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# Comparison of different absorption enhancers on the intranasal absorption of isosorbide dinitrate in rats

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ABSTRACT

The objective of this work was to study the influence of different absorption enhancers on the intranasal absorption of isosorbide dinitrate (ISDN). First of all, an *in situ* nasal perfusion technique in rats was used to investigate the effect of pH, concentration of drug solution and different absorption enhancers on the intranasal absorption of ISDN. The absorption enhancers investigated include hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD), chitosans (CS) of different molecular weight, and poloxamer 188. All of them enhanced the intranasal absorption of ISDN remarkably. It was found that poloxamer 188 had better permeation enhancing effect than that of HP-$\beta$-CD and CS of the same concentration. Thereafter, *in vivo* behaviors of the selected formulations were studied in rats and the pharmacokinetic parameters were calculated and compared with that of intravenous injection. Both *in situ* and *in vivo* studies demonstrated that poloxamer 188 played a key role in promoting intranasal absorption of ISDN. In nasal ciliotoxicity test, all the absorption enhancers investigated showed good safety profiles. Taking both enhancing effect and safety into account, we suggest poloxamer 188 is the most promising as an intranasal absorption enhancer.



## 1. Introduction

Isosorbide dinitrate (ISDN) is commonly used for therapy of stable angina pectoris and traditionally administrated via oral or sublingual, buccal routes. However, loss of consciousness appears in patients when angina pectoris breaks out, and thus it is difficult for patients to take medicine by themselves. Additionally, ISDN administrated orally has low bioavailability, only 30% of intravascular administration, due to its high first-pass metabolism in the gastrointestinal tract and liver (Zhao et al., 2007). The duration of buccal absorption was largely dependent on the tablet disintegration time. Moreover, the critical point of antianginal therapy depends, to a certain extent, on the ability of the drug to produce an immediate effect.

At present, intranasal administration represents a viable option for local and systemic delivery of diverse therapeutic compounds (Hussain, 1998; Wearley, 1991). Intranasal route allows a rapid onset of therapeutic effect, potential for direct-to-central nervous system delivery, avoidance of first-pass metabolism, and an easy way for drug administration. Besides, it can effectively avoid peak and valley phenomena and decrease toxicity and side effects (Behl et al., 1998). Therefore, intranasal (i.n.) administration may be an appropriate delivery route for ISDN.

However, there are two barriers limiting efficient nasal absorption of ISDN. One is the low membrane permeability, the other is the general rapid clearance of the administered formulation from the nasal cavity (Illum, 2003). So far, different intranasal absorption enhancers have been reported to increase nasal membrane permeability (Chandler et al., 1995). However, despite of their effectiveness, some of them were limited to use in nasal formulations due to their potentially unacceptable membrane toxicity (Chandler et al., 1995). Therefore, it is a great challenge to search for effective and safe intranasal absorption enhancers.

$\beta$-Cyclodextrins ($\beta$-CD) have been commonly studied as intranasal absorption enhancers due to their low toxicity. However, the promoting effect of $\beta$-CD itself in nasal cavity is not obvious. Methylated $\beta$-cyclodextrin (RM-$\beta$-CD), dimethyl-$\beta$-cyclodextrin (DM-$\beta$-CD) and HP-$\beta$-CD as its derivatives were reported to notably enhance intranasal absorption of drugs (Schipper et al., 1992, 1995; Gu et al., 2005). Regarding the safety properties of the three cyclodextrin derivatives, it can be described in the following order: HP-$\beta$-CD > RM-$\beta$-CD > DM-$\beta$-CD (Asai et al., 2002). Similarly, it was demonstrated that HP-$\beta$-CD had better safety profile and was well tolerated in the animal species tested (rats, mice and dogs) (Gould and Scott, 2005). As reported, HP-$\beta$-CD significantly promoted the

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absorption of acyclovir in nasal cavity (Chavanpatill and Vavia, 2004). In addition to the good safety profile, HP-$\beta$-CD has good solubility in water (as high as 50%) and can be used as a carrier to increase the solubility of poorly soluble drug. Therefore, HP-$\beta$-CD was selected in this study both as nasal absorption enhancer and as solubility enhancer of ISDN.

Compared to HP-$\beta$-CD, chitosan increased the AUC of plasma salmon calcitonin by more than onefold (Sinswat and Tengamnuay, 2003), implying that chitosan may have a better absorption-enhancing effect. Although chitosan derivative, N-trimethyl chitosan (TMC), was proved to be more effective in inducing immune responses to ovalbumin in mice following nasal administration in comparison to chitosan (Boonyo et al., 2007), it was demonstrated to be more toxic due to the increased positive charge (Mao et al., 2005). Moreover, TMC was effective at higher concentration than that of chitosan. Therefore, the homopolymer chitosan was chosen as the potential candidate to enhance the nasal absorption of ISDN.

In nasal absorption enhancers, surfactants are also very efficient. For example, Salzman et al. (1985) stated that insulin in nasal aerosol containing 1% (w/v) of the surfactant laureth-9 was rapidly absorbed within 15 min, and the plasma glucose was lowered by 50% in 45 min in patients. Similarly, ionic surfactants were licensed as potentially useful permeation enhancers for nasal delivery of fexofenadine (Lin et al., 2007). However, the safety of surfactants mentioned above was questionable (Salzman et al., 1985; Lin et al., 2007). Among all the surfactants, poloxamer 188 has been regarded as one of the safest in the field of pharmacy and can be used as the emulsifier in the preparation of submicron emulsion for intravenous injection. However, its potential application as an intranasal absorption enhancer has not been explored yet.

Therefore, in this study, taking ISDN as a model drug, the absorption-enhancing effect of different enhancers, HP-$\beta$-CD, chitosan and poloxamler 188, was studied and compared using *in situ* test and the absorption mechanism was illustrated. Safety properties of different enhancers were further confirmed and compared by using *in situ* toad palate model. Moreover, based on the *in situ* results, the selected intranasal formulations were evaluated *in vivo* by measuring drug blood concentration after intranasal administration in rats.

## 2. Materials and methods

### 2.1. Materials

ISDN was purchased from Shandong Keyuan Inc. Paracetamol (APAP, 99.6% purity, internal standard) was obtained from Shandong Xinhua Pharmaceutical Company, Ltd. (Jinan, China). The standard of isosorbide minitrate (ISMN) was purchased from National Institute for the Control of Pharmaceutical and Biological Products. Chitosan 400 kDa (CS 400) with a nominal degree of deacetylation of 85% was purchased from Weifang Kehai Chitin Co., Ltd. Chitosan 50 kDa (CS 50) and chitosan 100 kDa (CS 100) were prepared by oxidative degradation of CS 400 with $NaNO_2$ at room temperature (Mao et al., 2004). HP-$\beta$-CD (Degree of substitution 4–9) was purchased from Xi'an Deli biochemical company. Poloxamer 188 was a gift from BASF. Methanol and acetonitrile of chromatographic grade were purchased from Shangdong Yuwang Tech Reagent Company (Shandong, China). All other chemicals were of analytical grade.

### 2.2. Preparation of intranasal formulations

HP-$\beta$-CD and poloxamer 188 were dissolved in distilled water, and chitosan was dissolved in 0.5% (v/v) acetic acid saline solution to obtain desired concentrations for *in situ* test. ISDN was dissolved in the above-mentioned solution (0.5 mg mL$^{-1}$) and pH of the solution was adjusted to six unless specially indicated. Isotonicity was adjusted using sodium chloride. As for formulations used in *in vivo* studies, the preparation of HP-$\beta$-CD, poloxamer 188, chitosan solutions (0.5%) was the same as that for *in situ*, but the concentration of ISDN was 5 mg mL$^{-1}$. Intravenous injection solution was prepared by dissolving ISDN (0.25 mg mL$^{-1}$) in sterile saline solution and adjusting pH to 6.

### 2.3. In vitro analytical method of ISDN

The concentration of ISDN was analyzed by HPLC method. HPLC apparatus (Shimadzu, LC6-AS liquid chromatograph) connected to an ultraviolet variable wavelength detector (Model SPD-6A) with a C-18 reversed phase column (Bondapack, 5 μm, 4.6 mm × 200 mm, Shimadzu, Japan) and isocratic pump (Model LC6-AS, Shimadzu, Japan) was used. The mobile phase consisted of 40% of methanol and 60% of water. The flow rate was 1.0 mL min$^{-1}$. The UV detector wavelength was 230 nm and the oven temperature was 30 °C. The injection volume was 20 μL. The limits of detection and quantitation of ISDN were 1 and 3 μg mL$^{-1}$, respectively. A linear relationship between peak area and concentration was found in the concentration range of 3–100 μg mL$^{-1}$ ($A = 2363.5C - 1653.6$, $r = 0.9999$, $n = 7$). The inter-day relative standard deviations were less than 0.99% ($n = 5$).

### 2.4. Nasal perfusion studies in rats

The animal experiment was carried out in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985). Male Wistar rats (7 weeks old, 200 ± 20 g) were supplied by the Lab Animal Center of Shenyang Pharmaceutical University (Grade II, Certificate No. SYXK 2006-0064). The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals.

For the nasal absorption studies, male Wistar rats weighing 180–220 g were used and allowed free access to food and water. Before experimentation, the rats were anaesthetized with urethane at a dose of 4 mg kg$^{-1}$ and were placed in a supine position on a working surface. Then the rats were surgically treated by cutting trachea and the trachea was cannulated with a polyethylene tube to aid breathing (Hirai et al., 1981). Another tube was inserted through the esophagus into the posterior part of the nasal cavity. The nasopalatine duct was closed with an adhesive agent to prevent the drainage of the solution from the nasal cavity into the mouth. The tube inserted into the esophagus was connected to a reservoir of 5 mL drug solution under magnetic stirring and immersed in a water-bath at 37 °C. The solution was circulated, by means of a peristaltic pump from the reservoir through the nasal cavity and out of the nostrils back into the reservoir. Flow rate was set at 2.0 mL min$^{-1}$. Aliquots (100 μL) were sampled at various time intervals up to 2 h and replenished with an equal volume of saline. ISDN contents were analyzed by HPLC assay as described in Section 2.3.

### 2.5. Nasal ciliotoxicity of different absorption enhancers

Nasal ciliotoxicity studies were carried out using an *in situ* toad palate model (Jiang et al., 1995). In brief, the upper palate of the toads (30–40 g, male and female) was exposed and treated with 0.5 mL of 0.5% HP-$\beta$-CD, CS 50, CS 100 and poloxamer 188 saline solutions for 0.5 h, respectively. Thereafter, the test formulation was removed by washing the palate with saline, and then approximately 5 mm × 3 mm of the palate was dissected and the mucocilia

was examined with a Motic DMBA450 light microscopy (Motic China Group Co. Ltd., Beijing, China) and Camera (Nikon Fx-35A, Tokyo, Japan) at enlargement of 400× at regular intervals. The time from test solution administration to the stop of cilia movement was recorded and noted as the lasting time of cilia movement. Saline, ephedrine hydrochloride (1%, w/v solution) and sodium deoxycholate (one of the agents with serious nasal ciliotoxicity, 1%, w/v solution) were used as the blank, negative and positive controls, respectively. The relative inhibition rate of different absorption enhancers was calculated according to the following equation:

$$\text{Relative inhibition ratio} = \frac{\text{Lasting time of cilia movement of the test sample}}{\text{Lasting time of cilia movement of saline}} \times 100\%$$

### 2.6. In vivo analytical method of ISDN

In *in vivo* studies in rats, ISMN was determined as a metabolite of ISDN. Plasma samples were processed with the following steps: 100 μL of acetonitrile and 10 μL of internal standard (40 μg mL$^{-1}$ APAP in water) were added into 100 μL of serum samples. The mixture was vortexed for 3 min and centrifuged at 4000 rpm for 10 min. The supernatant was used for HPLC assay. The mobile phase consisted of 40% of acetonitrile and 60% of water. The flow rate was 1.0 mL min$^{-1}$. The UV detector wavelength was 210 nm and the oven temperature was 30 °C. The injection volume was 20 μL. The peak area ratio between ISMN and APAP was calculated. A linear relationship between the ratio of peak area and ISMN concentration was found in the concentration range of 0.5–40 μg mL$^{-1}$ ($A_{ISMN}/A_{APAP} = 0.0193C + 0.006$, $n = 7$). The extraction recovery was 82.19–86.28% and the method recovery was 96.60–101.90%. The inter-day RSDs were less than 4.26% ($n = 5$), and the intra-day relative standard deviations were less than 7.87% ($n = 5$).

### 2.7. In vivo studies in rats

Thirty-five healthy male Wistar rats weighing 180–220 g were divided into seven groups, 0.5% CS 50 group, 0.5% CS 100 group, 0.5% HP-β-CD group, 0.5% poloxamer 188 group, 0.5% poloxamer 188 combined with 30% HP-β-CD group, control group and intravenous administration group. For intravenous administration, an injection was administered via the tail vein (0.64 mg kg$^{-1}$), and in other groups the samples were administrated intranasally by inserting a polyethylene tube attached to a microsyringe approximately 0.7 cm into two nostrils (1.04 mg kg$^{-1}$) (Mao et al., 2006). For all the groups tested, blood samples of 0.3 mL were withdrawn at 0, 2, 5, 10, 20, 30, 60, 120 and 180 min after administration and were harvested immediately by centrifugation at 4000 rpm for 10 min. Then plasma samples were stored at −20 °C immediately after collection until analysis. Plasma samples were processed according to above described method. Each point represents the mean of five rats.

### 2.8. Statistical and pharmacokinetic data analysis

The peak plasma concentration ($C_{max}$) and the time to reach the peak concentration ($T_{max}$) were determined directly from the plasma concentration-time profiles. The absolute bioavailability ($F$) was calculated according to the following equation:

$$F_A = \frac{\text{AUC(i.n.)} \times \text{Dose(i.v.)}}{\text{AUC(i.v.)} \times \text{Dose(i.n.)}} \times 100\%$$

The pharmacokinetic parameters were obtained using 3p87 program. Data are presented as means ± standard deviations of five experiments. The statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Dunnett test. Probability values $P < 0.05$ were considered significant.

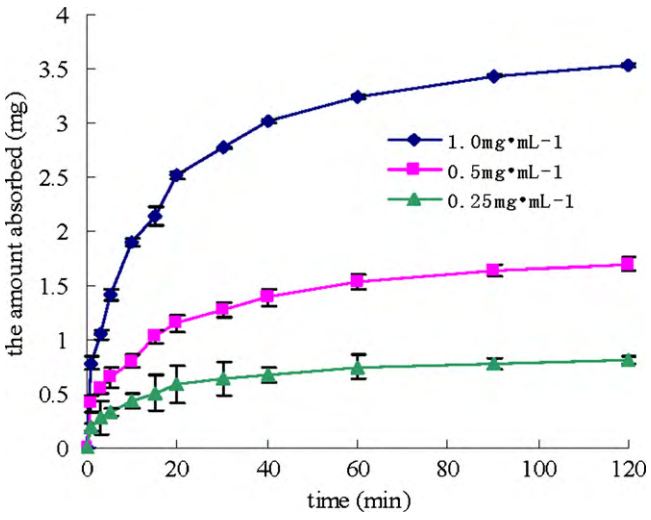


**Fig. 1.** Effect of drug concentration on the intranasal absorption of ISDN ($n = 3$).

## 3. Results

First of all, effect of pH, drug concentration, concentration of HP-β-CD, chitosan and poloxamer on the intranasal absorption of ISDN was investigated using *in situ* rat model. Thereafter, the selected formulations were used in *in vivo* studies in rats.

### 3.1. Effect of pH on intranasal absorption of ISDN

To optimize the solution pH, intranasal absorption of ISDN was investigated at pHs 5, 6 and 7, respectively. A slightly lower absorption was found at pH 5. However, no remarkable difference in absorption was found among pHs 5, 6 and 7 ($P > 0.05$). Considering that the pH of normal human nasal mucosa is in the range of 5.5–6.5 (Mei et al., 2008), pH 6 was selected for the following studies.

### 3.2. Effect of drug concentration

To investigate the effect of drug concentration on the nasal absorption, three concentration levels, 0.25, 0.5 and 1 mg mL$^{-1}$ were studied. As shown in Fig. 1, irrespective of the initial concentration, the amount absorbed increased linearly with time in the initial 20 min, and then the absorption rate slowed down and almost leveled off after 60 min. The absorption rate constants increased linearly with drug concentration and were found to be 0.0141, 0.0291, 0.0630 mg min$^{-1}$ for 0.25, 0.5 and 1 mg mL$^{-1}$ of drug solution, respectively ($y = 0.0656C - 0.0029$, $r = 0.999$). These results demonstrated that uptake of ISDN across nasal epithelium was dose-dependent. 0.5 mg mL$^{-1}$ was selected for the following studies to investigate the influence of absorption enhancers.

### 3.3. Effect of HP-β-CD on intranasal absorption of ISDN

HP-β-CD can be used as an absorption enhancer and also as a solubilizer to increase the solubility of hydrophobic drugs. Due to the poor solubility and poor membrane permeation of ISDN, HP-β-CD was selected for double effects in this study. To elucidate the effect of HP-β-CD concentration on the intranasal absorption of ISDN, HP-β-CD in the concentration range of 0.5–30% were studied and the results are shown in Fig. 2. It was noted that when the concentration of HP-β-CD was 0.5%, the absorption of ISDN increased remarkably after 20 min compared to the control group ($P < 0.05$). However, when the concentration was 3%, the absorption amount was even lower than that of the control

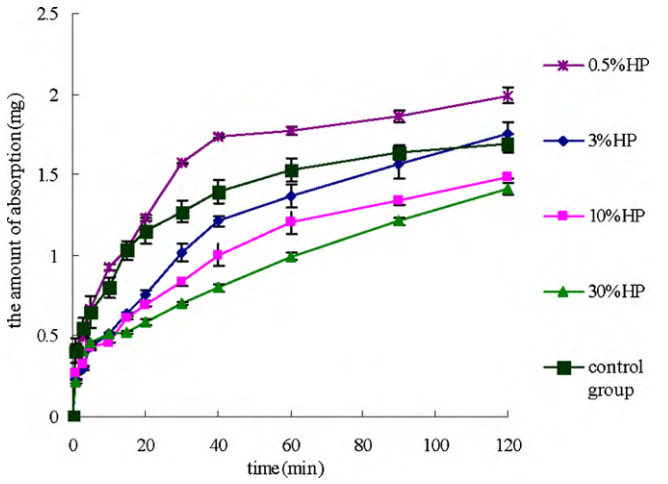


**Fig. 2.** Effect of HP-$\beta$-CD concentration on the intranasal absorption of ISDN ($n = 3$).

group before 90 min, and at 120 min the absorption amount of both groups was almost the same ($P > 0.05$). When further increasing HP-$\beta$-CD concentration up to 30%, the absorption rate of ISDN decreased exponentially (Fig. 2). The absorption rate constants were found to be 0.0336, 0.0269, 0.0208, 0.0151 mg min$^{-1}$ for 0.5%, 3%, 10% and 30% HP-$\beta$-CD group, respectively ($y = 0.031x^{-0.1924}$, $r = 0.98$).

### 3.4. Effect of different molecular weight chitosans on intranasal absorption of ISDN

Taking chitosan 50 kDa as an example, the influence of chitosan concentration on the intranasal absorption of ISDN was investigated in the range of 0.1–1.5% (w/v) (Fig. 3A). Compared to the control group, a significant increase in ISDN absorption was found at all the time points tested in chitosan containing groups, and the enhancement degree was chitosan concentration-dependent. When chitosan 50 kDa concentration was 0.1%, only a slight increase in ISDN absorption was observed. The absorption increased considerably when chitosan 50 kDa concentration was increased to 0.5%. A significant difference in absorption was found between 0.1% and 0.5% chitosan group ($P < 0.05$). Further increasing chitosan 50 kDa concentration to 1% caused no further absorption enhancement and no statistical difference was found between 0.5% chitosan and 1.0% chitosan group ($P > 0.05$). 1.5% chitosan expressed the highest absorption-enhancing effect. The similar absorption characteristics were found for chitosan 100 kDa, as shown in Fig. 3B.

Regarding to the influence of chitosan molecular weight, as shown in Fig. 3C, CS 100 kDa exhibited no significant increase in absorption compared to CS 50 kDa ($P > 0.05$) at the same concentration of 0.5%, and the absorption-enhancing effect of both groups was remarkable compared with that of the control group ($P < 0.05$).

### 3.5. Effect of poloxamer 188 on intranasal absorption of ISDN

Poloxamer 188 has not been investigated as an intranasal absorption enhancer in animal model to be best of our knowledge. In this study the absorption-enhancing effect of poloxamer 188 on the intranasal absorption of ISDN was investigated and the results are shown in Fig. 4. 0.5% poloxamer 188 had very significant absorption promoting effect compared to the control group ($P < 0.05$), and further increasing poloxamer 188 concentration to 1% caused a slight decrease in absorption, and no difference in

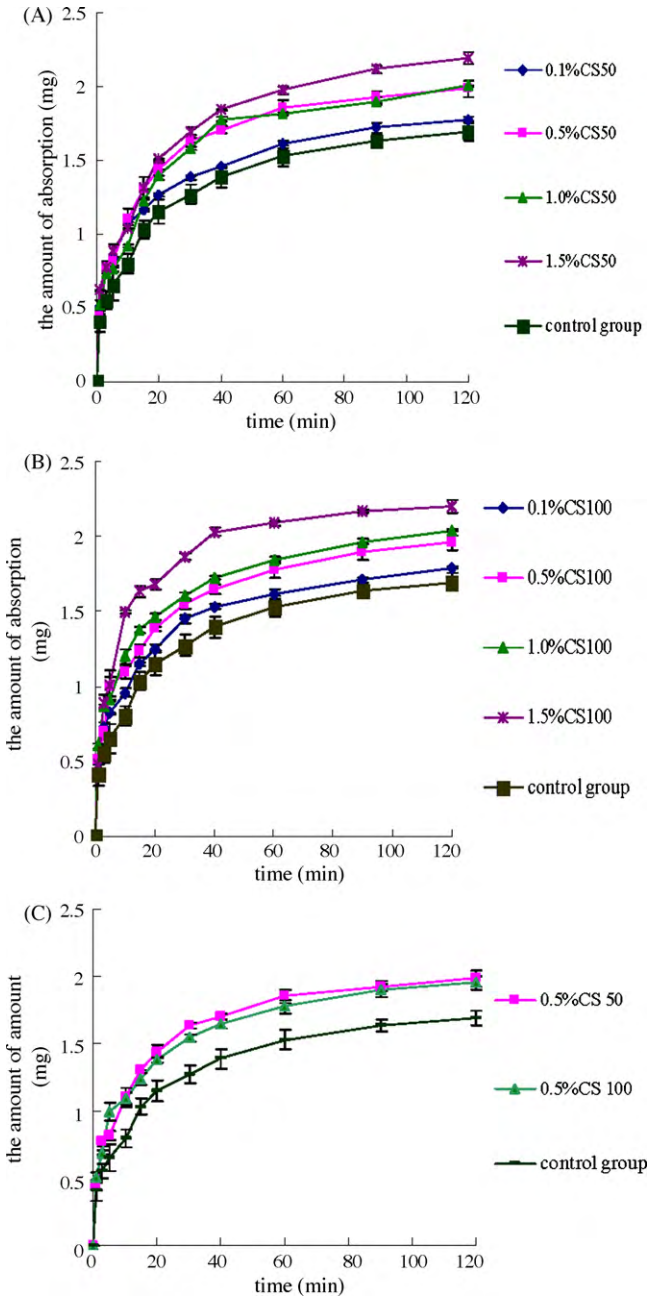


**Fig. 3.** Effect of different concentrations of CS 50 and CS 100 on the intranasal absorption of ISDN ($n = 3$).

absorption was found between 1% and 2% poloxamer 188 group ($P > 0.05$).

### 3.6. Comparison of different absorption enhancers and safety assessment

Fig. 5 showed the comparison of different absorption enhancers at the same concentration of 0.5%. It was showed that chitosan had a better absorption promoting effect in the first 30 min compared to that of HP-$\beta$-CD ($P < 0.05$), and the absorption rate constants were 0.061, 0.0336, respectively. However, after 30 min, their absorption promoting effect was comparable ($P > 0.05$). In contrast, compared to chitosan and HP-$\beta$-CD, poloxamer 188 displayed better promoting effect on intranasal absorption of ISDN in all the time points investigated ($P < 0.05$). The absorption rate constant of poloxamer

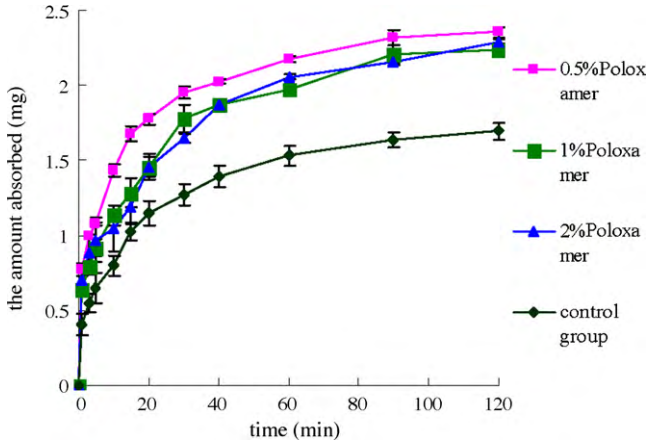


**Fig. 4.** Effect of poloxamer 188 concentration on the intranasal absorption of ISDN ($n = 3$).

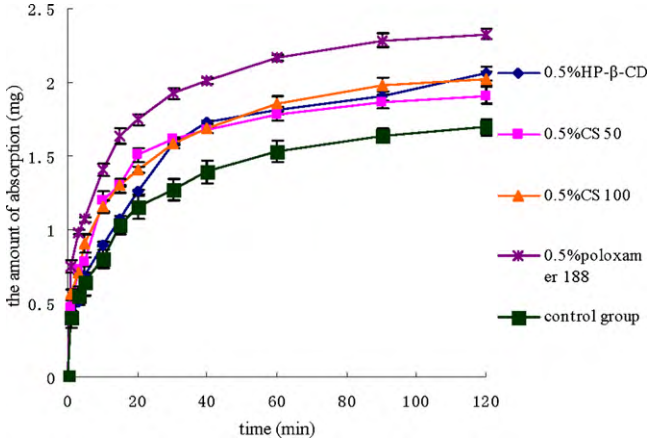


**Fig. 5.** Comparison of the absorption-enhancing effect of HP-$\beta$-CD, CS 50, CS 100 and poloxamer 188 ($n = 3$).

188 was 0.07, and the absorbed amount reached as high as 92.8% of the initial drug amount.

Safety of the absorption enhancers is of special importance for clinical application. Therefore, nasal ciliotoxicity of different enhancers was investigated and the results are shown in Fig. 6. The time of cilia movement for the control saline treated samples was

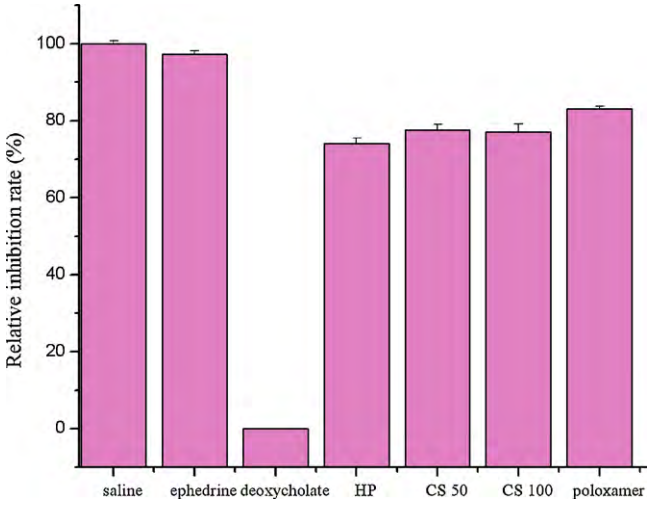


**Fig. 6.** Relative inhibition rate of different absorption enhancers ($n = 3$).

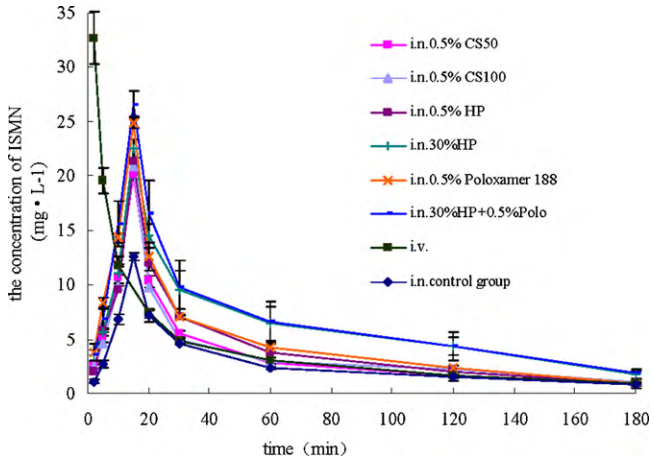


**Fig. 7.** Mean plasma concentration-time profiles after intranasal application of ISDN with 0.5% HP-$\beta$-CD, 30% HP-$\beta$-CD, 0.5% CS (50, 100), 0.5% poloxamer 188, and 30% HP-$\beta$-CD in combination with 0.5% poloxamer 188 in rats, compared with the control group and intravenous injection group. Indicated values are the mean of five experiments ($n = 5$).

711 min. The relative inhibition rates of HP-$\beta$-CD, CS 50, CS 100 and poloxamer 188 were 74.1%, 77.6%, 77.1% and 83.1%, respectively, all higher than 70%, indicating that the absorption enhancers selected in this study had good safety profiles. Among them, poloxamer 188 was much safer than chitosan and HP-$\beta$-CD.

### 3.7. *In vivo studies*

Based on the *in situ* results, samples containing the following absorption enhancers were selected for *in vivo* studies, that is, 0.5% HP-$\beta$-CD, 0.5% chitosan 100 kDa, 0.5% chitosan 50 kDa, and 0.5% poloxamer 188. In addition, solubility studies indicated that 30% HP-$\beta$-CD could be added in the formulation to increase the solubility of ISDN for clinical application, however, it exhibited absorption inhibition effect *in situ*. Therefore, its *in vivo* absorption was studied further to clarify this point. Also, the combination of 30% HP-$\beta$-CD with 0.5% poloxamer 188 was investigated. Plasma concentrations of ISMN after intranasal administration and intravenous injection are depicted in Fig. 7 and the main pharmacokinetic parameters are listed in Table 1. The pharmacokinetic profiles could be described with a two-compartment model. The drug concentration was measurable 2 min after adiminstration. $T_{max}$ of the intranasal groups was in the range of 11.55–18.2 min, indicating that ISDN was rapidly absorbed via intranasal route compared to that of 60 min per oral. Compared to the control group, all the absorption enhancers investigated increased the $C_{max}$ and AUC of ISDN significantly ($P < 0.05$). The absolute bioavailabilty of different group was calculated and it decreased with the following order: 0.5% poloxamer 188 combined with 30% HP-$\beta$-CD > 0.5% poloxamer 188 > 30% HP-$\beta$-CD > 0.5% HP-$\beta$-CD > CS 100, CS 50 > control group. The statistical analysis is shown in Table 1. In agreement with *in situ* study, no influence of chitosan molecular weight (50, 100 kDa) on absorption was found in the range studied ($P > 0.05$). For 30% HP-$\beta$-CD group, it had better absorption *in vivo* than that of 0.5% HP-$\beta$-CD group ($P < 0.05$) despite of its poor absorption *in situ*. Absolute bioavailability of 73% was achieved by using 0.5% poloxamer as an absorption enhancer. And the absorption could be further enhanced to 80% by the combination of 30% HP-$\beta$-CD.

Moreover, the correlation between *in situ* absorption results and *in vivo* absorption data at different time points was investigated (Table 2). Except for 30% HP-$\beta$-CD group, a good exponential correlation was established between *in situ* absorption amount ($M$) and plasma concentration ($C$) *in vivo* ($r > 0.96$). Fig. 8 exemplified a good

**Table 1**
Main pharmacokinetic parameters after intranasal (i.n.) administration of ISDN (1.04 mg kg$^{-1}$) with 0.5% HP-$\beta$-CD, 30% HP-$\beta$-CD, 0.5% CS (50, 100), 0.5% poloxamer 188, 30% HP-$\beta$-CD combined with 0.5% poloxamer 188 and the control formulations, as well as after intravenous (i.v.) injection of ISDN in rats (mean ± SD, $n$ = 5).

| Formulation | $C_{max}$ (μg mL$^{-1}$) | $T_{max}$ (min) | $AUC_{0\rightarrow 180\,min}$ (μg min mL$^{-1}$) | Absolute bioavailability (%) |
|---|---|---|---|---|
| i.n. Control group | 6.17 ± 0.58 | 16.5 | 572.0 ± 46.8 | 43.32 ± 2.88 |
| i.n. 0.5% HP-$\beta$-CD | 14.87 ± 2.15 | 14.5 | 757.4 ± 25.6 | 59.19 ± 1.58* |
| i.n. 0.5% CS 50 | 13.79 ± 1.79 | 13.1 | 640.3 ± 29.6 | 55.36 ± 2.68* |
| i.n. 0.5% CS 100 | 12.78 ± 1.68 | 12.4 | 661.9 ± 33.9 | 56.22 ± 1.78*,Δ |
| i.n. 30% HP-$\beta$-CD | 15.97 ± 2.18 | 18.2 | 800.1 ± 35.8 | 63.75 ± 2.31*,# |
| i.n. 0.5% Poloxamer | 18.77 ± 2.37 | 11.6 | 848.5 ± 58.2 | 73.36 ± 3.03* |
| i.n. 30% HP + 0.5% poloxamer | 22.91 ± 1.26 | 16.5 | 931.7 ± 42.2 | 80.55 ± 1.72* |
| i.v. (0.64 mg kg$^{-1}$) | – | – | 787.5 ± 26.7 | – |

* Significant difference compared to intranasal control group ($P$ < 0.05).
Δ No significant difference compared to CS (50) group ($P$ > 0.05).
\# Significant difference compared to 0.5%HP-$\beta$-CD ($P$ < 0.05).

**Table 2**
Equations between ISDN plasma concentration *in vivo* and drug absorption amount *in vitro* for each group ($n$ = 5).

| Formulation | C–$M$ equation | $r$ |
|---|---|---|
| Control group | $C = 338.96e^{-3.3078M}$ | 0.994 |
| 0.5% HP-$\beta$-CD | $C = 242.99e^{-2.3516M}$ | 0.993 |
| 30% HP-$\beta$-CD | $C = 35.032e^{-1.6451M}$ | 0.867 |
| 0.5%CS 50 | $C = 2434.6e^{-3.7116M}$ | 0.995 |
| 0.5%CS 100 | $C = 679.85e^{-2.9941M}$ | 0.976 |
| 0.5%poloxamer | $C = 4005.7e^{-3.2204M}$ | 0.986 |

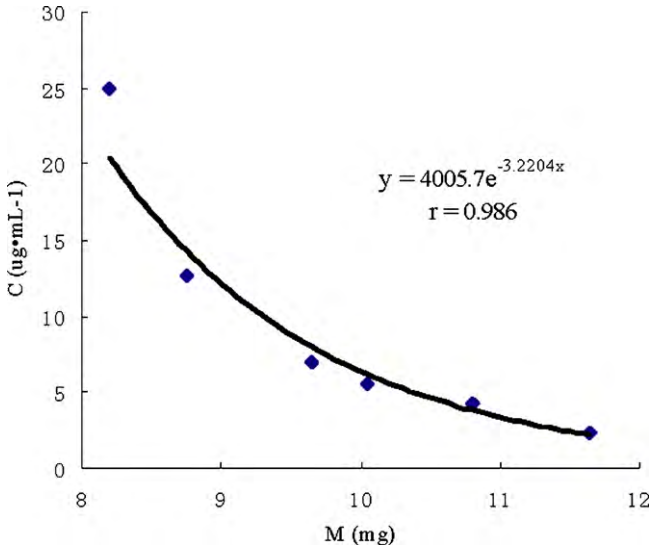


**Fig. 8.** The relationship between ISDN plasma concentration *in vivo* ($C$) and drug absorption amount *in vitro* ($M$) for poloxamer 188 ($n$ = 5).

*in situ–in vivo* correlation for poloxamer 188. This result indicates that *in situ* absorption can be a useful tool for *in vivo* absorption predication in most cases.

## 4. Discussion

The absorption of ISDN via intranasal route was proved to be pH-independent. This can probably be explained by the fact that the main transport mechanism of lipophilic drug across nasal mucosa is by lipoidal pathway (Behl et al., 1998). As an ester, ISDN is neutral in water and the breaking of ester bond only happens in the presence of strong acid or alkali. When the pH is 5, 6 and 7, it has no effect on the structure of ISDN and lipid solubility. Therefore, pH has no significant effect on the intranasal absorption of ISDN in this range.

Due to the dose-dependent cell influx phenomenon (Sanico et al., 1997), the intranasal absorption of ISDN was concentration-dependent. Irrespective of the initial concentration, the amount absorbed increased linearly with time in the initial 20 min, and then the absorption rate slowed down and almost leveled off after 60 min. This proved that a saturation phenomenon existed in intranasal absorption of ISDN. Similarly, a saturation phenomenon in *in situ* test has been reported with analgin (Mao et al., 1997) and leucine enkephalin (Hussain et al., 1995). This can be explained by the fact that hydration between drug solution and nasal mucosa reduced effective lipoidal pathway on the membrane as a result of long time contact (Mei et al., 2008). Thus, further intranasal absorption of ISDN was impeded.

Compared to the control group, HP-$\beta$-CD exhibited good absorption-enhancing effect at the concentration of 0.5% in both *in situ* test and *in vivo* studies. This may be partly explained by the fact that HP-$\beta$-CD was able to open tight junctions between epithelium cells on nasal mucosa (Hussain and Paulson, 2004). Also, this may relate to the cholesterol depleting efficacy of HP-$\beta$-CD (Deli, 2009). Shao et al. (2004) also discovered that lipid constituents were extracted by HP-$\beta$-CD. In *in situ* test, as the concentration of HP-$\beta$-CD increased to 30%, the absorption of ISDN decreased significantly. In contrast, it exhibited a good absorption *in vivo*. This can probably be explained by the different *in situ* and *in vivo* environment, which influenced ISDN release from the formulation. In *in situ* condition, most of ISDN was included in the cavity of HP-$\beta$-CD, and as we know, only the drug existing in the free molecular state could get through the mucosal member, therefore, the delayed drug absorption may be explained by the slow drug release from the cyclodextrin cavity due to the limited fluid. However, absorption *in vivo* was considered to be proceeded under sink condition and ISDN could be released faster from the cavity of HP-$\beta$-CD, and dissolution is no more a limiting step. Similarly, 40% HP-$\beta$-CD was proved to be able to increase the bioavailability of baicalein from 52.3% to 86.4% (Liu et al., 2006).

It was found that chitosan had a good promoting effect on intranasal absorption of ISDN and the effect was apparent even 3 min after application. In our previous study with tetramethylpyrazine phosphate (Mei et al., 2008), it was shown that the absorption-enhancing effect of chitosan was apparent only 40 min after application. Therefore, this followed study indicated that the time of onset of chitosan is also affected by the properties of the active drug substance. Moreover, this study showed that the intranasal absorption promoting effect of chitosan depended on its concentration. A slight increase in absorption was observed when 0.1% chitosan was applied, and the absorption was increased remarkably when chitosan concentration increased to 0.5%. Similarly, it was reported that from 0.1% to 0.5%, the absorption-enhancing effect of chitosan was concentration-dependent (Sinswat and Tengamnuay, 2003). When the concentration of chitosan was further increased to 1.0%, no significant increase in absorption was found compared with that of

0.5% chitosan group. In general, the absorption-enhancing effect of chitosan is explained by the fact that chitosan could transiently open tight junctions between nasal epithelial cells by interaction with the Protein Kinase C pathway when it was applied to confluent cell cultures (Kast et al., 2002). The chitosan-induced reversible increase in TJ permeability involves the redistribution of occludin, ZO-1 and the actin-cytoskeleton (Deli, 2009). 0.5% CS may be sufficient to open all the available tight junctions on the nasal mucosa (Borchard et al., 1996). This is in agreement with our previous studies (Mei et al., 2008). However, it was noticed that the intranasal absorption of ISDN was further improved when the concentration of chitosan increased to 1.5%. It was reported that when the concentration of chitosan was used at lower than 1.0%, the cells expressed undamaged and functionally intact. However, cells were damaged by 1.5% chitosan due to its high viscosity and adhesive characteristic (Borchard et al., 1996). Therefore, this result can probably be explained by the high permeation of the member due to cell damage. In agreement with our results, it was reported previously that no change in transepithelial electrical resistance (TEER) value was found when increasing chitosan–glutamate concentration from 0.5% to 1.0%, however, TEER decreased to 59.6% of the initial value when the concentration of chitosan–glutamate increased to 1.5% (Borchard et al., 1996), indicating that cells were damaged. Therefore, chitosan could be used as a safe absorption enhancer when its concentration was below 1%.

In addition, effect of chitosan molecular weight on the intranasal absorption of ISDN was studied. No significant difference in absorption was found between chitosan 50 and 100 kDa in this study both *in situ* and *in vivo*. However, by using tetramethylpyrazine phosphate (Mei et al., 2008) as a model drug, it was found that chitosan 100 kDa has a better enhancing effect than that of chitosan 50 kDa. This can probably be explained by the different properties of the active substance, implying that the influence of chitosan molecular weight on absorption might be drug property dependent. In accordance with this study, no difference in nasal absorption of insulin was found between chitosan 50 and 130 kDa group in rats (Aspden et al., 1996).

Poloxamer 188 is a water-soluble, nonionic surfactant and has extensive application in the field of pharmacy. The critical micelle concentration (CMC) of poloxamer 188 is 0.105% (Maskarinec et al., 2002) and it was non-toxic at concentrations higher than its critical micelle concentration (CMC) on the human nasal epithelial cells (Lin et al., 2007). However, only limited information is available regarding the potential of poloxamer 188 as an intranasal absorption enhancer. So far, there was only one report indicating that poloxamer 188 was ineffective as a permeating enhancer of hydrophilic compounds in nasal epithelial cells (Lin et al., 2007) and no study in animal model has been performed. In our present study, 0.5% poloxamer 188 showed a significant improvement in the intranasal absorption of ISDN both *in situ* and *in vivo*. This can probably be explained by the fact that poloxamer 188 could form stable colloid in water at concentration of 0.5% and might encapsulate lipophilic drugs by chemical, physical and electrostatic interaction (Cjoappetta and Sosnik, 2007). Moreover, Pimienta et al. (1992) reported that the mucoadhesion of poloxamer proceeds via an immediate adhesion of the polyoxyethylene chains to mucus through secondary forces, followed by their diffusion into mucus where they undergo molecular interactions. However, when the concentration of poloxamer 188 increased to 1–2%, no further increase in ISDN absorption was found but it decreased slightly, probably the increased viscosity of poloxamer 188 slowed down ISDN release from the micelles.

Compared to HP-$\beta$-CD and chitosan, poloxamer 188 was the most effective absorption enhancer both *in situ* and *in vivo*, and it also owned the best safety profile based on the ciliotoxicity test, implying its potential as a promising intranasal absorption enhancer. However, it should be noted that the occurrence of pharmacological and immunological responses *in vivo* raises several questions that must be addressed regarding the commercially available poloxamers, if they are to be used safely and be amenable to both scientific and clinical rigor (Moghimi and Hunter, 2000).

In *in vivo* studies in rats, ISMN was determined as a metabolite of ISDN, because ISDN cannot be detected even 5 min after intranasal administration. A study (All and Blume, 1985) reported that the concentration of ISMN was 20 times of ISDN and 10–15 times of isosorbide 2-mononitrate (2-ISMN), the other metabolite. That is to say, ISDN and 2-ISMN were negligible compared to ISMN. Therefore, the *in vivo* metabolic characteristic of the prototype drug was depicted by determining the concentration of its main metabolite in plasma.

The good correlation between *in situ* results and *in vivo* absorption data at different time points implied that *in situ* perfusion method could be used as an effective tool to predict intranasal absorption *in vivo*. However, when drug absorption is release rate dependent, as in the case of 30% HP-$\beta$-CD group, the *in situ* model is not predictable.

## 5. Conclusions

In this paper, the intranasal absorption of ISDN was found to be pH-independent in the range of pHs 5–7 but dose-dependent. The intranasal absorption-enhancing capacity of three kinds of enhancers, HP-$\beta$-CD, chitosan and poloxamer 188, were investigated and compared based on *in situ* and *in vivo* studies. Safety of the three absorption enhancers was investigated using *in situ* toad palate model. Among them, poloxamer 188 was found to be the most effective for the intranasal absorption of ISDN with marginal ciliotoxicity. Moreover, mechanisms of different enhancers were illustrated. The *in situ*/*in vivo* correlation study indicated that *in situ* perfusion method could be used as an effective tool to predict intranasal absorption *in vivo* when drug absorption is release rate independent.

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