to determine the effect of additives on the transport of VB₁₂. The VB₁₂ complex prepared by G44/14 could potentially be applied to improve the VB₁₂ intestinal absorption, which could be readily applied commercially.

Declarations of interest: none.

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Legends of Figures

Fig. 1. Caco-2 cell viability after incubation with Vitamin B₁₂ (a), G44/14 (b) or Labrasol (c) at 37°C for 24 h compared to a positive (0.2% Triton X-100) and negative control (DMEM). Data are shown as mean ± SD (n = 6). The concentration of G44/14 (Fig. 1b) above 500 µg/mL and Labrasol (Fig. 1c) above 1000 µg/mL were started to show significant (p<0.05) cell viability decrease, respectively.

Fig. 2. Apparent permeability coefficient (P_{app}) of Vitamin B₁₂ across Caco-2 cell monolayers using Transwell® Permeable Supports. The data are presented as the Mean ± SD (n=3). The P_{app} value of the test compounds of Vitamin B₁₂ without Caco-2 cell monolayers, Vitamin B₁₂ with G44/14 (50 µg/mL), Vitamin B₁₂ with G44/14 (200 µg/mL), Vitamin B₁₂ with verapamil (50 µg/mL) and Vitamin B₁₂ with cyclosporin A (50 µg/mL) are significantly different from Vitamin B₁₂ (500 µg/mL) ($P<0.01$).

Fig. 3. DSC of Vitamin B₁₂ (a), G44/14 (b), Vitamin B₁₂/ G44/14 physical mixture (c) and Vitamin B₁₂ dispersion (d).

Fig. 4. FT-IR of Vitamin B₁₂ reference standard (a), Vitamin B₁₂ sample (b) and Vitamin B₁₂ dispersion (c).

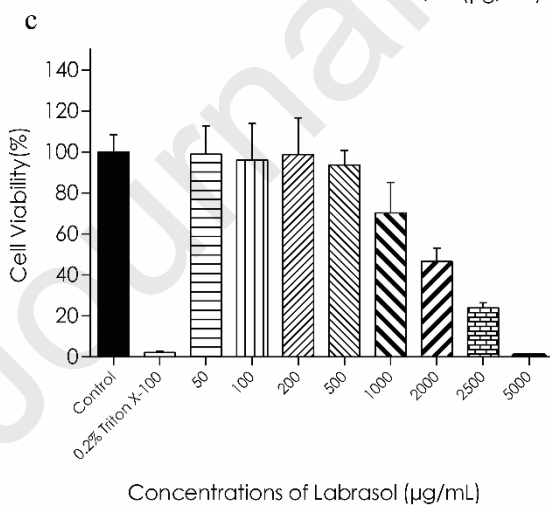
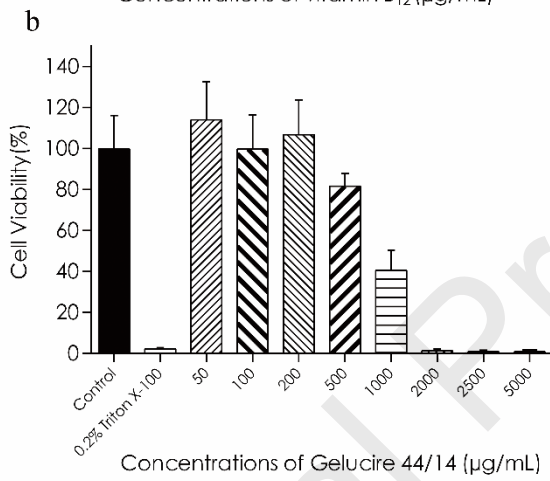
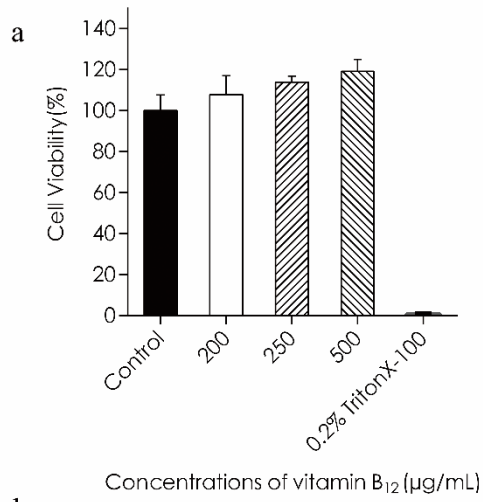
Fig. 5. Scanning electron microscopy pictures of Vitamin B₁₂ (a) and Vitamin B₁₂ solid dispersion (b).

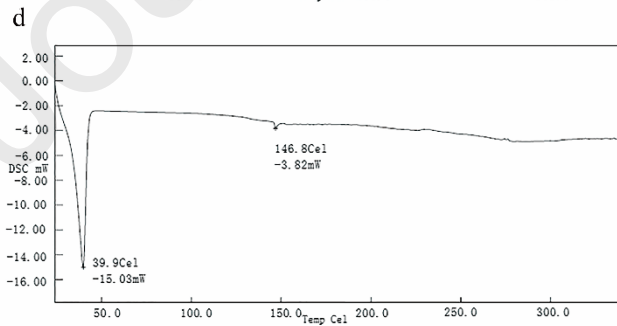
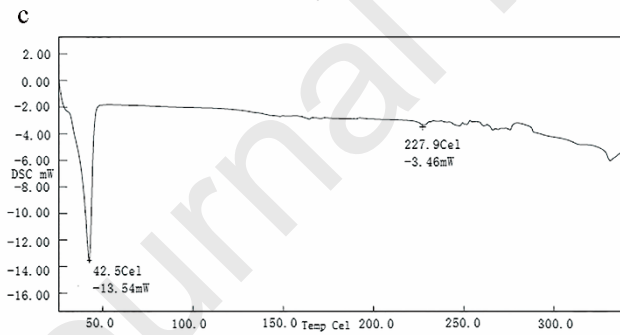
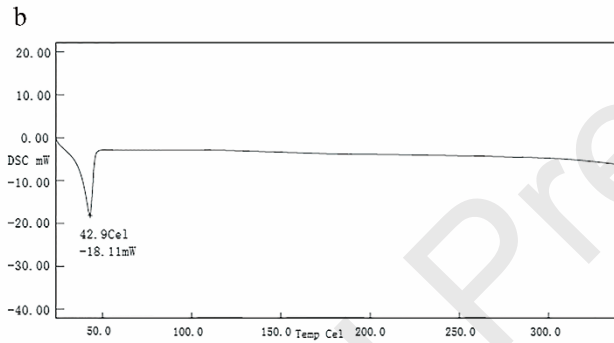
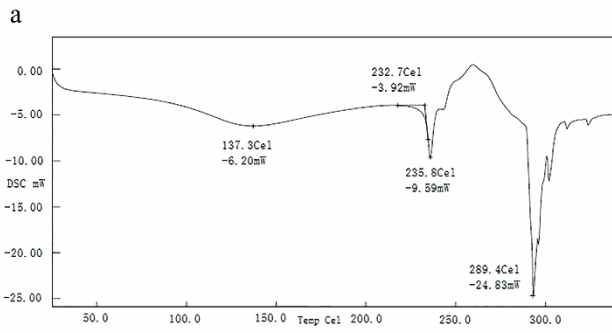
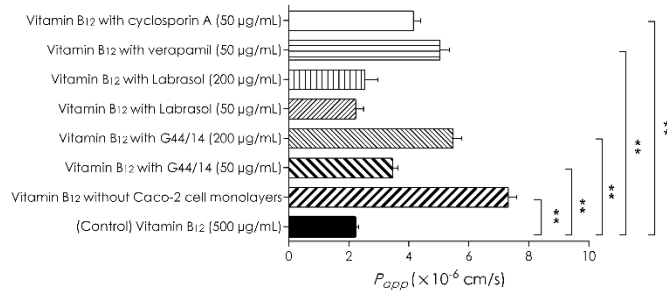
Fig. 6. (a). D-glucose absorption by using everted rat gut sacs. Values are presented as Mean ± SD (n = 5); (b). The transport of Vitamin B₁₂ from mucosa to serosa side using *ex vivo* rat everted gut sac method. The P_{app} values of Vitamin B₁₂ combined with verapamil (50 µg/mL), quinidine (422.5 µg/mL), and Gelucire 44/14 (10000 µg/mL) are significantly different from Vitamin B₁₂ (500 µg/mL) ($P<0.01$), respectively. The data are presented as the Mean ± SD (n=5).

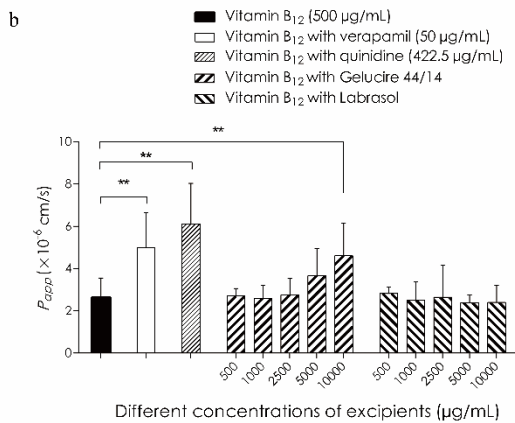
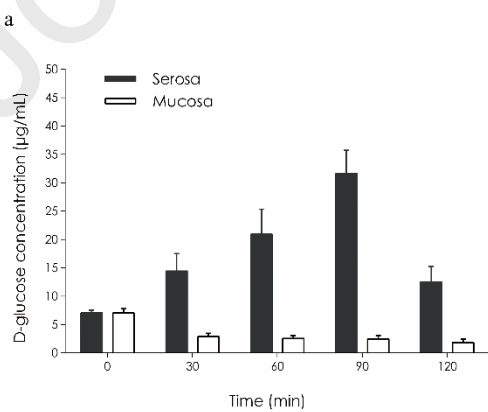
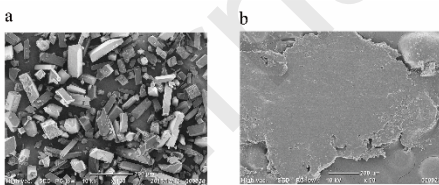
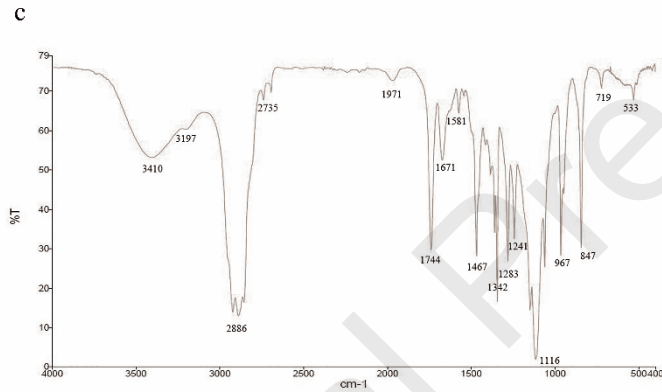
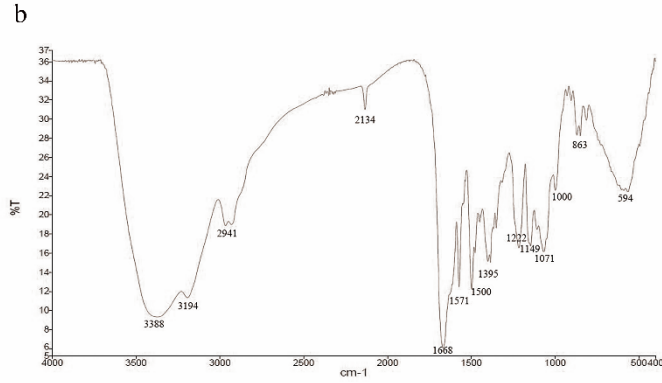
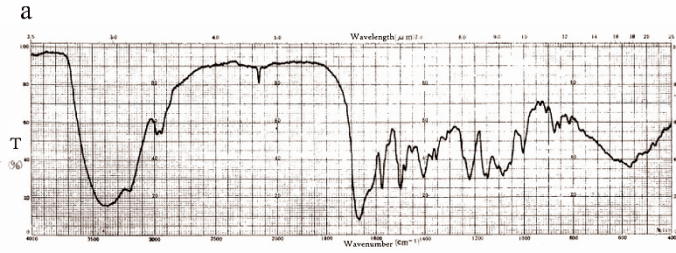
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Precision, accuracy, and recoveries for assay of Vitamin B₁₂ and fluoresceinsodium.







Journal Pre-proofs