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A novel transdermal patch incorporating isosorbide dinitrate with bisoprolol: *In vitro* and *in vivo* characterization

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Abstract

The combination therapy of nitrate and selective β-adrenoceptor antagonist has shown benefits for treatment of hypertension and heart disease than either drug alone. The objectives of the present study were to define effects on the antihypertension activity and pharmacokinetics of a novel transdermal patch incorporating isosorbide dinitrate (ISDN) with bisoprolol (BP). The 3:2 ratio of ISDN to BP (mg/mg) in the transdermal patches exhibited better anti-hypertension effect synergistically with a similar inhibiting heart rates effect to that of BP alone in renovascular hypertensive rats, and was therefore selected as a final formulation. The *in vitro* transdermal penetration of both ISDN and BP from the patches displayed a zero-order process, and the penetration rate constants were 7.4 μg/(cm^2 h) for ISDN, and 5.9 μg/(cm^2 h) for BP, respectively. After transdermal administration at single dose or multiple doses, the synergistic anti-hypertension effect was confirmed in spontaneously hypertensive rats also. The effect of each patch lasts for 3 days, and increased with the total dose of two drugs (2 mg/cm^2, ISDN:BP = 3:2, mg/mg), showing a dose dependant manner. After transdermal administration to rabbits, the absolute bioavailabilities were 33.6% for ISDN, and 31.3% for BP, respectively. The maximal concentrations (C_{max}) of both drugs were significantly reduced while the areas under the plasma concentration–time curve (AUC), and mean residence times (MRT) were evidently increased and extended, respectively. As a patient-friendly, convenient, and multi-day dosing therapeutic system, the transdermal patches incorporating ISDN and BP could be promising for prevention and treatment of hypertension.

Keywords: Transdermal delivery; Hypertension; Isosorbide dinitrate; Bisoprolol; Pharmacodynamics; Pharmacokinetics

1. Introduction

Heart diseases mortality increases dramatically with age. Heart disease deaths that occur before the age of 65 are generally considered premature, preventable deaths, and are therefore of particular public health significance. Hypertension is one of the main causes of heart disease, and in recent years, the age-adjusted hypertension and hypertensive disease death rates are increasing (Hoyert et al., 2005). Consequently, the prevention and treatment of hypertension are of social significance (Kim and Kitakaze, 2004).

A transdermal patch is a medicated adhesive patch that is placed on the skin to deliver a time-released dose of medication through the skin for treating systemic illnesses. Since early 1980s, this dosage form of transdermal therapeutic system (TTS) has been available in the pharmaceutical market. Such a system offers a variety of significant clinical benefits over others, such as tablets and injections. For examples, it provides controlled release of the drug into the patient, and enables a steady blood-level profile, leading to reduced systemic side effects and, sometimes, improved efficacy over other dosage forms (Ranade, 1991; Modamio et al., 2000; Ke et al., 2005). In addition, the dosage form of transdermal patches is user-friendly, convenient,

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painless, and offers multi-day dosing, it is generally accepted that they offer improved patient compliance (Audet et al., 2001). In these regards, the transdermal therapeutic system is of particular clinical significance for prevention and long-term treatment of the chronic diseases like hypertension.

Isosordide dinitrate (ISDN), a vasodilator, is used primarily to prevent and treat angina, and in the treatment of acute heart attacks and heart failure (http://www.nlm.nih.gov/medlineplus/druginfo/uspdi/202411.html). After oral administration, ISDN is subject to extensive first pass elimination, thus leading to a lower oral bioavailability in human subject (30%). In addition, it exhibits larger inter- and intra-subject variability in plasma concentrations (Fung, 1985; Taylor et al., 1985). The duration of the effect after oral ISDN is only 3–4 h, which is not long enough to meet therapeutic needs (Johnson et al., 1981).

Bisoprolol is a highly selective beta 1-adrenoceptor antagonist (beta-blocker), which has no partial agonist (intrinsic sympathomimetic activity) (Brodde, 1986) or membrance-stabilizing (local anesthetic) activity (Harting et al., 1986). In animals, bisoprolol is more potent than atenolol or metoprolol, but less potent than propranolol, and betaxolol. Bisoprolol has a long duration of action, with significant reduction in exercise tachycardia (about 20%) being observed in subjects with stable angina pectoris 24 h after oral administration of 5 and 10 mg (de Muinck et al., 1987). Both systolic and diastolic blood pressure are reduced by bisoprolol (by up to about 20%, respectively, in healthy subjects and in patients with mild to moderate essential hypertension) as well as mycocardial oxygen demand (by up to 34%) (Lancaster and Sorkin, 1988).

The combination of nitrate and beta-blocker has been used in the therapies of hypertension and heart disease. It was reported that the application by combination of nitrate and beta-blocker (Bosch et al., 1993) caused a significant greater reduction in hepatic venous pressure gradient (HVPG) as compared to application of either drug alone. This combination application was superior to beta-blocker alone in maintaining liver perfusion and hepatic function, while the beneficial effect of reducing azygos blood flow was unchanged (Garcia-Pagan et al., 1990, 1991). When compared to administration of beta-blocker alone, the addition of a controlled release formulation of nitrate once daily to long term beta-blocker therapy in patients with angina pectoris increased excise capacity, and provided significant anti-anginal and anti-ischaemic effects. The combination was well tolerated, and the only significant side effect was headache, which rapidly disappeared during the continued therapy. There was no sign of a decrease in the effect of treatment during a follow-up period of 1 year (Uusitalo, 1987).

The endothelium is confirmed as a potential therapeutic target. Endothelial dysfunction has been demonstrated in resistance and conduction vessels. The study of anti-hypertensive therapy on endothelial vasodilation is regarded as a new pharmacological approach (Chamontin, 2006). The chest pain of coronary heart disease (angina) usually occurs when the heart requires more blood and oxygen than its coronary vessels can deliver. This demand for oxygen is related, in part, to blood pressure. The demand for oxygen can be reduced in one of two ways. First, dilating the veins and pooling the blood lowers the blood pressure. Second, dilating the arteries reduces the pressure that the heart has to pump against. ISDN reduces angina by dilating both the veins and the arteries. Therefore, we hypothesize that the combination therapy using ISDN and BP would provide more beneficial effect for anti-hypertension. Accordingly, ISDN and BP were first incorporated together into the transdermal patches, named as ISDN-BP-TTS, by our institution in collaboration with Beijing Kangbeide Pharmaceuticals, Ltd. (Beijing, China). The objectives of the present study were to define effects on the antihypertension activity and pharmacokinetics in animals following transdermal administration of a novel patch incorporating isosorbide dinitrate (ISDN) with bisoprolol (BP).

2. Materials and methods

2.1. Formulations

The formulation of each patch was designed for 2 days' clinical application at least. The target delivery rates of isosorbide dinitrate and bisoprolol were tailored according to the rationale (Babu and Pandit, 2005), a zero-order release kinetic equation: $AK_0 = C_pCl$, where A represents the area of the transdermal patch; K_0 represents target zero-order delivery rate; C_p represents the effective therapeutic plasma concentration; Cl represents the total body clearance rate.

The best formula of the patches prepared is shown in Table 1. An acrylic resin composition (invented and patented by Beijing Kangbeide Pharmaceuticals, Ltd., Beijing, China) was dissolved in ethyl acetate at ambient temperature. ISDN, BP and penetration enhancers were then added to the above solution that was agitated at room temperature for 20 min, and then homogenized for 10 min. The mixture was applied to the surface of a flexible backing membrane (tailored by Beijing Kangbeide Pharmaceuticals, Ltd., Beijing, China), placed at static state for 10 min, and dried at 40 °C for 0.5 h and at 60 °C for 1.5 h. After cooling, the patches were cut, covered by a protective membrane layer, and packed by aluminium–plastic membrane. The structure of the patches consisted of three layers, namely, backing membrane, pressure sensitive adhesive layer that acted as the reservoir of drugs together with the adhesive, and protective membrane, as illustrated in Fig. 1.

Various formulations of the transdermal patches were prepared for screening a suitable ratio of isosorbide dinitrate (ISDN)

Table 1
Composition and characteristics of the patches prepared

Acrylic resin composition[a] as a matrix and drug reservoir	mg	765
ISDN	mg	48
BP	mg	32
Enhancers consisting of azone, Tween 80, and glycerol (6.7:38.7:54.6, w/w/w)	% (w/w)	Quabtum satis
Patch thickness	μm	~700
Area	cm^2	40

[a] Invented and patented by Beijing Kangbeide Pharmaceuticals, Ltd., Beijing, China.

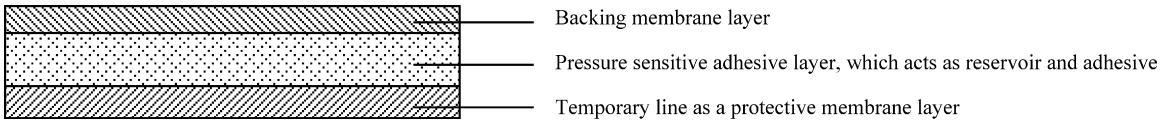


Fig. 1. Cross-sectional scheme of the ISDN-BP-TTS.

Table 2
Formulation screening design for the ISDN-BP-TTS

Formulation	ISDN plus BP (per cm^2)	Mark
1	Blank patch, as a control	0:0
2	2.0 mg + 0 mg	1:0, ISDN alone
3	0 mg + 2.0 mg	0:1, BP alone
4	0.67 mg + 0.33 mg	1:2
5	0.50 mg + 1.50 mg	1:3
6	0.80 mg + 1.20 mg	2:3
7	1.33 mg + 0.67 mg	2:1
8	1.50 mg + 0.50 mg	3:1
9	1.20 mg + 0.80 mg	3:2
10	1.0 mg + 1.0 mg	1:1

to bisoprolol (BP) for the pharmacodynamic and pharmacokinetic studies, consisting of 10 formulations (1–10), in which one patch contained 2 mg of drug(s) in total per cm^2. ISDN and BP were constructed in the specified ratios, marked as 0:0, 1:0, 0:1, 1:2, 1:3, 2:3, 2:1, 3:1, 3:2, 1:1, respectively, as indicated in Table 2.

2.2. Animals

Male guinea pigs (450 ± 30 g) and Japanese white rabbits (2.2 ± 0.1 kg) were provided by Peking University Animal Center (Beijing, China). Adult spontaneously hypertensive rats (SHR) (180–220 g) were purchased from Beijing Weitong Lihua Experimental Animals Ltd. Co. (Beijing, China). Sprague–Dawley rats (150–200 g) were obtained from Fudan University Animal Center (Shanghai, China). All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

2.3. In vitro release

The *in vitro* release of transdermal patches, formulation-9 (ISDN:BP = 3:2), was performed with a disk-assembly method, using an apparatus according to the Chinese Pharmacopoeia (2005, Part II, Appendix XD method III). Briefly, a volume of 900.0 ml fresh degassed purified water (dissolution medium) was placed in the vessel and the temperature of the medium was equilibrated to 32 ± 0.5 °C. A patch sample of 20.0 cm^2 was applied to the stainless steel disk-assembly (SSDA), with the release surface facing up. The back of the patch was attached on the SSDA by using a cyanocarylate adhesive. Then the paddle was rotated at 100 revolutions/min, immediately. Sampling time-points were set at 0.5, 1.0, 3.0, 6.0, and 12.0 h, respectively. The same volume of fresh degassed purified water was supplemented to the receiver after each sampling. The samples were filtered through a 0.2 μm filter membrane and analyzed by the HPLC method. Briefly, an aliquot of 10 μl sample containing isosorbide dinitrate and bisoprolol was assayed on a Shimadzu's model LC-2010A HPLC system (Tokyo, Japan) at 35 °C. The mobile phase consisted of a binary mixture of methanol and ammonium bicarbonate buffer (pH 9.2) (40:60, v/v). The mobile phase was delivered at a flow rate of 1.0 ml/min. A reversed-phase X-Terra RP_{18} column (150 mm × 4.6 mm, 5 μm, Waters Corporation, Milford, MA, USA) was used, and the detection wavelength was set at 230 nm. The limits for quantification of isosorbide dinitrate and bisoprolol were 0.1 and 0.5 μg/ml, respectively. The between-day coefficients of variation for both drugs were less than 2%.

2.4. In vitro transdermal penetration

Male guinea pig skin (approximate 1 mm in thickness) was carefully excised. After removing the hypodermal adipose tissue, the skin was used as a barrier membrane for the *in vitro* transdermal penetration. The transdermal penetration of formulation-9 (ISDN:BP = 3:2) was performed in Franz-type glass single-diffusion cells with 'a' 5 cm^2 penetration area and a 100.0 ml receptor volume, as reported previously (Ke et al., 2005). Briefly, the receptor medium (degassed water containing 1% sodium azide as a preservative, w/v) in Franz-type glass single-diffusion cells was magnetically stirred at 32.0 ± 0.5 °C. Sampling time-points were set at 3.0, 6.0, 12.0, 24.0, 48.0, 72.0, and 96.0 h. The same volume of fresh degassed purified water containing 1% sodium azide (w/v) was supplemented to the receptor after each sampling. The samples were filtered through a 0.2 μm filter membrane, and an aliquot of 10 μl the filtrate containing isosorbide dinitrate and bisoprolol was assayed as above.

2.5. Effect on pharmacodynamics

2.5.1. Renovascular hypertensive rat model

Renovascular hypertensive rat model was constructed as reported previously (Burrell et al., 1997; Jung et al., 2004). Briefly, 6 weeks prior to experiment, each Sprague–Dawley rat was clipped at the left rental artery with a sliver clip having an internal gap of 0.25 mm under ketamine (50 mg/kg, injected intraperitoneally) anesthesia. The control group was operated with the same condition as the experimental group, except that no clipping was made.

2.5.2. Ratio of ISDN to BP screening

The renovascualr hypertensive rats were randomly divided into 10 groups (9–10 rats for each group): groups 1–10. Rats were acclimatized to a BESN-II system (Fu and Luo, 2004) that is a computer-based multi-channel instrument for measuring

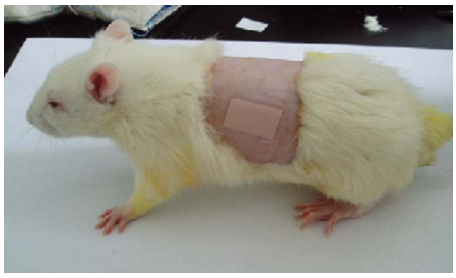


Fig. 2. Transdermal administration. During the pharmacodynamic studies, the transdermal patch (ISDN-BP-TTS) was applied on one site of the two flanks of the shaven rats.

tail-artery blood pressure and heart rates (invented by Dr. Ji-Hua Fu, China Pharmaceutical University, Nanjing, China) for 2 weeks prior to administration. In the period of acclimation, the rat systolic pressure (SAP), diastolic arterial pressure (DAP) and heart rate (HR) were measured at least for triplicate times.

The rats in group 1 were administered transdermally with the blank patches (formulation-1) as a control, and those in other groups (groups 2–10) were with drug containing patches (formulations-2–10), respectively. One rat was given with one patch, as illustrated in Fig. 2. The total dose (ISDN plus BP) administered to each rat in groups 2–10 was 10 mg/kg, in varied ratios of ISDN to BP as indicated in Table 2, namely, 1:0, 0:1, 1:2, 1:3, 2:3, 2:1, 3:1, 3:2, 1:1, respectively. After administration, the blood pressures (SAP and DAP), and heart rates were measured at 0 (prior to dosing), 5.0, 10.0, 15.0, 20.0, 28.0, 35.0, 45.0, 50.0, 60.0, and 72.0 h, respectively.

Lowering effects on the SAP and DAP values, and inhibiting effect on the HR values were expressed by the differences between the value after administration and before administration, respectively. By combining the difference values obtained at 15.0, 20.0, and 28.0 h, the mean value in the interval of 15.0–28.0 h were calculated as the representative value for SAP, DAP, and HR, respectively.

2.5.3. Effect of ISDN-BP-TTS on adult spontaneously hypertensive rats

2.5.3.1. Single dose. Adult spontaneously hypertensive rats (SHR) were randomly divided into five groups (9–10 rats for each group): groups I–V, respectively. The rats in group I were administered transdemally with the formulation-1 (each administered as one patch), as a blank control, and the rats in group II were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions, in which bisoprolol fumarate tablets (Beijing Sihuan Pharmaceutical Company, Beijing, China) were ground into powders and suspended in 0.5% CMC-Na (carboxymethylcellulose sodium), as a positive control. The rats in groups III–V were administered transdermally with the formulation-9 (each administered as one patch) at 5.0, 10.0, 20.0 mg/kg of total drugs (ratio of ISDN to BP, 3:2), respectively. The patches administered in all groups were removed from the rats at 72 h, respectively. After administration, the blood pressures (SAP and DAP), and heart rates were measured at 0 (prior to dosing), 5.0, 10.0, 15.0, 20.0, 28.0, 35.0, 45.0, 50.0, and 60.0 h, respectively.

2.5.3.2. Multiple doses. Adult spontaneously hypertensive rats (SHR) were randomly divided into five groups (9–10 rats for each group): groups VI–X, respectively. The rats in group VI were administered transdemally with formulation-1 (each administered as one patch) in every 2 days for eight consecutive days, as a blank control, and the rats in group VII were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions once daily for eight consecutive days, as a positive control. The rats in groups VIII–X were administered transdermally with formulation-9 (each administered as one patch) at 5.0, 10.0, 20.0 mg/kg of total drugs (ratio of ISDN to BP, 3:2) in every 2 days for eight consecutive days, respectively. During multiple dosing, a used patch was replaced with a new one with the same dose, and the administration site for each dosing was changed but the site should be in the area of two flanks of the rats.

2.6. Effect on pharmacokinetics

2.6.1. Administration and sampling

Rabbits were equally divided into two groups (10 for each): groups A and B. Before administration, rabbits were housed individually in standard cages, kept on a standard diet, and had free access to water.

The rabbits in group A were administered intravenously with a sterile physiological saline solution containing isosorbide dinitrate (3 mg) and bisoprolol fumarate (1.93 mg bisoprolol) through marginal ear vein, respectively. After administration, a volume of 2.0 ml blood samples were drawn from the marginal vein of the ear at 0 (prior to dosing), 0.083, 0.167, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 10.0, and 24.0 h, and collected with the heparinized tubes, respectively. Plasma samples were separated by immediately centrifugation at 3000 revolutions/min for 10 min, and stored at −70 °C until analysis. The rabbits in group B were administered transdermally with formulation-9 (each administered as one patch) at 40.0 mg of total drugs (ratio of ISDN to BP, 3:2), respectively. The patch was stuck onto one dorsal flank of rabbit after carefully shaving the hair, and removed at 96 h after administration. A volume of 2.0 ml blood samples were drawn from the marginal vein of the ear at 0 (prior to dosing), 0.33, 0.67, 2.0, 6.0, 12.0, 24.0, 48.0, 72.0, 96.0, and 120.0 h, and the following procedures were the same as above.

2.6.2. Preparation of plasma samples

A volume of 1.0 ml plasma was mixed with 10 μl of 2,4-dinitrochlorobenzene (internal standard) by a vortex mixer as the sample. The Waters oasis cartridge (Waters Corporation, Milford, MA, USA) was pre-conditioned by 1.0 ml methanol, and 1.0 ml water in turn. The sample was applied onto the cartridge, followed by washing with 1.0 ml water, and 1.0 ml 5% methanol solution, respectively. ISDN and BP were then eluted by 1.0 ml methanol. The eluted fraction by the methanol was evaporated to dryness under a gentle nitrogen stream. The residue was reconstituted in 100 μl methanol for the below measurements.

2.6.3. Measurement of ISDN in plasma

Measurement method for ISDN concentration in plasma was modified according to the previous report (Gabiga et al., 2000).

Briefly, an aliquot of 10 μl the reconstituted residue sample was injected onto a Hewlett-Packard Model 6890 gas chromatography system equipped with a Ni-63 electron-capture detector, a split–splitless injector containing a deactivated splitless liner, and a Hewlett-Packard Model 7363A autosampler (Hewlett-Packard Company, South Taft Loveland, CO, USA). The measurement conditions were as follows: a HP-1 30 m × 0.25 mm fused silica capillary column; ultrahigh purity nitrogen as the carrier gas; the flow rate, 2.5 ml/min; the injection mode, split; the injection port temperature, 220 °C; and the detector temperature, 250 °C. The column temperature was initially set at 110 °C, then the oven temperature was increased at a speed of 5 °C/min to 170 °C and maintained for 2 min. After each analysis, the oven temperature was increased to 230 °C at a speed of 30 °C/min, and maintained for 3 min to purge residuals. The lower limit of quantification (LOQ) of the assay was 1.0 ng/ml, and linearity was obtained for ISDN concentration ranging from 2.0 to 30.0 ng/ml ($R^2 = 0.993$). The coefficients of variation of the inter-day and intra-day precision of the quality control samples ranged from 1.1% to 5.3% and accuracy ranged from 93.8% to 96.2%.

2.6.4. Measurement of bisoprolol in plasma

Measurement method for BP concentration in plasma was modified according to the previous report (Braza et al., 2002). Briefly, an aliquot of 20 μl the reconstituted residue sample was analyzed using a Waters Alliance 2690 HPLC system equipped with a 474-fluorescence detector, and a 717 type of auto-sampler (Waters Corporation). The mobile phase consisted of a binary mixture of acetonitrile and ammonium bicarbonate buffer (pH 9.7) (30:70, v/v). The mobile phase was delivered at a flow rate of 1.0 ml/min. The analytical column was X-Terra RP_{18} (150 mm × 4.6 mm, 5 μm, Waters Corporation), and the column temperature was 25 °C. The excitation and emission wavelengths were set at 275 and 305 nm, respectively. The lower limit of quantification (LOQ) of the assay was 4.0 ng/ml, and linearity was obtained for BP concentration ranging from 5.0 to 150.0 ng/ml ($R^2 = 0.999$). The coefficients of variation of the inter-day and intra-day precision of the quality control samples ranged from 0.8% to 5.3% and accuracy ranged from 90.1% to 99.3%.

2.7. Calculations and statistics

Non-compartmental pharmacokinetics was used for calculating the parameters using the 3p87 software, a practical pharmacokinetic program compiled by Chinese Association of Mathematic Pharmacology (Beijing, China). The area under the plasma concentration–time curve (AUC) was calculated according to the trapezoidal rule. C_{max}, $t_{1/2}$, and MRT values were obtained from the non-compartmental analyses, and denote the maximal concentration, terminal half-life, and mean residence time, respectively. Absolute bioavailability (F) was calculated with the following equation: $F = (AUC_{transdermal} \times dose_{i.v.})/(AUC_{i.v.} \times dose_{transdermal})$, where $AUC_{transdermal}$ and $AUC_{transdermal}$ represent the area under the plasma concentration–time curve after transdermal administration, and after intravenous bolus injection, respectively. $Dose_{transdermal}$ and $dose_{transdermal}$ are the dose of transdermal administration, and intravenous bolus injection, respectively.

Data are expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was used to determine significance among groups, after which post hoc tests with the Bonferroni correction were used for comparison between individual groups. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Transdermal patch design

According to the published data, the lower C_p and total body clearance Cl values for ISDN were 1 ng/ml (Assinder et al., 1977) and 3.2 l/min (Laufen and Leitold, 1992), the target delivery rate for transdermal administration of ISDN was estimated to be above 4.8 μg/(cm^2 h). Similarly, the lower C_p and total body clearance Cl values for BP were 10 ng/ml and 16 l/h (Lancaster and Sorkin, 1988), the target delivery rate for transdermal administration of BP estimated should be above 4.0 μg/(cm^2 h). In the above calculations, the area of patches was 40 cm^2.

3.2. In vitro release and transdermal penetration

The *in vitro* released percentage of ISDN and BP *versus* time profiles from formulation-9 are illustrated in Fig. 3A and B, respectively. Results showed that the released percentage of ISDN was higher than that of BP at the same time-point, respectively. The release kinetics *in vitro* for both of ISDN and BP exhibited a first order process, and the equations of released percentage (y, %) *versus* time (x, h) were $y = 16.8 \ln x + 59.3$ ($R^2 = 0.91$) for ISDN, and $y = 19.4 \ln x + 41.4$, $R^2 = 0.99$ for BP, respectively.

The *in vitro* cumulative penetration amount per cm^2 time profiles of the transdermal patches containing isosorbide dinitrate (ISDN) and bisoprolol (BP) from formulation-9 are illustrated in Fig. 4A and B, respectively. The results showed that the penetration kinetics for ISDN or BP was a zero-order process. The equations of cumulative penetration amount (μg/cm^2, y) *versus* time (h, x) were $y = 53.84x + 7.39$ ($R^2 = 0.99$) for ISDN, and $y = 23.84x + 5.86$ ($R^2 = 0.99$) for BP, respectively. The calculated delivery rates (penetration rate constants) for transdermal administration were 7.4 μg/(cm^2 h) for ISDN, and 5.9 μg/(cm^2 h) for BP, thus meeting the designed target delivery rats for both drugs, respectively.

3.3. Ratio of ISDN to BP screening

After transdermal administrations of various formulations (formulations-3–10), the SAP, DAP, and HR values of renovascular hypertensive rats were obviously decreased at 5–60 h, but slightly at 72 h, as compared to those before administration or those after administration of formulation-1 (the blank patch as a control), respectively.

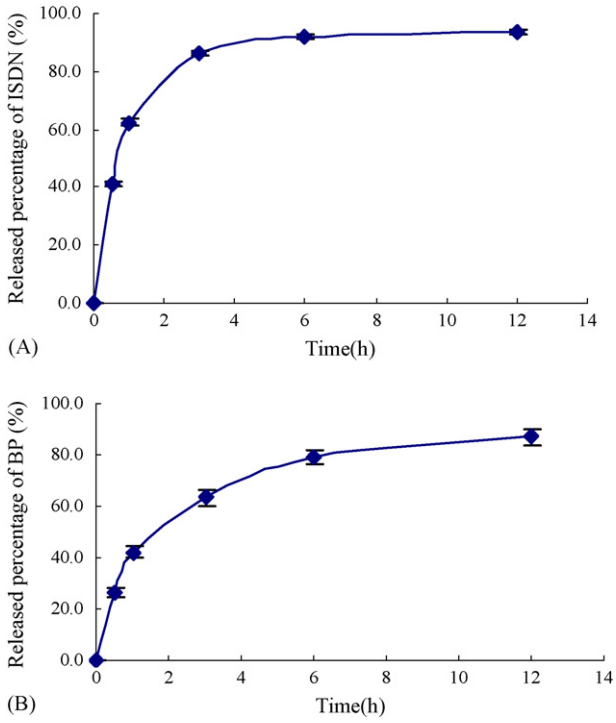


Fig. 3. Released percentages-time profiles *in vitro* of the transdermal patches containing isosorbide dinitrate (ISDN) and bisoprolol (BP). Keys: (A) released percentage of ISDN *vs*. time; (B) released percentage of BP *vs*. time. The *in vitro* release of patches of formulation-9 (containing 1.2 mg ISDN with 0.8 mg BP/cm^2 of the patch) was performed with a disk-assembly apparatus with 'a' 20 cm^2 release area and a volume of 900.0 ml dissolution medium, according to the Chinese Pharmacopoeia (2005 edition, Part II, Appendix XD method III). Data are presented as the mean ± standard deviation (S.D.).

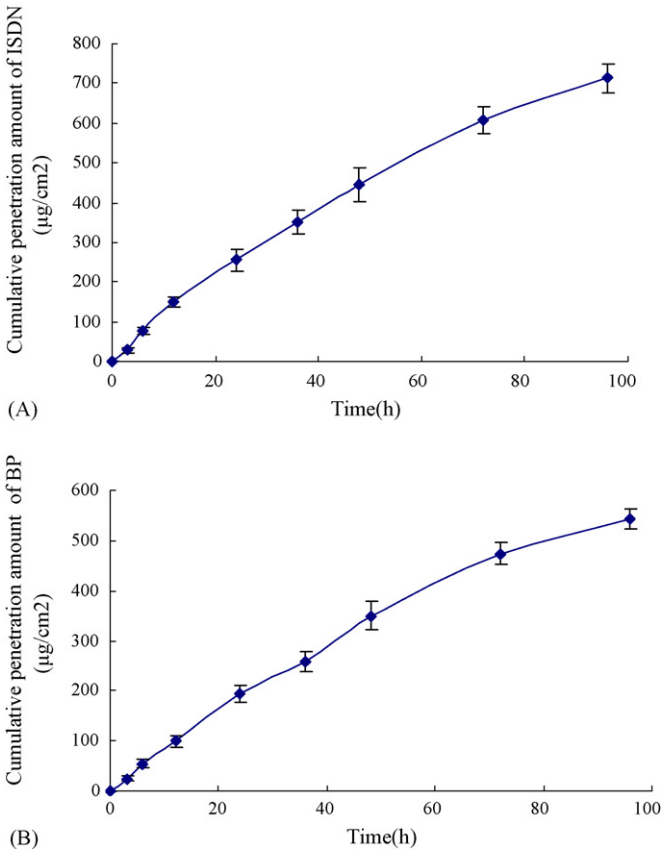


Fig. 4. The *in vitro* cumulative penetration amount per cm^2 time profiles of the transdermal patches containing isosorbide dinitrate (ISDN) and bisoprolol (BP). Keys: (A) the cumulative penetration amount per cm^2 of ISDN *vs*. time; (B) the cumulative penetration amount per cm^2 of BP *vs*. time. The excised skin by removing adipose tissue from male guinea pigs was used as a barrier membrane for transdermal penetration *in vitro*. The transdermal penetration was performed with formulation-9 (containing 1.2 mg ISDN with 0.8 mg BP/cm^2 of the patch) in Franz-type glass single-diffusion cells with 'a' 5 cm^2 penetration area and a 100.0 ml receptor volume. Data are presented as the mean ± standard deviation (S.D.).

Results in Fig. 5A showed that the rank order in the decreased difference values of SAP after administrations was formulation-5 > formulation-9 > formulation-4 > formulation-6 > formulation-10 > formulation-8 > formulation-3 > formulation-7 > formulation-2, as compared to the SAP baseline value before administration. In contrast, the difference value of SAP was slightly increased after administration of formulation-1 (as a blank control).

Results in Fig. 5B showed that the rank order in the decreased difference values of DAP after administrations was formulation-9 > formulation-5 > formulation-10 > formulation-4 > formulation-6 > formulation-3 > formulation-7 > formulation-8, as compared to the DAP baseline value before administration. In contrast, the difference values of SAP were slightly increased after administration of formulations-2, and -1, respectively.

Results in Fig. 5C showed that the rank order in the decreased difference values of HR after administrations was formulation-4 > formulation-6 > formulation-5 > formulation-10 > formulation-9 > formulation-3 > formulation-7 > formulation-8 > formulation-2, as compared to the HR baseline value before administration. In contrast, the difference value of HR was slightly increased after administration of formulation-1 as a blank control.

Taken together, the final formulation-9 (ratio of ISDN to BP, 3:2) was selected according to the obviously lowered effects on SAP and DAP values, and the medium inhibited effect on the HR values of renovascular hypertensive rats.

3.4. Effect on adult spontaneously hypertensive rats

3.4.1. Single dose

After transdermal administration at a single dose (one patch of the formulation-9), the effects of ISDN-BP-TTS on the SAP, DAP, and HR values of adult spontaneously hypertensive rats are described in Tables 3–5, respectively.

Results in Table 3 showed that, after transdermal administration of the blank patches (group I), the mean SAP values at various time-points (0–60 h) remained at the same levels as those before administration, exhibiting a hypertensive characteristic. In contrast, after intragastrical administration of bisoprolol fumarate suspensions as a positive control (group II), the mean SAP values in the interval between 5 and 28 h were significantly decreased. However, the mean SAP values were gradually recovered to the values before administration from 35 to 60 h. After transdermal administration of the drug-loaded patches at low

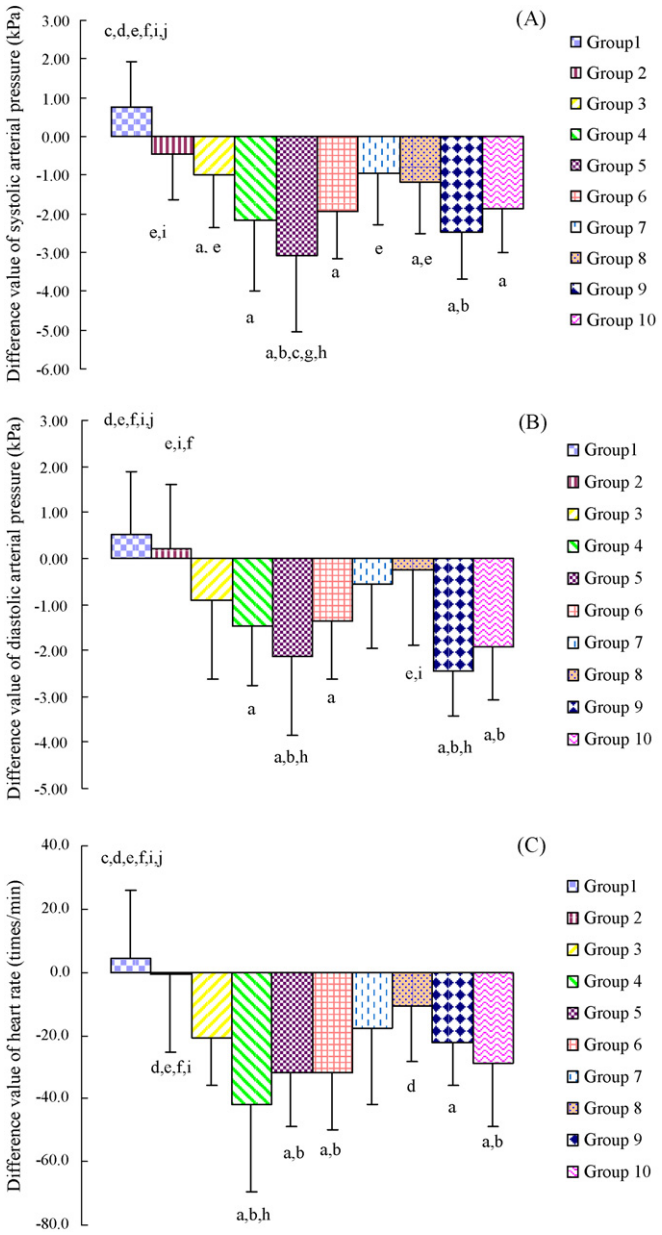


Fig. 5. Lowering effects on the systolic pressure (SAP) and diastolic arterial pressure (DAP), and inhibiting effect on the heart rate (HR) of renovascular hypertensive rats following transdermal administration of formulations-1–10 as a single patch for 72 h, respectively. (A) The difference value of SAP between after and before administration; (B) the difference value of DAP between after and before administration; (C) the difference value of HR between after and before administration. The above difference values were the mean values by combining the ones obtained at 15, 20, and 28 h, respectively. One rat in group 1 was given with a blank transdermal patch; one rat in group 2, with a patch containing ISDN alone (10 mg/kg); and each rat in the group 3, with a patch containing BP (10 mg/kg) alone, respectively. One rat in group 4 was given with a patch at a total dose of 10 mg/kg both drugs (ISDN:BP = 1:2, mg/mg). Accordingly, one rat in groups 5–10 was given with a patch at a total dose of 10 mg/kg containing ISDN and BP in varied ratios of ISDN to BP: 1:3, 2:3, 2:1, 3:1, 3:2, and 1:1 mg/mg, respectively. Data are presented as the mean ± standard deviation (S.D.), (a) $p < 0.05$ *vs.* group 1; (b) $p < 0.05$, *vs.* group 2; (c) $p < 0.05$ *vs.* group 3; (d) $p < 0.05$ *vs.* group 4; (e) $p < 0.05$ *vs.* group 5; (f) $p < 0.05$ *vs.* group 6; (g) $p < 0.05$ *vs.* group 7; (h) $p < 0.05$ *vs.* group 8; (i) $p < 0.05$ *vs.* group 9; (j) $p < 0.05$ *vs.* group 10.

dose (group III), the mean SAP values in the interval between 5.0 and 60.0 h were significantly decreased as compared to those before administration, showing a long action effect. Similar effects were observed after transermal administration of the drug-loaded patches at medium (group IV) and high dose (group V), respectively. Among groups from III to V, the SAP lowering effects showed a dose-dependent manner.

Results for DAP values in Table 4 showed a similar tendency to those for SAP values in Table 3. Slight different phenomena were that the lowering effects were only lasted for 20.0 h in the positive control (group II), and for 45.0 h in the low dose group (group III), respectively.

Results in Table 5 showed that, after transdermal administration of the blank patches (group I), the mean HR values at various time-points (0–60.0 h) were the same as those before administration. In contrast, after intragastrical administration of bisoprolol fumarate suspensions as a positive control (group II), the mean HR values in the interval between 5.0 and 45.0 h were significantly decreased. The mean HR values were gradually recovered to the values before administration from 50.0 to 60.0 h. After transdermal administration of the drug-loaded patch at low dose (group III), the mean HR values in the interval between 5.0 and 35.0 h were significantly decreased as compared to those before administration, while the mean HR values were gradually recovered to the values before administration from 45.0 to 50.0 h. Irregularly, the mean HR values at 60.0 h were decreased. Similarly, lowered HR values were observed and lasted for 60.0 h after transermal administration of the drug-loaded patches at medium (group IV) and high dose (group V), respectively. Among groups from III to V, the HR lowering effects showed a dose-dependent characteristic.

3.4.2. Multiple doses

After transdermal administrations as a single patch of ISDN-BP-TTS in every 2 days for eight consecutive days at low, medium, and high dose, the effects on the SAP, DAP, and HR values of adult spontaneously hypertension rats are depicted in Tables 6–8, respectively.

Results in Table 6 showed that, after transdermal administrations of the blank patches (group VI), the mean SAP values at various time-points during the 8 days fluctuated slightly but kept at a higher level, similar to that before administration, demonstrating a hypertensive characteristic. On the contrary, after multiple intragastrical administrations of bisoprolol fumarate suspensions as a positive control (group VII), the mean SAP values in the period between days 2 and 8 were significantly decreased. After multiple transdermal administrations of the drug-loaded patches at low dose (group VIII), the mean SAP values in the period between days 2 and 8 were significantly decreased as compared to those before administration. More obvious pressure lowering effects were observed in groups IX and X, respectively. Among groups from VIII to X, the SAP lowering effects showed a dose-dependent manner.

Similar lowering effects were observed in the DAP, and HR values, as shown in Tables 7 and 8, respectively.

Table 3
Effects on the systolic arterial pressure (SAP) of adult spontaneously hypertensive rats ($n = 9$–10 for each group) after transdermal administration as a single patch of ISDN-BP-TTS at low, medium, and high dose, respectively

Group	SAP value after administration and the SAP difference value (Δ in parentheses) between before and after the administration (kPa)				
	Before	5.0 h	10.0 h	15.0 h	20.0 h
Group I	26.29 ± 0.90	26.52 ± 0.64 (0.23 ± 0.95)	26.65 ± 0.77 (0.37 ± 0.93)	26.47 ± 0.85 (0.18 ± 0.91)	26.67 ± 0.66 (0.39 ± 0.83)
Group II	26.34 ± 0.44	24.29 ± 1.01 a2,b2 (−2.05 ± 1.16) a2	23.89 ± 0.63 a2,b2 (−2.45 ± 0.75) a2	24.17 ± 0.43 a2,b2 (−2.17 ± 0.47) a2	24.51 ± 0.14 a2,b2 (−1.83 ± 0.45) a2
Group III	26.05 ± 0.82	24.03 ± 0.68 a2,b2 (−2.03 ± 0.61) a2	24.12 ± 0.74 a2,b2 (−1.93 ± 0.84) a2	23.79 ± 0.71 a2,b2 (−2.26 ± 0.91) a2	24.17 ± 0.43 a2,b2 (−1.88 ± 0.84) a2
Group IV	26.50 ± 0.76	23.81 ± 1.23 a2,b2 (−2.69 ± 1.12) a2	23.72 ± 0.80 a2,b2 (−2.78 ± 1.02) a2	23.70 ± 0.89 a2,b2 (−2.79 ± 0.94) a2	23.84 ± 0.64 a2,b2 (−2.65 ± 0.67) a2
Group V	26.92 ± 0.83	23.79 ± 1.69 a2,b2 (−3.13 ± 1.47) a2	23.22 ± 0.76 a2,b2 (−3.70 ± 0.78) a2	23.18 ± 0.69 a2,b2 (−3.74 ± 0.78) a2	23.39 ± 0.54 a2,b2 (−3.53 ± 0.73) a2

Group	SAP value after administration and the SAP difference value (Δ in parentheses) between before and after the administration (kPa)				
	28.0 h	35.0 h	45.0 h	50.0 h	60.0 h
Group I	26.20 ± 0.76 (−0.08 ± 0.73)	26.56 ± 1.05 (0.27 ± 0.76)	26.77 ± 0.63 (0.48 ± 1.00)	26.23 ± 0.59 (−0.06 ± 1.18)	26.30 ± 1.48 (0.01 ± 1.53)
Group II	25.31 ± 0.89 a1,b2 (−1.03 ± 0.99) a1	25.92 ± 0.84 (−0.42 ± 0.68)	26.54 ± 0.73 (0.20 ± 0.92)	26.5 ± 0.84 (0.16 ± 0.93)	26.27 ± 0.64 (−0.07 ± 0.93)
Group III	25.13 ± 0.76 a1,b1 (−0.92 ± 1.13)	24.83 ± 0.49 a2,b2 (−1.22 ± 0.90) a2	25.52 ± 0.85 a2 (−0.54 ± 1.17)	24.96 ± 0.56 a2,b2 (−1.09 ± 0.84)	24.93 ± 0.65 a1,b2 (−1.12 ± 0.65)
Group IV	24.35 ± 0.49 a2,b2 (−2.14 ± 0.71) a2	24.39 ± 0.54 a2,b2 (−2.11 ± 0.88) a2	24.39 ± 0.47 a2,b2 (−2.11 ± 0.59) a2	25.17 ± 0.66 a2,b2 (−1.32 ± 0.99) a1	24.82 ± 1.10 a1,b2 (−1.68 ± 1.38) a1
Group V	23.32 ± 0.39 a2,b2 (−3.6 ± 0.72) a2	23.75 ± 0.48 a2,b2 (−3.17 ± 0.80) a2	24.26 ± 1.43 a2,b2 (−2.66 ± 1.58) a2	25.26 ± 0.98 a1,b2 (−1.66 ± 1.19) a2	24.68 ± 1.22 a1,b2 (−2.23 ± 1.60) a2

Notes: The rats in group I were administered transdemally with the formulation-1 (each administered as one patch), as a blank control, and the rats in group II were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions, in which bisoprolol fumarate tablets (Beijing Sihuan Pharmaceutical Company, Beijing, China) were ground into powders and suspended in 0.5% CMC-Na (carboxymethylcellulose sodium), as a positive control. The rats in groups III–V were administered transdermally with the formulation-9 (each administered as one patch) at 5, 10, 20 mg/kg of total drugs (ratio of ISDN to BP, 3:2), respectively. Data are expressed as the mean ± standard deviation (S.D.). a1: $p < 0.05$ *vs*. blank control group; a2: $p < 0.01$ *vs*. blank control group; b1: $p < 0.05$ *vs*. the group before administration; b2: $p < 0.01$ *vs*. the group before administration.

Table 4
Effects on the diastolic arterial pressure (DAP) of adult spontaneously hypertensive rats ($n = 9$–10 for each group) after transdermal administration as a single patch of ISDN-BP-TTS at low, medium, and high dose, respectively

Group	DAP value after administration and the DAP difference value (Δ in parentheses) between before and after the administration (kPa)				
	Before	5.0 h	10.0 h	15.0 h	20.0 h
Group I	19.86 ± 0.57	20.14 ± 0.54 (0.27 ± 0.54)	20.10 ± 0.78 (0.24 ± 0.89)	20.62 ± 0.31 (0.75 ± 0.53)	20.39 ± 0.66 (0.53 ± 0.91)
Group II	19.95 ± 0.64	18.65 ± 0.42 a2,b2 (−1.3 ± 0.71) a2	18.15 ± 0.65 a2,b2 (−1.81 ± 0.48) a2	18.57 ± 0.49 a2,b2 (−1.39 ± 0.74) a2	18.93 ± 0.47 a2,b2 (−1.03 ± 0.71) a2
Group III	20.03 ± 0.57	18.56 ± 0.80 a2,b2 (−1.48 ± 0.64) a2	18.53 ± 0.60 a2,b2 (−1.50 ± 0.84) a2	18.55 ± 0.77 a2,b2 (−1.49 ± 0.63) a2	18.96 ± 0.41 a2,b2 (−1.07 ± 0.41) a2
Group IV	20.13 ± ±0.37	18.07 ± 0.53 a2,b2 (−2.06 ± 0.52) a2	17.68 ± 0.61 a2,b2 (−2.45 ± 0.57) a2	17.58 ± 0.63 a2,b2 (−2.55 ± 0.74) a2	17.96 ± 0.55 a2,b2 (−2.17 ± 0.48) a2
Group V	20.46 ± 0.66	17.76 ± 1.10 a2,b2 (−2.71 ± 1.30) a2	17.76 ± 0.51 a2,b2 (−2.71 ± 0.91) a2	17.35 ± 0.47 a2,b2 (−3.11 ± 0.70) a2	17.49 ± 0.64 a2,b2 (−2.97 ± 1.07) a2

Group	DAP value after administration and the DAP difference value (Δ in parentheses) between before and after the administration (kPa)				
	28.0 h	35.0 h	45.0 h	50.0 h	60.0 h
Group I	20.26 ± 0.61 (0.40 ± 0.56)	20.05 ± 0.72 (0.19 ± 0.68)	19.98 ± 0.75 (0.12 ± 0.44)	20.08 ± 0.27 (0.21 ± 0.60)	19.35 ± 1.27 (−0.52 ± 1.18)
Group II	19.85 ± 0.74 (−0.10 ± 0.64)	19.92 ± 0.86 (−0.03 ± 0.67)	20.36 ± 0.89 (0.40 ± 0.74)	20.01 ± 0.58 (0.06 ± 0.58)	19.71 ± 0.48 (−0.24 ± 0.52)
Group III	19.20 ± 0.52 a2,b1 (−0.84 ± 0.79) a2	19.06 ± 0.31 a2,b2 (−0.97 ± 0.42) a2	19.44 ± 0.50 b1 (−0.60 ± 0.69) a1	19.61 ± 0.46 a1 (−0.43 ± 0.76)	19.58 ± 0.61 (−0.46 ± 0.89)
Group IV	18.46 ± 0.43 a2,b2 (−1.67 ± 0.31) a2	18.50 ± 0.74 a2,b2 (−1.63 ± 0.82) a2	18.58 ± 0.43 a2,b2 (−1.54 ± 0.39) a2	18.87 ± 0.56 a2,b2 (−1.26 ± 0.50) a2	18.71 ± 1.07 b2 (−1.75 ± 1.04) a1
Group V	18.14 ± 0.30 a2,b2 (−2.33 ± 0.74) a2	18.19 ± 0.36 a2,b2 (−2.27 ± 0.88) a2	18.58 ± 1.18 a2,b2 (−1.88 ± 1.33) a2	19.32 ± 0.84 a1,b2 (−1.14 ± 1.00) a2	18.64 ± 1.14 b2 (−1.49 ± 1.29)

Notes: The rats in group I were administered transdemally with the formulation-1 (each administered as one patch), as a blank control, and the rats in group II were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions, in which bisoprolol fumarate tablets (Beijing Sihuan Pharmaceutical Company, Beijing, China) were ground into powders and suspended in 0.5% CMC-Na (carboxymethylcellulose sodium), as a positive control. The rats in groups III–V were administered transdermally with the formulation-9 (each administered as one patch) at 5, 10, 20 mg/kg of total drugs (ratio of ISDN to BP, 3:2), respectively. Data are expressed as the mean ± standard deviation (S.D.). a1: $p < 0.05$ *vs*. blank control group; a2: $p < 0.01$ *vs*. blank control group; b1: $p < 0.05$ *vs*. the group before administration; b2: $p < 0.01$ *vs*. the group before administration.

Table 5
Effects on the heart rate (HR) of adult spontaneously hypertensive rats ($n = 9$–10 for each group) after transdermal administration as a single patch of ISDN-BP-TTS at low, medium, and high dose, respectively

Group	HR value after administration and the HR difference value (Δ in parentheses) between before and after the administration (beats/min)				
	Before	5.0 h	10.0 h	15.0 h	20.0 h
Group I	396.6 ± 13.6	385.1 ± 21.9 (−11.5 ± 16.3)	399 ± 22.1 (2.39 ± 10.6)	390.9 ± 19.2 (−5.7 ± 17.1)	395.7 ± 18.7 (−0.9 ± 13.3)
Group II	400.6 ± 9.2	353.2 ± 12.6 a2,b2 (−47.4 ± 12.6) a2	358.7 ± 16.7 a2,b2 (−41.9 ± 18.5) a2	369.6 ± 14.4 a1,b2 (−31 ± 14.9) a2	376.2 ± 16.3 a1,b2 (−24.4 ± 15.4) a2
Group III	398.2 ± 11.5	377.7 ± 12.4 b1 (−20.6 ± 20.3)	382.7 ± 5.6 a1,b2 (−15.6 ± 13.4) a2	383 ± 8.0 b2 (−15.2 ± 13.3)	380.6 ± 8.4 a1,b2 (−17.7 ± 5.6) a2
Group IV	400.4 ± 8.4	376.9 ± 12.4 b2 (−23.1 ± 13.9)	376.4 ± 6.9 a2,b2 (−23.6 ± 9.70) a2	374.9 ± 8.4 a1,b2 (−25.1 ± 12.9) a1	378 ± 7.6 a1,b2 (−22 ± 11.1) a2
Group V	403.1 ± 10.4	355.5 ± 21.4 a2,b2 (−47.6 ± 21.9) a2	361.4 ± 8.8 a2,b2 (−41.7 ± 10.5) a2	355 ± 18.6 a2,b2 (−48.1 ± 13.0) a2	357.9 ± 15.7 a2,b2 (−45.2 ± 11.0) a2

Group	HR value after administration and the HR difference value (Δ in parentheses) between before and after the administration (beats/min)				
	28.0 h	35.0 h	45.0 h	50.0 h	60.0 h
Group I	399.3 ± 12.3 (2.7 ± 14.2)	404.9 ± 4.6 (8.3 ± 13.6)	391.9 ± 17.6 (−4.7 ± 14.6)	406.4 ± 15.3 (9.8 ± 8.7)	393.6 ± 25.9 (−3.1 ± 21.7)
Group II	388.4 ± 13.9 b2 (−12.2 ± 11.3) a1	389.5 ± 5.6 a2,b2 (−11.1 ± 8.4) a2	391 ± 8.4 b2 (−9.6 ± 8.2)	398.8 ± 10.8 (−1.8 ± 8.6) a2	403.1 ± 14.5 (2.5 ± 14.7)
Group III	388.3 ± 8.3 a1,b1 (−9.9 ± 11.7)	384.7 ± 10.1 a2,b2 (−13.6 ± 11.4) a2	389.7 ± 11.9 (−8.6 ± 11.3)	388.6 ± 12.3 a1 (−9.7 ± 17.0) a2	385.7 ± 10.6 b1 (−12.6 ± 14.3)
Group IV	382.8 ± 8.8 a2,b2 (−17.2 ± 11.1) a2	388 ± 13.1 a2,b1 (−12 ± 12.3) a2	383.7 ± 4.5 b2 (−16.3 ± 6.1) a1	387 ± 4.5 a2,b2 (−13.0 ± 8.1) a2	381 ± 19.7 b1 (−18.9 ± 22.3)
Group V	358.5 ± 13.2 a2,b2 (−44.6 ± 14.6) a2	364.5 ± 15.8 a2,b2 (−38.6 ± 19.60) a2	370.8 ± 9.6 a2,b2 (−32.3 ± 12.5) a2	382.3 ± 12.3 a2,b2 (−20.8 ± 11.9) a2	374.2 ± 12.0 a1,b2 (−28.9 ± 13.3) a2

Notes: The rats in group I were administered transdemally with the formulation-1 (each administered as one patch), as a blank control, and the rats in group II were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions, in which bisoprolol fumarate tablets (Beijing Sihuan Pharmaceutical Company, Beijing, China) were ground into powders and suspended in 0.5% CMC-Na (carboxymethylcellulose sodium), as a positive control. The rats in groups III–V were administered transdermally with the formulation-9 (each administered as one patch) at 5, 10, 20 mg/kg of total drugs (ratio of ISDN to BP, 3:2), respectively. Data are expressed as the mean ± standard deviation (S.D.). a1: $p < 0.05$ *vs*. blank control group; a2: $p < 0.01$ *vs*. blank control group; b1: $p < 0.05$ *vs*. the group before administration; b2: $p < 0.01$ *vs*. the group before administration.

Table 6
Effects on the systolic arterial pressure (SAP) of adult spontaneously hypertensive rats ($n = 9$–10 for each group) after transdermal administrations as a single patch of ISDN-BP-TTS in every 2 days for eight consecutive days at low, medium, and high dose, respectively

Group	SAP value after administration and the SAP difference value (Δ in parentheses) between before and after the administration (kPa)				
	Before	2nd day	4th day	6th day	8th day
Group VI	27.02 ± 0.54	26.59 ± 0.65 (−0.43 ± 0.45)	26.81 ± 0.43 (−0.21 ± 0.66)	26.65 ± 0.49 (−0.36 ± 0.66)	27.07 ± 0.49 (0.05 ± 0.76)
Group VII	26.48 ± 0.52	25.72 ± 0.54 a2,b2 (−0.76 ± 0.40)	25.39 ± 0.67 a2,b2 (−1.09 ± 0.90) a1	25.25 ± 0.74 a2,b2 (−1.22 ± 0.83) a1	25.24 ± 0.72 a2,b2 (−1.23 ± 0.86) a2
Group VIII	26.30 ± 0.89	25.94 ± 1.02 (−0.36 ± 1.01)	25.24 ± 0.80 a2,b2 (−1.06 ± 0.86) a1	25.20 ± 0.91 a2,b1 (−1.10 ± 1.09)	24.71 ± 0.57 a2,b2 (−1.59 ± 0.470 a2
Group IX	26.55 ± 0.63	25.84 ± 0.55 a1,b2 (−0.71 ± 0.25)	24.65 ± 0.90 a2,b2 (−1.90 ± 0.87) a2	24.20 ± 0.68 a2,b2 (−2.35 ± 1.07) a2	24.09 ± 0.56 a2,b2 (−2.46 ± 0.79) a2
Group X	26.62 ± 0.71	24.43 ± 0.74 a2,b2 (−2.19 ± 0.66) a2	24.18 ± 0.86 a2,b2 (−2.44 ± 0.81) a2	24.17 ± 0.67 a2,b2 (−2.45 ± 0.66) a2	23.44 ± 0.53 a2,b2 (−3.18 ± 0.86) a2

Notes: The rats in group VI were administered transdemally with formulation-1 (each administered as one patch) in every 2 days for eight consecutive days, as a blank control, and the rats in group VII were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions once daily for eight consecutive days, as a positive control. The rats in groups VIII–X were administered transdermally with formulation-9 (each administered as one patch) at 5, 10, 20 mg/kg of total drugs (ratio of ISDN to BP, 3:2) in every 2 days for eight consecutive days, respectively. During multiple dosing, a used patch was replaced with a new one with the same dose, and the administration site for each dosing was changed but the site was in the area of two flanks of the rats. Data are expressed as the mean ± standard deviation (S.D.). a1: $p < 0.05$ *vs*. blank control group; a2: $p < 0.01$ *vs*. blank control group; b1: $p < 0.05$ *vs*. the group before administration; b2: $p < 0.01$ *vs*. the group before administration.

Table 7
Effects on the diastolic arterial pressure (DAP) of adult spontaneously hypertensive rats ($n = 9$–10 for each group) after transdermal administrations as a single patch of ISDN-BP-TTS in every 2 days for eight consecutive days at low, medium, and high dose, respectively

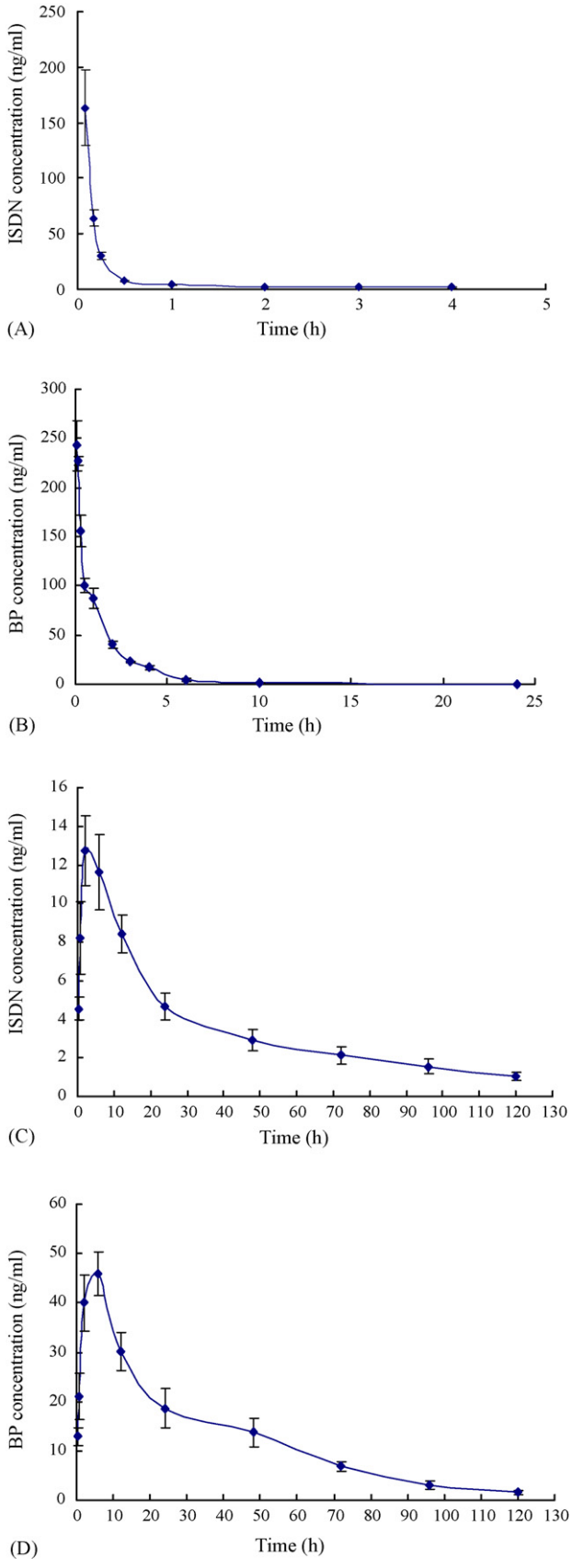
Group	DAP value after administration and the DAP difference value (Δ in parentheses) between before and after the administration (kPa)				
	Before	2nd day	4th day	6th day	8th day
Group VI	19.98 ± 0.43	20.50 ± 0.92 (0.52 ± 0.74)	20.27 ± 0.49 (0.29 ± 0.47)	20.77 ± 0.42 (0.79 ± 0.64)	20.43 ± 0.73 (0.45 ± 0.98)
Group VII	19.53 ± 0.49	19.00 ± 0.77 a2,b1 (−0.52 ± 0.63) a2	18.71 ± 0.49 a2,b2 (−0.82 ± 0.67) a2	18.92 ± 0.47 a2,b2 (−0.61 ± 0.47) a2	18.74 ± 0.39 a2,b2 (−0.78 ± 0.48) a2
Group VIII	19.68 ± 0.59	19.68 ± 1.22 (0.01 ± 1.19)	19.04 ± 0.77 a2,b2 (−0.63 ± 0.54) a2	18.52 ± 0.80 a2 (−1.16 ± 0.81) a2	18.65 ± 0.63 a2 (−1.03 ± 0.62) a2
Group IX		19.86 ± 0.68 (−0.80 ± 1.41) a1	19.06 ± 1.17 a1 (−1.48 ± 1.74) a2	18.38 ± 1.50 a2,b1 (−1.68 ± 0.83) a2	18.18 ± 0.73 a2,b2 (−1.92 ± 0.44) a2
Group X	19.88 ± 0.54	18.96 ± 0.78 a2,b2 (−1.82 ± 0.59) a2	18.06 ± 0.70 a2,b2 (−2.21 ± 0.61) a2	17.68 ± 0.73 a2,b2 (−2.45 ± 0.58) a2	17.43 ± 0.53 a2,b2 (−0.93 ± 0.61) a2

Notes: The rats in group VI were administered transdemally with formulation-1 (each administered as one patch) in every 2 days for eight consecutive days, as a blank control, and the rats in group VII were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions once daily for eight consecutive days, as a positive control. The rats in groups VIII–X were administered transdermally with formulation-9 (each administered as one patch) at 5, 10, 20 mg/kg of total drugs (ratio of ISDN to BP, 3:2) in every 2 days for eight consecutive days, respectively. During multiple dosing, a used patch was replaced with a new one with the same dose, and the administration site for each dosing was changed but the site was in the area of two flanks of the rats. Data are expressed as the mean ± standard deviation (S.D.). a1: $p < 0.05$ *vs*. blank control group; a2: $p < 0.01$ *vs*. blank control group; b1: $p < 0.05$ *vs*. the group before administration; b2: $p < 0.01$ *vs*. the group before administration.

Table 8
Effects on the heart rate (HR) of adult spontaneously hypertensive rats ($n = 9$–10 for each group) after transdermal administrations as a single patch of ISDN-BP-TTS in every 2 days for eight consecutive days at low, medium, and high dose, respectively

Group	HR value after administration and the HR difference value (Δ in parentheses) between before and after the administration (beats/min)				
	Before	2nd day	4th day	6th day	8th day
Group VI	402.2 ± 11.3	401.2 ± 14.8 (−0.9 ± 4.8)	398.4 ± 10.4 (−3.7 ± 11.4)	398.1 ± 21.3 (−4.1 ± 14.0)	401.7 ± 11.4 (−0.5 ± 11.3)
Group VII	401.6 ± 10.5	393.2 ± 13.4 (−8.3 ± 12.2)	384.1 ± 11.5 a1,b2 (−17.5 ± 12.0) a1	383.7 ± 15.5 b2 (−17.9 ± 11.8) a1	380.4 ± 9.5 a2,b2 (−21.1 ± 9.2) a2
Group VIII	396.3 ± 13.8	389.7 ± 23.6 (−6.7 ± 18.1)	385.0 ± 13.7 a1,b1 (−11.3 ± 10.9)	380.4 ± 14.6 a1,b2 (−15.9 ± 9.2) a1	377.3 ± 12.3 a2,b2 (−19.0 ± 11.7) a2
Group IX	408.6 ± 8.0	397.9 ± 15.4 b1 (−10.8 ± 13.7)	382.3 ± 11.6 a2,b1 (−26.3 ± 8.1) a2	384.6 ± 11.9 b2 (−24.1 ± 10.4) a2	382.1 ± 12.1 a2,b2 (−26.5 ± 12.5) a2
Group X	411.6 ± 9.1	393.1 ± 16.8 b2 (−18.5 ± 9.7) a2	380.0 ± 14.9 a2,b2 (−31.6 ± 12.1) a2	372.6 ± 14.4 a2,b2 (−39.1 ± 10.5) a2	376.8 ± 14.2 a2,b2 (−34.9 ± 13.6) a2

Notes: The rats in group VI were administered transdemally with formulation-1 (each administered as one patch) in every 2 days for eight consecutive days, as a blank control, and the rats in group VII were given intragastrically with bisoprolol fumarate (10 mg/kg for each rat) suspensions once daily for eight consecutive days, as a positive control. The rats in groups VIII–X were administered transdermally with formulation-9 (each administered as one patch) at 5, 10, 20 mg/kg of total drugs (ratio of ISDN to BP, 3:2) in every 2 days for eight consecutive days, respectively. During multiple dosing, a used patch was replaced with a new one with the same dose, and the administration site for each dosing was changed but the site was in the area of two flanks of the rats. Data are expressed as the mean ± standard deviation (S.D.). a1: $p < 0.05$ *vs*. blank control group; a2: $p < 0.01$ *vs*. blank control group; b1: $p < 0.05$ *vs*. the group before administration; b2: $p < 0.01$ *vs*. the group before administration.



3.5. Effect on pharmacokinetics

3.5.1. Concentration–time profiles

After intravenous (i.v.) injection of the sterile physiological saline solution containing isosorbide dinitrate (3.0 mg) plus bisoprolol fumarate (1.93 mg bisoprolol) the mean plasma concentrations for ISDN were 163.5 ± 34.3 ng/ml at 0.083 h, 30.4 ± 3.6 ng/ml at 0.25 h, 4.1 ± 0.2 ng/ml at 1 h, and 1.7 ± 0.2 ng/ml at 4 h, while those for BP were 242.6 ± 26.0 ng/ml at 0.083 h, 87.4 ± 10.8 ng/ml at 1 h, 4.6 ± 1.0 ng/ml at 6 h, and minimal (around the detection limit) at 24 h, as illustrated in Fig. 6A and B, respectively.

After transdermal administration as a single patch containing isosorbide dinitrate (24.0 mg) plus bisoprolol (16.0 mg), the mean plasma concentrations for ISDN were 4.6 ± 0.6 ng/ml at 0.33 h, 12.7 ± 1.8 ng/ml (maximal concentration) at 2.0 h, 4.7 ± 0.7 ng/ml at 24.0 h, 2.1 ± 0.5 ng/ml at 72.0 h, and 1.0 ± 0.2 ng/ml at 120.0 h, while those for BP were 12.9 ± 1.7 ng/ml at 0.33 h, 45.9 ± 4.5 ng/ml (maximal concentration) at 6.0 h, 18.6 ± 4.1 ng/ml at 24.0 h, 6.8 ± 1.0 ng/ml at 72.0 h, and minimal (around detection limit) at 120.0 h, as depicted in Fig. 6C and D, respectively.

3.5.2. Pharmacokinetic parameters

The pharmacokinetic parameters of isosorbide dinitrate (ISDN) and bisoprolol (BP) are presented in Table 9. As compared to intravenous administration without dose normalization, the C_{max} values of transdermal administration were significantly decreased (13.1 ± 1.6 ng/ml for $ISDN_{transdermal}$ *versus* 147.9 ± 70.4 ng/ml for $ISDN_{i.v.}$, and 47.4 ± 3.0 ng/ml for $BP_{transdermal}$ *versus* 323.1 ± 174.0 ng/ml for $BP_{i.v.}$, respectively). On the contrary, the $AUC_{0-t\,h}$ values were significantly increased (432.0 ± 43.1 h ng/ml for $ISDN_{transdermal}$ *versus* 160.9 ± 40.5 h ng/ml for $ISDN_{i.v.}$, and 1547.9 ± 197.4 h ng/ml for $BP_{transdermal}$ *versus* 597.1 ± 94.5 h ng/ml for $BP_{i.v.}$, respectively), and the MRT values evidently extended (57.3 ± 7.8 h for $ISDN_{transdermal}$ *versus* 0.6 ± 0.4 h for $ISDN_{i.v.}$, and 36.3 ± 2.6 h for $BP_{transdermal}$ *versus* 1.93 ± 0.7 h for $BP_{i.v.}$, respectively). Similarly, the apparent $T_{1/2}$ values were also markedly increased (46.4 ± 7.3 h for $ISDN_{transdermal}$ *versus* 1.5 ± 0.8 h for $ISDN_{i.v.}$, and 23.0 ± 3.3 h for $BP_{transdermal}$ *versus* 2.5 ± 1.7 h for $BP_{i.v.}$, respectively). After dose normalization, the absolute bioavailabilities of transdermal administration were 33.6% for ISDN, and 31.3% for BP, respectively.

Fig. 6. Plasma concentration–time profiles of isosorbide dinitrate (ISDN) and bisoprolol (BP) after intravenous (i.v.) injection of the sterile physiological saline solution containing isosorbide dinitrate (3.0 mg) and bisoprolol fumarate (1.93 mg bisoprolol) through ear marginal vein of rabbit (group A, $n = 10$) or transdermal administration as a single patch of ISDN-BP-TTS containing isosorbide dinitrate (24.0 mg) and bisoprolol (16.0 mg) onto one flank of rabbit (group B, $n = 10$), respectively. (A) Mean plasma isosorbide dinitrate–time profile after i.v. injection; (B) mean plasma bisoprolol–time profile after i.v. injection; (C) mean plasma isosorbide dinitrate–time profile after transdermal administration; (D) mean plasma isosorbide dinitrate–time profile after transdermal administration. Data are presented as the mean ± standard deviation (S.D.).

Table 9
Pharmacokinetic parameters of isosorbide dinitrate (ISDN) and bisoprolol (BP) after intravenous (i.v.) injection of the sterile physiological saline solution containing isosorbide dinitrate (3.0 mg) and bisoprolol fumarate (1.93 mg bisoprolol) through ear marginal vein of rabbit (group A, $n = 10$) or transdermal administration as a single patch of ISDN-BP-TTS containing isosorbide dinitrate (24.0 mg) and bisoprolol (16.0 mg) onto one flank of rabbit (group B, $n = 10$), respectively

Parameter	Group A		Group B	
	$ISDN_{i.v.}$	$BP_{i.v.}$	$ISDN_{transdermal}$	$BP_{transdermal}$
C_{max} (ng/ml)	147.9 ± 70.4	323.1 ± 174.0	13.1 ± 1.6 a	47.4 ± 3.0 b
T_{max} (h)			3.3 ± 2.1	4.7 ± 2.1
$AUC_{0-t\,h}$ (h ng/ml)	160.9 ± 40.5	597.1 ± 95.4	432.0 ± 41.3 a	1547.9 ± 197.4 b
MRT (h)	0.6 ± 0.4	1.9 ± 0.7	57.3 ± 7.8 a	36.3 ± 2.6 b
$T_{1/2}$ (h)	1.5 ± 0.8	2.5 ± 1.7	46.4 ± 7.3 a	23.0 ± 3.3 b
F (%)			33.6	31.3

Notes: $AUC_{0-t\,h}$ represents the area under the plasma concentration–time curve (from 0 to t hours), where t is the last time-point of measurement for each, namely, 4 h for $ISDN_{i.v.}$, 24 h for $BP_{i.v.}$, and 120 h for $ISDN_{transdermal}$ or $BP_{transdermal}$, respectively. C_{max}, $t_{1/2}$, and MRT values denote maximal concentration, terminal half-life, and mean residence time, respectively. F was the absolute bioavailability, which was calculated with the following equation: $F = (AUC_{transdermal} \times dose_{i.v.})/(AUC_{i.v.} \times dose_{transdermal})$, where $AUC_{transdermal}$ and $AUC_{transdermal}$ represent the area under the plasma concentration–time curve after transdermal administration, and after intravenous bolus injection, respectively. $dose_{transdermal}$ and $dose_{transdermal}$ are the dose of transdermal administration, and intravenous bolus injection, respectively. a: $p < 0.01$ *vs*. $ISND_{i.v.}$ in group A; b: $p < 0.01$ *vs*. $BP_{i.v.}$ in group A.

3.6. Skin irritation

In both pharmacodynamic and pharmacokinetic studies, the total irritation scores were evaluated according to Draize method (Draize et al., 1944). Basically, there were no obvious irritation effects as the total irritation score was identified to be zero in all animals.

4. Discussion

In the present study, we developed a single-layer drug-in-adhesive type of trandermal patches, in which the adhesive layer not only serves as an adhere layer to the skin but also is responsible for the releasing of the drug, as reported previously (Liang, 2005). On the two sides of the adhesive layer, there are a temporary liner-layer and a permanent backing. The *in vitro* characterization demonstrated that such a system was stable for 24 months at 4.0–40.0 °C (data not shown).

The *in vitro* release result shows that the release rate of ISDN is faster than that of BP while release kinetics of both drugs is the first-order process, suggesting that the outwards moving of both drugs from the adhesive is associated with the passive diffusion process (Ruan et al., 2006). The differences in the moving speed could be related to the discrepancy of their physicochemical properties. In addition, the difference in the interaction between drug and the pressure sensitive adhesive may contribute to this difference in moving speed (Kokubo et al., 1994). However, the *in vitro* transdermal penetration result indicates both ISDN and BP are synchronously released, exhibiting a zero-order process for each. Taken together, the results of *in vitro* release and *in vitro* transdermal penetration suggest that the skin is the rate-limiting step for both drugs when applying the transdermal patches.

The results of the ratio of ISDN to BP screening demonstrate that ISDN alone after transdermal administration exhibits slight lowering effect for SAP but minimal for DAP and HR values. In contrast, BP alone after transdermal administration shows obvious lower effects for SAP, DAP and HR values, respectively. When the co-administration of ISDN and BP as a patch in varied ratio of ISDN to BP, the lowering effects for SAP, DAP, and HR values are significantly enhanced, respectively, indicating an obviously synergistic effect for each indicator. However, the different ratios of ISDN to BP have varied effects, among which formulation-5 (ISDN:BP = 1:3) and formulation-9 (ISDN:BP = 3:2) result in the most strong effects for SAP and DAP values, respectively. As compared to formulations-5, the -9 shows less inhibiting effect to heart rates (HR), and is therefore selected as the final one. The different synergistic effects on the SAP, DAP and HR values could be derived from the additive effect of the varied action mechanisms from both drugs, namely, ISDN dilates the blood vessels (Ishibashi et al., 2003) and changes hemodynamic parameters (Orban Schiopu et al., 2005) in hypertension, and BP is a β-adrenoceptor antagonist (β-blocker), which, by binding to cardiac β-adrenoceptors, is able to block the binding (and therefore action) of the endogenous catecholamines adrenaline and noradrenaline, resulting in a reduction in the rate and force of cardiac contraction (Baker, 2005).

For further elucidating the antihypertensive effect of the transdermal patch (ISDN:BP = 3:2, named as ISDN-BP-TTS), the spontaneously hypertensive rats were included in the present study. Results from single dose administration indicate that the transdermal administration of ISDN-BP-TTS is effective for treatment of hypertension, showing a long action pattern as compared with the positive control administered intragastrically. Similar results are further confirmed with the multiple doses for eight consecutive days. These results suggest that such a therapeutic system would be feasible for the future clinical therapy. In view of the responses of low, medium and high dose groups, the medium and dose administration may be the suitable for keeping antihypertensive effect for 2 days. However, the high dose causes more strong dose-related reduction in the heart rates, suggesting that the medium dose may be suitable for the future clinical trials.

Pharmacokinetic study indicate that the multi-day dosing given as one patch is possible as the concentrations of both ISDN and BP after transdermal administration of one patch last at least for 3–5 days. On the contrary, the concentrations of ISDN and BP once given intravenously maintain at detectable levels only

for several hours. Results of pharmacokinetic parameters show that, after transdermal administration, the maximal concentrations for both of ISDN and BP are significantly reduced while their AUC and MRT values enhanced as compared to those after intravenous injection, respectively, suggesting that the possible side effects from ISDN and BP may be attenuated due to the lowered peak concentrations (Ranade, 1991), and pharmacological effects can be enhanced and extended. Actually, the pharmacodynamic study evidenced such effects, albeit the toxicity needs to be further investigated.

After transdermal administration of the present patch, the absolute bioavailability for ISDN was two times higher than that for a matrix type ISDN transdermal therapeutic system (33.6% for ISDN-BP-TTS in rabbits *versus* 11.7% for the matrix type patch reported in rats) (Gabiga et al., 2000). This may be due to two aspect reasons: the difference in the absorption extent of animal species, and difference in the drug delivery system in which the absorption enhancer, as a predominant factor, may influence the absorption extent (Walker and Smith, 1996; Pfister and Hsieh, 1990a,b; Aqil et al., 2005). There is no report regarding transdermal patch of BP. Nevertheless, 31.3% absolute bioavailability of BP in the present study demonstrates that, after transdermal administration, BP can be well absorbed to the systemic circulation.

In addition, the elimination half-life ($T_{1/2}$) of ISDN given intravenously to rabbit is similar to that reported value of ISDN administered intravenously to human (Schaumann, 1989), suggesting that the difference in species does not alter the elimination of ISDN. In contrast, an obvious difference is observed in elimination half-life of BP after intravenous administration in various species: 1 h in rats, 3 h in monkeys, 5 h in dogs, and 11 h in humans (Buhring et al., 1986), respectively. In the present study, the observed elimination half-life of BP in rabbits following the intravenous route was similar to that reported value in rats (2.5 h in rabbits *versus* 3.0 h in rats). After transdermal administration, the half-life values of both ISDN and BP are prolonged as compared to those after intravenous injection. These may be caused by the longer duration of absorption when given as the transdermal patches, therefore, the values may most likely represent the concentration dropping half-lives for two drugs at the whole disposition phase rather than the elimination half-life values at the terminal phase. According to the common understanding, the elimination half-life values are not altered with the different formulations (such as injections and transdermal patches) but dependant on the characteristic of chemical entities themselves.

In summary, the *in vitro* transdermal penetration of both ISDN and BP from the newly developed transdemal patches shows a zero-order process for each, and the penetration rate constants were 7.4 μg/(cm^2 h) for ISDN, and 5.9 μg/(cm^2 h) for BP, respectively. The transdermal patches incorporating ISDN with BP provide obvious anti-hypertension effect in spontaneously hypertensive rats. The effect after administration of one patch lasts for 3 days, and increases with the total dose of both drugs (ISDN:BP = 3:2, mg/mg), showing a dose dependant manner. When compared with either drug alone, the anti-hypertension effect of the combination of ISDN and BP is significantly enhanced. Pharmacokinetics demonstrates that, as compared to the intravenous injection, the C_{max} values of both drugs after transdermal administration are significantly reduced while their AUC, and MRT values are evidently increased and extended, respectively. The transdermal patches incorporating ISDN and BP would provide a useful strategy for prevention and treatment of hypertension.

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