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Transdermal permeation of drugs with differing lipophilicity: Effect of penetration enhancer camphor

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ABSTRACT

The aim of the present study was to investigate the potential application of (+)-camphor as a penetration enhancer for the transdermal delivery of drugs with differing lipophilicity. The skin irritation of camphor was evaluated by *in vitro* cytotoxicity assays and *in vivo* transdermal water loss (TEWL) measurements. A series of model drugs with a wide span of lipophilicity (log *P* value ranging from 3.80 to −0.95), namely indometacin, lidocaine, aspirin, antipyrine, tegafur and 5-fluorouracil, were tested using *in vitro* transdermal permeation experiments to assess the penetration-enhancing profile of camphor. Meanwhile, the *in vivo* skin microdialysis was carried out to further investigate the enhancing effect of camphor on the lipophilic and hydrophilic model drugs (i.e. lidocaine and tegafur). SC (stratum corneum)/vehicle partition coefficient and Fourier transform infrared spectroscopy (FTIR) were performed to probe the regulation action of camphor in the skin permeability barrier. It was found that camphor produced a relatively low skin irritation, compared with the frequently-used and standard penetration enhancer laurocapram. *In vitro* skin permeation studies showed that camphor could significantly facilitate the transdermal absorption of model drugs with differing lipophilicity, and the penetration-enhancing activities were in a parabola curve going downwards with the drug log *P* values, which displayed the optimal penetration-enhancing efficiency for the weak lipophilic or hydrophilic drugs (an estimated log *P* value of 0). *In vivo* skin microdialysis showed that camphor had a similar penetration behavior on transdermal absorption of model drugs. Meanwhile, the partition of lipophilic drugs into SC was increased after treatment with camphor, and camphor also produced a shift of CH_2 vibration of SC lipid to higher wavenumbers and decreased the peak area of the CH_2 vibration, probably resulting in the alteration of the skin permeability barrier. This suggests that camphor might be a safe and effective penetration enhancer for transdermal drug delivery.

1. Introduction

Transdermal drug delivery offers many advantages over oral administration, such as the avoidance of hepatic first pass metabolism and gastrointestinal effects, reduced side effects, and improved patients compliance (Goswami, 2013; Williams and Barry, 2012). However, drug delivery to and across the skin remains one of the major challenges in the development of drug delivery systems, which is largely attributed to the highly-organized and poor permeability of stratum corneum (SC), the uppermost layer of the skin (Hadgraft and Lane, 2011). Despite numerous permeation-enhancing techniques like microneedles (Bariya et al., 2012) and microemulsion(Wang et al., 2012), were widely studied and applied in transdermal drug delivery system, a kind of well-established approach for improving drug delivery is the use of penetration enhancer in the transdermal formulations.

Camphor, a naturally occurring terpene compound (as shown in Fig. 1), is isolated from the wood of the camphor laurel tree (*Cinnamomum camphora*) and commonly applied to the skin for counterirritant, analgesic, and antipruritic characteristics(Burkhart and Burkhart, 2003). Currently, camphor is reported to improve the transdermal absorption of certain drugs as a penetration enhancer (Cui et al., 2011). However, more compounds with differing physicochemical properties, were not yet tested to

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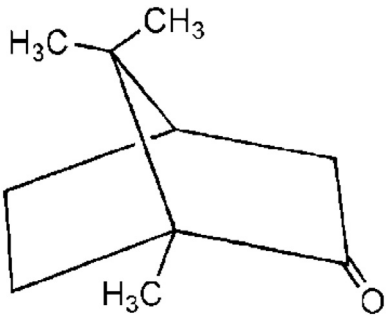

Fig. 1. Chemical structure of camphor.

further clarify the penetration-enhancing characteristic of camphor and its relevant mechanism. Quantitative structure-activity relationship (QSAR) studies revealed that the skin permeability of drug was substantially determined by its physiochemical properties, especially the lipophilicity denoted by log *P* (Patel et al., 2002; Potts and Guy, 1992). Thus, a series of model drugs with a wide span of lipophilicity, namely indometacin (log *P* = 3.80)(Lan et al., 2014b), lidocaine (log *P* = 2.56)(Zhao et al., 2008), aspirin (log *P* = 1.23)(Lien and Gaot, 1995), antipyrine (log *P* = 0.23) (Lee et al., 1994), tegafur (log *P* = −0.48)(Lee et al., 1994) and 5-fluorouracil (log *P* = −0.95)(Chen et al., 2013), were employed to investigate the penetration-enhancing properties of camphor.

Meanwhile, structurally the SC is a thin and multi-laminate tissue and it comprises layers of keratin-rich corneocytes separated by an intercellular lipid domain. This arrangement has been compared to a "brick-and-mortar" model, with the corneocytes as the bricks and the intercellular lipids providing the mortar (Lane, 2013; Moser et al., 2001). It is commonly accepted that the major barrier to drug permeation is principally attributed to the complex and orderly-arranged structure of the intercellular lipids. The transdermal penetration will enter the SC and have numerous interactions with the different components of the skin, especially the structured skin lipids. The enhancers usually change the environment of the lipids to promote the diffusion of drug molecules or influence their solubility (Hadgraft and Lane, 2013; Williams and Barry, 2012). Thus the interaction between penetration enhancer and SC will contribute to shed light on its underlying mechanisms of action. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) has advantages on obtaining information about the lipids and keratin conformation in the SC (Lan et al., 2014b). Consequently, SC/vehicle partition coefficient and FTIR studies were carried out to probe the penetration-enhancing mechanisms of camphor.

2. Materials and methods

2.1. Drugs and chemicals

(+)-Camphor was supplied by Tokyo Chemical Industry Co., Ltd., (Shanghai, China). Laurocapram, propylene glycol (PG) and Trypsin were obtained from Sinopharm Chemical Reagent Co.Ltd (Beijing, China). 3-(4,5-Dimethylthiahiazo-2-y1)-2, 5-diphenytetrazoliumromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co., Ltd. (Shanghai, China). Indometacin, 5-fluorouracil, lidocaine, aspirin, antipyrine and tegafur were supplied by J&K Chemical Ltd (Shanghai, China). Methanol and acetonitrile of HPLC grade were obtained from Fisher Chemical (Pittsburgh, PA). Dulbecco's Modified Eagles Medium (4.5 g/l Glucose, DMEM-H), Minimum Essential Medium (MEM) and fetal bovine serum (FBS) were supplied by Fisher Scientific (Pittsburgh, PA).

2.2. Cell viability assay

Epidermal keratinocytes (HaCaT cell lines) and dermal fibroblasts (HFF cell lines) were supplied from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. MTT assay was used to evaluate the cellular toxicity of camphor on epidermal keratinocytes and dermal fibroblasts. The well-established and standard penetration enhancer laurocapram was employed to compare and evaluate the cytotoxicity of camphor. The keratinocytes or fibroblasts were seeded into 96-well plates at a density of 9000 cells/100 μL/well. After 24 h incubation, the cells were incubated with different concentrations of camphor or laurocapram for 24 h at 37 °C. Subsequently, the medium was removed and supplemented with fresh medium containing 20 μL MTT. Following 4 h of incubation in CO_2 incubator (37 °C), the medium containing MTT reagent was replaced with 150 μL DMSO to dissolve the formazan crystals. Then the absorbance was read at 490 nm (Multiskan Go microplate spectrometer, Thermo Scientific, Finland) to determine cell viability.

2.3. Transepidermal water loss (TEWL) determinations

The TEWL assays were carried out to determine whether camphor have certain impact on the skin integrity. The data on the TEWL changes were obtained from Sprague-Dawley rats (190–210 g, 5 weeks old), which were provided by the Experimental Animal Center of the first affiliated hospital of Chinese PLA general hospital. All the animal procedures were carried out in accordance with the NIH guidelines for the care and use of laboratory animals and also were approved by the Committee on Animal Research of the first affiliated hospital of Chinese PLA general hospital. The rats were anesthetized with urethane (1.5 g/kg, i.p.), then the abdominal hair was shaved off by an electric clipper and a heating pad was placed under the rat back to keep the normal body temperature. The central abdomen of the rats was divided into two symmetrical zones with each area of 2.0 cm^2. One of marked zones was treated with 3% v/v camphor or laurocapram solution for 90 min, and the mixture of propylene glycol (PG): water (70:30, v/v) was used as the solvent of enhancer. The untreated zone was used as the baseline standard for each measurement. The rats served as control was treated with the solvent only. The treated skin zone was carefully cleaned with a cotton swab moistened with warm water prior to the TEWL determination. A TM300 Tewameter (C&K Co. Germany) was employed to record the TEWL value, which was expressed in g m^{-2} h^{-1}. Measurement was carried out at a stable level of 60 s after application of the detecting probe to the skin. After 6 h, the TEWL values were again recorded to check the recovery of skin integrity.

The TEWL value of the rat skin treated with camphor or laurocapram was expressed as ΔTEWL (g/m^2/h), which was calculated by subtracting the baseline value of untreated zone from the achieved TEWL measurement. The ER_{TEWL} value was the ratio of ΔTEWL value in the enhancer group to that in the control group. The ΔTEWL(6 h gap, g/m^2/h) was the TEWL value of the skin which the enhancer had been removed off for 6 h, and the recovery of TEWL value was expressed as R_{TEWL}, which was calculated by using the following equation: $R_{TEWL} = (\Delta TEWL_{(6\,hgap)} - \Delta TEWL)/\Delta TEWL$.

2.4. HPLC methodology of model drugs

The drug analysis was performed on an Agilent 1260HPLC system (Santa Clara, USA), which mainly consisted of variable wavelength detector, quaternary pump and OpenLAB CDS chemstation workstation. Separation was carried out within different

chromatographic columns (250 mm × 4.6 mm, 5 μm particle size) as shown in Table 1. For all drug analysis, the injection volume of samples was 10 μL, and the flow rate and column temperature was set at 1.0 mL min^{-1} and 25 °C, respectively. The chromatographic conditions of all drugs were listed in Table 1. The retention times for indomethacin, lidocaine, aspirin, antipyrine, tegafur and 5-fluorouracil were 10.5, 6.2, 7.8, 11.4, 6.5, and 5.7 min, respectively. These HPLC methods were validated with specificity, linearity, recovery, within-day and between-day precision. Good linearity with a correlation coefficient r^2 exceeding 0.995 was observed for all HPLC methods. All method recovery of the low, medium and high control samples kept in the range of 95–105%. The with-in and between-day precision, calculated as RSD of successive injection of six solutions carried out on 3 different days, was below 3% for all model drugs, which could achieve the quantitative test request of drug analysis.

2.5. Preparation of excised skin and stratum corneum (SC)

The excised skins were gained from Male Sprague-Dawley rats (190–210 g, 5 weeks old). Prior to the experiment, the abdominal hair were shaved off by an electric clipper, after the rats were anesthetized with urethane (1.5 g/kg, i.p.). The full-thickness, which consisted of dermis and epidermis with SC, were excised from the shaved abdomen zones. The adhering subcutaneous fat and other extraneous tissue were carefully removed with cotton swab, and the excised skin was washed with phosphate buffer saline (PBS, pH 7.2).

The SC was prepared with reference to the method previously reported (Jain et al., 2002; Lan et al., 2014a). The excised skin was soaped in 0.5% (v/v) trypsin solution for 8 h. The SC sheets were carefully separated by using a moistened cotton swab, and thoroughly cleaned with distilled water. Then the prepared SC sheets were dried in a vacuum desiccator until use.

2.6. In vitro *transdermal permeation studies*

The *in vitro* transdermal permeation studies were carried out using TK-20B diffusion apparatus (Shanghai KaiKai Co., Ltd., Shanghai, China). The diffusion cells with a volume of 7 mL and an effective diffusion area of 1.77 cm^2 were used in this experiment. The excised skin was sandwiched between the diffusion cells with the SC side up. The isotonic PBS (pH 7.2) served as receiver solution was filled into the receiver cells. The stirring rate and temperature were set as 200 r/min and 32 °C, respectively. The donor cells were filled with 2 mL saturated drug solution, which was prepared by dissolving enough drugs in 70/30 (v/v) PG/water. After application of drug solution and 3% (v/v) camphor or laurocapram, an aliquot (2 mL) of sample was withdrawn from the diffusion cells at predetermined time intervals, and sample volume was replaced with receiver solution. Then the drug content in the samples was analyzed using the HPLC method described above.

2.7. Microdialysis system

The microdialysis system consisted of an 11-Elite micro-infusion pump (Harvard Apparatus, USA) with a 2.5 mL glass syringe (Hamilton, USA) and a CMA 30 linear microdialysis probe (inner diameter, 0.24 mm; outer diameter, 0.38 mm; molecular cut-off, 6000 Da). The inlet tube of the probe was connected to the microinjection pump using polyethylene tubing. In all the experiments, the length of the membrane accessible to dialysis was 10 mm, and the perfusate flow rate was 1.0 μL/min. Cannulas were used as insertion guides, and vials were used to collect the dialysate samples.

2.8. *Recovery validation* in vitro *and correlation* in vivo

In vitro recovery was estimated prior to the microdialysis studies to ensure that the retrodialysis method was suitable for this *in vivo* recovery study and to ensure that the probes used would provide reproducible and efficient sampling.

The *in vitro* recovery was assessed by a concentration difference method. A linear microdialysis probe was placed in a 25 mL Franz diffusion cell containing different concentrations (0.625, 1.25, 2.5, 5.0, 10.0, 20.0 μg/mL^{-1}) of model drug in sequence, and the dialysis membrane portion of the probe was completely immersed in the solution at 32 °C. The probe was perfused with drug solution (3.0 μg/mL^{-1}) at a flow of 1.0 μL/min. After an equilibration period of 1 h, the dialysate was collected into a HPLC vial for another 30 min. Dialysate samples were analyzed using HPLC to determine the drug concentration. *In vitro* relative recovery was calculated as the slope of the linear regression of drug concentration difference between dialysate (C_d) and perfusate (C_p) as a function of drug concentration in the perfusate (C_p). When the drug concentration in dialysate (C_d) is higher than that in perfusate (C_p), the slope of the linear regression actually refers to the delivery of the microdialysis probe, whereas the slope should be the recovery of the probe (Ping-tian et al., 2001).

In vivo recovery ratio was determined using the retrodialysis method, which relies on the assumption that net drug transport from the perfusate into the surrounding tissues through the microdialysis membrane is equal to that from the tissues into the perfusate. The diffusive loss of model drug was determined, and recovery ratio was calculated from the following equation:

Table 1
Chromatography conditions for the analysis of the model drugs.

Samples	HPLC Columns	Mobile phase	UV wavelength(nm)
Indometacin	Agilent ZORBAX 300StableBond SB-C18Column	B: D1 (55:45,v/v)	232
Lidocaine	Merck Lichrospher WP 300 RP-18Column	A:C (65:35,v/v)	230
Aspirin	Agilent ZORBAX 300StableBond SB-C18Column	B:C (30:70,v/v)	228
Antipyrine	Merck Lichrospher WP 300 RP-18Column	B:D2 (25:75,v/v)	254
Tegafur	Merck Purospher STAR RP-18 endcapped Column	B:C (15:85,v/v)	254
5-fluorouracil	Merck Purospher STAR RP-18 endcapped Column	A:C (10:90,v/v)	266

A: methanol; B: acetonitrile; C: distilled water; D1: 0.01 mol/l acetic acid; D2: 0.01 mol/L phosphate buffer

Recovery(*in vivo*) = $(C_p - C_d)/C_p$. To determine the diffusive loss of model drug, a rat was anesthetized with intraperitoneal urethane aqueous solution (1.5 g/kg). The linear probe was inserted into the dermis of the abdominal skin. After perfusion with PBS for 1 h, the standard drug solution was used as perfusate. HPLC assays were conducted to determine the loss of drug from the standard drug solutions.

2.9. In vivo *microdialysis studies*

Prior to administration of model drug, the rat were anesthetized during *in vivo* microdialysis. The fur at the application site was shaved with a shaver in each rat. The microdialysis probe was implanted in the dermis, and the active dialysis window was placed immediately below the site of topical drug administration. The probe was continuously perfused with PBS. The skin was allowed to equilibrate for 1 h before a blank sample was taken from the microdialysis probe and 1.5 h after the start of perfusion. A flat cylindrical glass cover of about 1.5 cm in height, 15 mm in diameter, with an edge width of 1.5 mm was glued above the application site by using UHU all-purpose adhesive. During the experiment, the application site and the probe were kept level. Dialysate samples were collected into HPLC vials, which were replaced every 30 min. Dialysis sampling was continued for 10 h. As with the *in vitro* skin permeation studies, 2 mL saturated drug solutions with or without 3% camphor of laurocapram were used to investigate the *in vivo* penetration-enhancing role of the enhancers on model drugs.

2.10. SC/vehicle partition coefficient studies

Partition coefficient of model drugs between SC and vehicle (PG: water, 70/30, v/v) with or without camphor was determined as reported previously (Lan et al., 2014b). The dried SC sheets were pulverized in a mortar with a pestle, weighed (20 mg) and mixed by vortexing with 1 mL of 3% (v/v) camphor or laurocapram in vehicle containing 100 mg/mL model drugs. The SC treated with solvent alone was used as control. The mixture was equilibrated for 12 h at 32 °C, and centrifuged at 16,000 r/min for 5 min. Subsequently, the drug content in the supernatant solution was analyzed by HPLC as described above. The amount of model drug bound to the SC was calculated by subtracting the amount of the drug in supernatant from the total amount added. The SC/vehicle partition coefficient was obtained using the following equation: SC/vehicle partition coefficient = (drug content in SC)/(drug content in vehicle).

2.11. Fourier transform infrared (FTIR) experiments

The dried SC sheets was cut into proper pieces with each area of 1 cm^2, and treated with 3% (v/v) camphor or laurocapram for 12 h at 32 °C. The SC sample treated with solvent (70/30, v/v, PG: water) alone was used as control. After treatment, the SC samples were carefully washed with distilled water and dried in vacuum desiccator at room temperature overnight for complete dehydration. A FTIR spectrometer (Thermo Nicolet Nexus, Madison, USA) equipped with an attenuated total reflectance (ATR) attachment was employed to obtain the FITR spectra of SC samples. The spectra were recorded with the following parameters: resolution of 2 cm^{-1} and scanning times of 100. The FTIR spectra were obtained and analyzed using OMNIC 6.2 software attached to the FTIR instrument itself.

2.12. Data and statistical analysis

The parameters of the *in vitro* transdermal penetration studies were obtained by plotting the cumulative amount of permeated drug per unit area against time (*h*). The slope of the linear portion of the plot (between 8 and 14 h) was presented as steady state flux (J_{ss}, μg/cm^2/h), and the lag time (T_{lag}, h) was obtained from the x-intercept of the linear portion of the plot. The Q_{24}(μg/cm^2) was the cumulative amount of drug penetrated across the skin after 24 h. Enhancer activity was expressed as enhancement ratio (ER_{Jss}), which was obtained calculated using the following equation: $ER_{Jss} = J_{ss}$ (enhancer treatment)/J_{ss} (control).

As with the *in vitro* data analysis, the apparent absorption profiles though the skin were constructed by plotting the cumulative amount of model drugs in the dermal dialysate against time. The *in vivo* transdermal penetration rate was obtained from the slope of linear portion of the absorption profile. The lag time (T_{lag}, h) was defined as the intercept on the time axis extrapolated from the absorption profile as described previously (Matsuyama et al., 1994; Nakashima et al., 1996).

Data was expressed as mean ± S.D. (standard deviation). A two-tailed Student's *t*-test was carried out using SPSS 16.0 software when comparing two different conditions. A signification level was taken as $p < 0.05$.

3. Results and discussion

3.1. Cytotoxicity of camphor in vitro

The epidermal keratinocytes and dermal fibroblasts were employed to gain insights into the cytotoxicity of camphor and roughly evaluate its skin irritation. A frequently-used and standard transdermal penetration enhancer laurocapram (Hadgraft et al., 1996; Williams and Barry, 2012), was also used to compare the cytotoxicity of camphor. The result of keratinocytes and fibroblasts treated with different concentrations of camphor or laurocapram are presented in Table 2 and Fig. 2, respectively. Camphor or laurocapram showed concentration-dependent reductions in cell viability on both keratinocytes and fibroblasts. However, it existed significant difference between camphor and laurocapram in terms of IC_{50} value (concentration of drugs inducing a 50% decrease in cell viability).

Though most transdermal penetration enhancers showed fairly satisfactory performance in facilitating the permeation of drug molecule across the skin, few of them have been approved for clinical use on account of their skin irritation. Camphor was derived from natural products, so it was expected to possess low irritancy potential. Based on the results of cytotoxicity assay, the IC_{50} values of camphor were significantly higher in both keratinocytes and fibroblasts in comparison to those of laurocapram, and it further implied that camphor had relatively low skin irritation.

3.2. TEWL evaluations

Determination of TEWL can be an effective marker to reflect on the health and efficiency of skin barrier function or skin integrity (Elmahjoubi et al., 2009; Kalia et al., 2000), and TEWL method is commonly used as a good technique to assess the structural alteration of skin samples. Therefore, TEWL was measured in the investigation to further evaluate the skin irritation induced by

Table 2
Cytotoxicity of camphor and laurocapram for keratinocytes and fibroblasts.

Samples	IC_{50}-keratinocytes (mmol/L)	IC_{50}-fibroblasts (mmol/L)
Camphor	5.35 ± 0.17	5.21 ± 0.16
Laurocapram	0.20 ± 0.02	0.33 ± 0.01

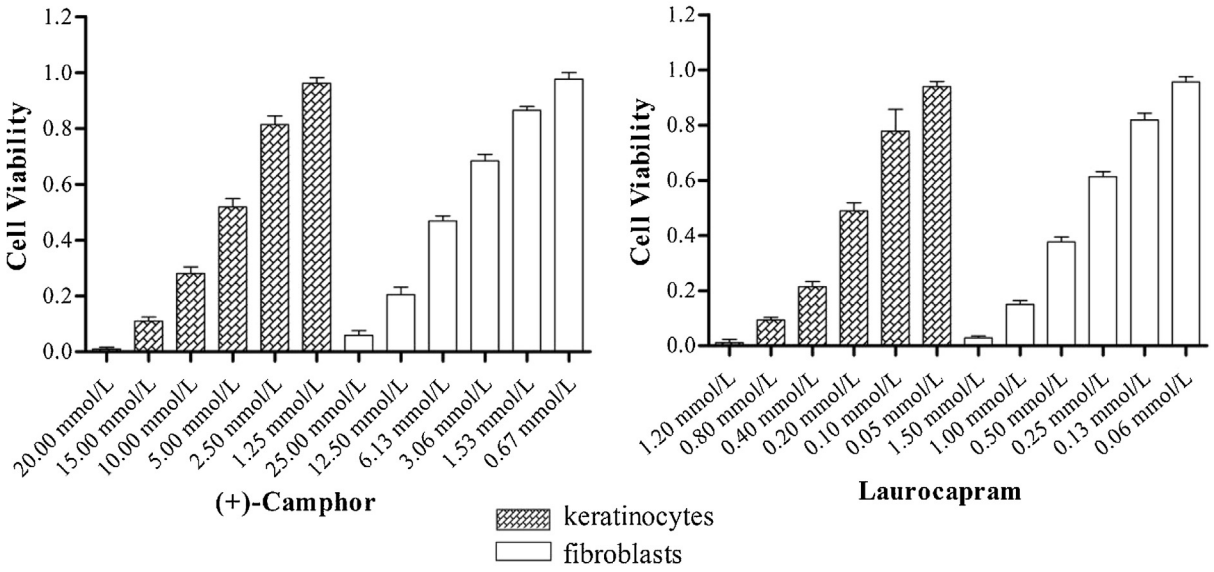

Fig. 2. Cell viabilities of keratinocytes and fibroblasts after treated with different concentrations of camphor and laurocapram.

camphor. Meanwhile, in many other skin penetration enhancement studies (Lakshmi et al., 2014; Williams and Barry, 2012), the terpene penetration enhancer or laurocapram was employed typically at low concentrations (between 1% and 5%, often 3%). Camphor or laurocapram at 3% (v/v) concentration was thus tested in this study.

As presented in Table 3 and Fig. 3, significantly different increases in ΔTEWL were observed after the 90 min application of camphor and laurocapram, and the ΔTEWL value in the camphor group was markedly lower than that in the laurocapram group. Meanwhile, when the enhancers were removed from the skin for 6 h, the chosen skin zone appeared to recover the ΔTEWL value to certain extent. The recovery of ΔTEWL values for camphor and laurocapram group reached up to 44.95% and 17.16%, respectively. Based on these founding, it was reasonable to verify that camphor had a relative low skin irritation, and could be used as a more safer transdermal penetration enhancer.

3.3. In vitro *skin permeation studies*

Considering that enough human skin for a large number of skin permeation tests was usually not available, the rat skin was used in this study to investigate the transdermal penetration profiles of camphor. An estimate of actual skin permeation across human skin could be obtained from the rat skin permeation experiments, even though the rat skin was more permeable to all compounds with different lipophilicity than human skin (approximately 10.9-fold) (Godin and Touitou, 2007; Van Ravenzwaay and Leibold, 2004). Hence, the skin permeation characteristic through human skin can be predicted from the excised rat skin models. In addition, each model drug was mixed with 3% v/v of camphor or laurocapram dissolved in a solvent mixture of PG: water (70:30, v/v). The solvent mixture of PG and water was chosen as a base solvent on account of its ability of solubilize all tested components, including the poor water-soluble drug indometacin and the lipophilic enhancer camphor. Moreover, the solvent system of 70/30(v/v) PG/water had a negligible impact on the skin integrity (Batheja et al., 2011; Panchagnula et al., 2001).

Table 3
Changes of TEWL values of rat skin after treatment with different agents.

Samples	ΔTEWL ($g/m^2/h$)	ER_{TEWL}	ΔTEWL(6 h gap) ($g/m^2/h$)	R_{TEWL}
Control	0.74 ± 0.16	–	0.26 ± 0.11	65.05%
Camphor	2.64 ± 0.79[a]	3.55	1.45 ± 0.24[a]	44.95%
Laurocapram	11.12 ± 2.48[a]	14.94	9.21 ± 2.73[a]	17.16%

Values are expressed as the means with S.D. ($n = 3$).
[a] Indicates statistically significant difference in comparison to the control at $p < 0.05$.

The *in vitro* skin permeation profiles and parameters of all drugs are presented in Fig. 4 and Tables 4–5, respectively. It was clear that the *in vitro* skin permeability of all model drugs was significantly enhanced by camphor or laurocapram compared to the control, and the highest increase in ER_{Jss} was observed with antipyrine (17.80-fold) after treated with 3% v/v camphor. This indicated that camphor could markedly facilitate the skin permeation of drugs with different lipophilicity. Although 3% v/v laurocapram exhibited higher efficiency in enhancing the permeation of all model drugs compared with 3% v/v camphor, the *in vitro* skin permeation profiles of 3% v/v camphor were similar to that of 3% v/v laurocapram. Meanwhile, the incorporation of camphor and laurocapram resulted in the increase of the lag time (T_{lag}) compared to the control. T_{lag} value can be related to diffusivity according to the following equation: $T_{lag} = h^2/6D$, where D refers to the diffusion coefficient, h is the length of the permeation path (Song et al., 2005). This indicated that camphor or laurocapram could produce the changes in the diffusion coefficient or the length of the permeation path.

As shown in Fig. 5, a parabola curve going downwards was observed after plotting the ER_{Jss} against the drug log P values. Therefore, correlation analysis was carried out between the ER_{Jss} and corresponding drug lipophilicity. The suitable regression equations was obtained from regression analysis as follows: Camphor: $ER_{Jss} = -0.43 \log P^2 - 1.26 \log P + 13.95$, $r = 0.86$ ($P = 0.13$); laurocapram: $ER_{Jss} = -1.21 \log P^2 - 0.25 \log P + 21.21$, $r = 0.68$ ($P = 0.39$).

Based on the results of correlation analysis, within the range of drug log P value from 0 to 4, the transdermal permeation ER values of model drugs gradually increased with the decrease of log P

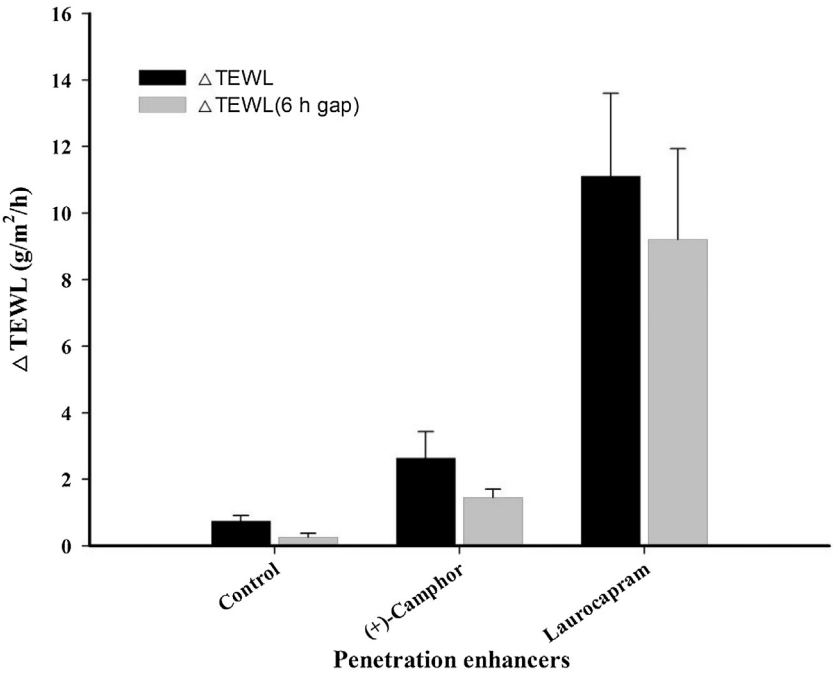

Fig. 3. Changes of TEWL values of rat skin after treated with different agents.

value. When the drug log *P* value was below 0, the ER values started to decrease with drug log *P* value reducing. This indicated that camphor could exhibit the optimal penetration-enhancing activity for weak lipophilic or hydrophilic drugs (an estimated log *P* value of 0). In addition, camphor also displayed roughly similar penetration-enhancing characteristics, compared with that of the standard enhancer laurocapram.

3.4. In vitro *and* in vivo *recovery validation of microdialysis*

From the *in vitro* recovery validation studies, a linear relationship was found between C_p and $C_d - C_p$ as displayed in Fig. 6 (Lidocaine: $C_d - C_p = -0.6379C_p + 1.7853$, $r^2 = 0.9999$; Tegafur: $C_d - C_p = -4041C_p + 1.1854$, $r^2 = 0.9995$). The results showed that the *in vitro* recovery of probe was equal to the delivery of the microdialysis probe, implying that no interaction between the drugs and dialysis membrane occurred in isotonic PBS, and the drug transport from the perfusate into the surrounding tissues through the dialysis membrane was equal to that from the tissues into the perfusate (Ping-tian et al., 2001).

The *in vivo* recovery of positive dialysis cannot be directly determined, a general practice is to use the retrodialysis method, but this must insure consistent results from the two dialysis methods. Results from the *in vitro* studies had shown that the microdialysis probe was not the rate-limiting factor for drug diffusion in the dialysis, the *in vivo* recovery by retrodialysis could be used to correct the data from *in vivo* microdialysis samples. According to the *in vivo* recovery studies using the retrodialysis method, the *in vivo* recovery of lidocaine and tegafur kept a stable value of 45.30 ± 0.48% and 20.31 ± 0.76% in 12 h (as shown in Fig. 6), respectively.

(A) Loss of lidocaine assessed by retrodialysis; (B) *in vitro* probe characterization of lidocaine by concentration difference method; (C) loss of tegafur assessed by retrodialysis; (D) *in vitro* probe characterization of tegafur by concentration difference method.

3.5. In vivo *skin microdialysis*

The *in vivo* skin microdialysis was employed to further investigate the penetration-enhancing effect of camphor. Although *in vivo* skin microdialysis has many advantages, it is also easily affected by some factors, such as drug physicochemical properties, low drug concentrations (Holmgaard et al., 2011). The *in vivo* recovery for the strongly lipophilic or hydrophilic drugs is often very low, thus the sampling by microdialysis is not favorable for these drugs. Therefore, the moderately lipophilic (lidocaine, log *P* = 2.56) and hydrophilic (tegafur, log *P* = −0.48) were chosen as model drugs to investigate the *in vivo* penetration-enhancing role of camphor.

The drug concentration-time curves and cumulative amount profiles of model drugs in dialysate collected from the dermis with or without 3% (v/v) camphor were displayed in Fig. 7, respectively. The *in vivo* penetration parameters of model drugs were listed in Table 7. Similar to the *in vitro* skin permeation studies, camphor or laurocapram significantly promoted the *in vivo* transdermal flux or cumulative amounts of two model drugs in comparison to the control, and 3% (v/v) also exhibited relative high efficiency compared with the same concentration of camphor. Despite the *in vitro* and *in vivo* skin penetration studies exhibited different transdermal parameters due to the difference of *in vitro* and *in vivo* environment, these enhancers exhibited a similar penetration behavior on the transdermal absorption of model drugs, which further verified the results of the *in vitro* skin penetration studies.

3.6. SC/vehicle partition coefficient

Results of SC/vehicle partition coefficients of model drugs with and without 3% v/v camphor are listed in Tables 4–6, respectively. Compared to the control group, the enhancement ratios of SC/vehicle partition coefficients of model drugs were 1.68 (indometacin), 2.04 (lidocaine), 1.21 (aspirin), 0.98 (antipyrine), 1.05

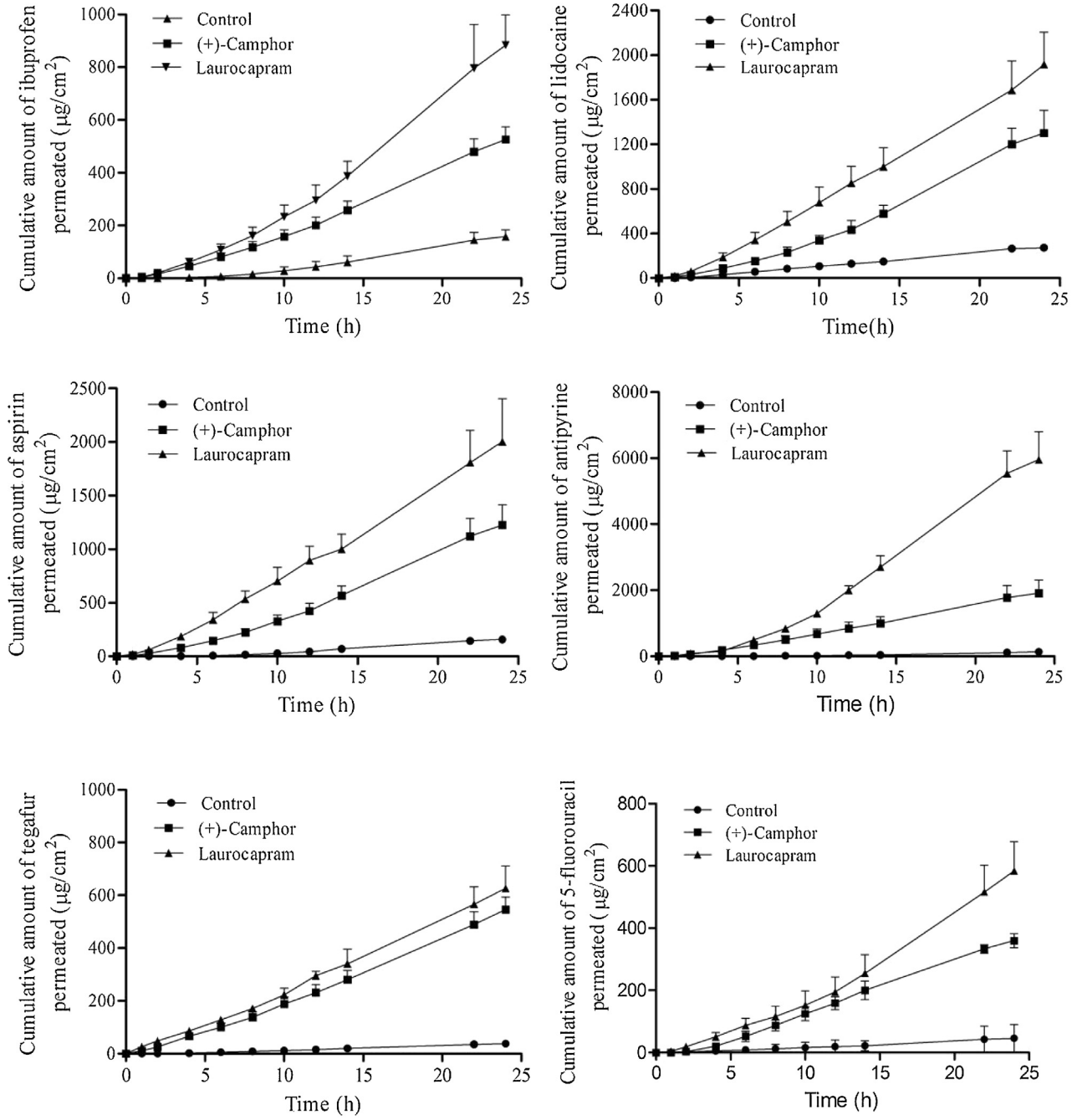

Fig. 4. *In vitro* transdermal permeation curves of model drugs across the rat skin ($n = 6$).

(tegafur) and 0.96 (5-fluorouracil), and 3% v/v camphor could significantly increase the partition coefficients of indomethacin and lidocaine. This indicated that camphor, perhaps owing to its lipid-soluble characteristics, could facilitate the partition of lipophilic drugs into SC to some extent, which was similar to the results of other studies (Lan et al., 2014b).

Table 4
In vitro transdermal permeation parameters and partition coefficients of indometacin and lidocaine after treated with different enhancer samples.

Samples	Indometacin					Lidocaine				
	J_{ss} (μg/cm^2/h)	Q_{24} (μg/cm^2)	T_{lag} (h)	ER_{Jss}	*SC/vehicle*	J_{ss} (μg/cm^2/h)	Q_{24}(μg/cm^2)	T_{lag}(h)	ER_{Jss}	*SC/vehicle*
Control	5.43 ± 1.04	217.92 ± 28.29	2.04 ± 0.51	–	0.058 ± 0.008	11.25 ± 2.31	317.81 ± 40.58	2.79 ± 0.82	–	0.076 ± 0.009
Camphor	21.55 ± 4.13[a]	761.49 ± 52.91[a]	3.52 ± 0.76[a]	3.97	0.097 ± 0.011[a]	63.9 ± 9.73[a]	1783.61 ± 118.73[a]	3.59 ± 0.74	5.68	0.155 ± 0.012[a]
Laurocapram	30.46 ± 5.08[a]	1171.73 ± 89.23[a]	4.17 ± 0.98[a]	5.61	0.080 ± 0.009[a]	82.01 ± 11.89[a]	2140.62 ± 139.58[a]	4.28 ± 0.85	7.29	0.115 ± 0.010[a]

Values are expressed as the means with S.D. ($n = 6$).
[a] Indicates statistically significant difference in comparison to the control at $p < 0.05$.

Table 5
In vitro transdermal permeation parameters and partition coefficients of aspirin and antipyrine after treated with differing enhancer samples.

Samples	Aspirin					Antipyrine				
	J_{ss} (μg/cm²/h)	Q_{24}(μg/cm²)	T_{lag} (h)	ER_{Jss}	*SC/vehicle*	J_{ss} (μg/cm²/h)	Q_{24}(μg/cm²)	T_{lag} (h)	ER_{Jss}	*SC*/vehicle
Control	6.41 ± 0.95	289.65 ± 32.72	2.52 ± 0.78	–	0.071 ± 0.016	4.58 ± 0.79	176.89 ± 14.73	1.34 ± 0.45	–	0.097 ± 0.014
Camphor	62.94 ± 9.58[a]	2387.52 ± 119.67[a]	3.28 ± 0.69	9.82	0.086 ± 0.009	81.52 ± 9.32[a]	2984.92 ± 159.75[a]	1.97 ± 0.38	17.80	0.095 ± 0.016
Laurocapram	82.24 ± 12.57[a]	3258.61 ± 289.48[a]	3.97 ± 1.04	12.83	0.081 ± 0.011	163.78 ± 21.57[a]	5438.19 ± 443.98[a]	3.64 ± 0.68[a]	35.76	0.103 ± 0.009

Values are expressed as the means with S.D. ($n = 6$).
[a] Indicates statistically significant difference in comparison to the control at $p < 0.05$.

Table 6
In vitro transdermal permeation parameters and partition coefficients of tegafur and 5-fluorouracil after treated with differing enhancer samples.

Samples	Tegafur					5-fluorouracil				
	J_{ss} (μg/cm²/h)	Q_{24}(μg/cm²)	T_{lag} (h)	ER_{Jss}	*SC/vehicle*	J_{ss} (μg/cm²/h)	Q_{24}(μg/cm²)	T_{lag} (h)	ER_{Jss}	*SC*/vehicle
Control	1.78 ± 0.14	36.21 ± 8.56	2.39 ± 0.78	–	0.037 ± 0.002	1.64 ± 0.75	69.21 ± 10.93	2.14 ± 0.70	–	0.019 ± 0.005
Camphor	28.44 ± 3.97[a]	548.47 ± 45.82[a]	3.84 ± 1.05	15.98	0.039 ± 0.005	19.46 ± 3.91[a]	513.98 ± 34.59[a]	3.46 ± 0.84	11.87	0.018 ± 0.007
Laurocapram	35.04 ± 5.83[a]	683.73 ± 63.91[a]	4.31 ± 0.94[a]	19.69	0.036 ± 0.004	25.20 ± 4.71[a]	975.21 ± 50.47[a]	3.95 ± 0.58[a]	15.37	0.019 ± 0.003

Values are expressed as the means with S.D. ($n = 6$).
[a] Indicates statistically significant difference in comparison to the control at $p < 0.05$.

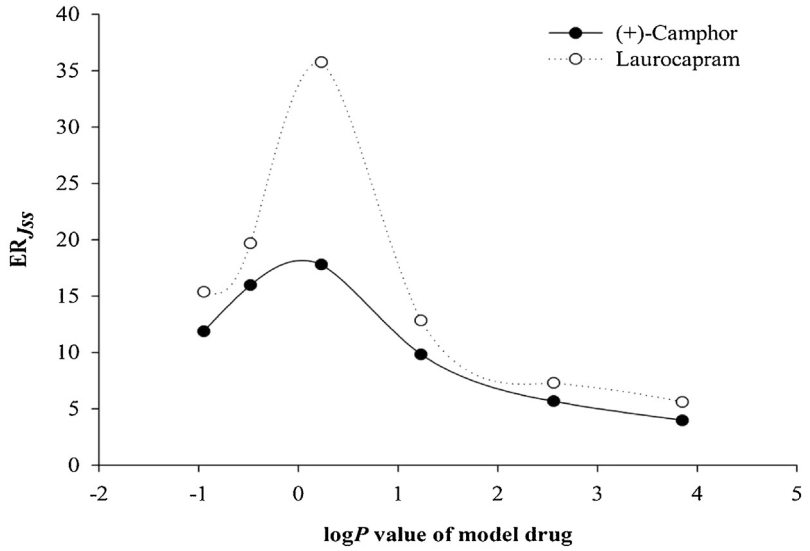

Fig. 5. Relationship between ER_{Jss} and log *P* values of model drugs.

Drug molecules are initially released from the vehicle followed by partitioning into the uppermost layer of epidermis, i.e. SC, and these molecules will subsequently diffuse across the SC as a result of a concentration gradient, which helps the further diffusion across the viable epidermis and dermis (Lane, 2013). Therefore, the partition of drugs into SC is the first step of their delivery across the skin, and contributes to improve the permeability of the drugs. Based on these results, camphor might enhance the skin permeability of lipophilic drugs to certain extent, partly by increasing their partition into SC.

3.7. FTIR studies

To further investigate the camphor-induced alteration in molecular organization of skin SC, ATR-FTIR measurements were conducted to probe the structure changes of SC lipids and keratin, as well as to understand the possible enhancement mechanism of the enhancer. After scanned by ATR-FTIR, SC lipid exhibits two strong CH_2 asymmetric ($\nu_{as}CH_2$, ~2918 cm^{-1}) and symmetric vibrations (ν_sCH_2, ~2850 cm^{-1}) in the range of 2800 ~ 3000 cm^{-1}. Meanwhile, SC keratins show two sharp amide vibrations in the range of 1500–1700 cm^{-1}, namely amide I (~1651 cm^{-1}) and amide II (~1538 cm^{-1}), which are derived from the amide bonds of keratin. These absorption peaks are usually used as key parameters to investigate the structure alterations of SC lipids and keratin induced by penetration enhancers (Moore and Rerek, 2000; Narishetty and Panchagnula, 2005). The alterations in the peak position of CH_2 vibrations or amides absorption peaks suggest that the changes of molecular organization of SC lipids and keratins occur under the application of penetration enhancers. Meanwhile, the peak areas of these absorption bands are proportional to the amount of SC lipids or keratins; the decrease in peak areas of these absorption bands is always accompanied by the loss of the corresponding components (Jain et al., 2002; Van Ravenzwaay and Leibold, 2004).

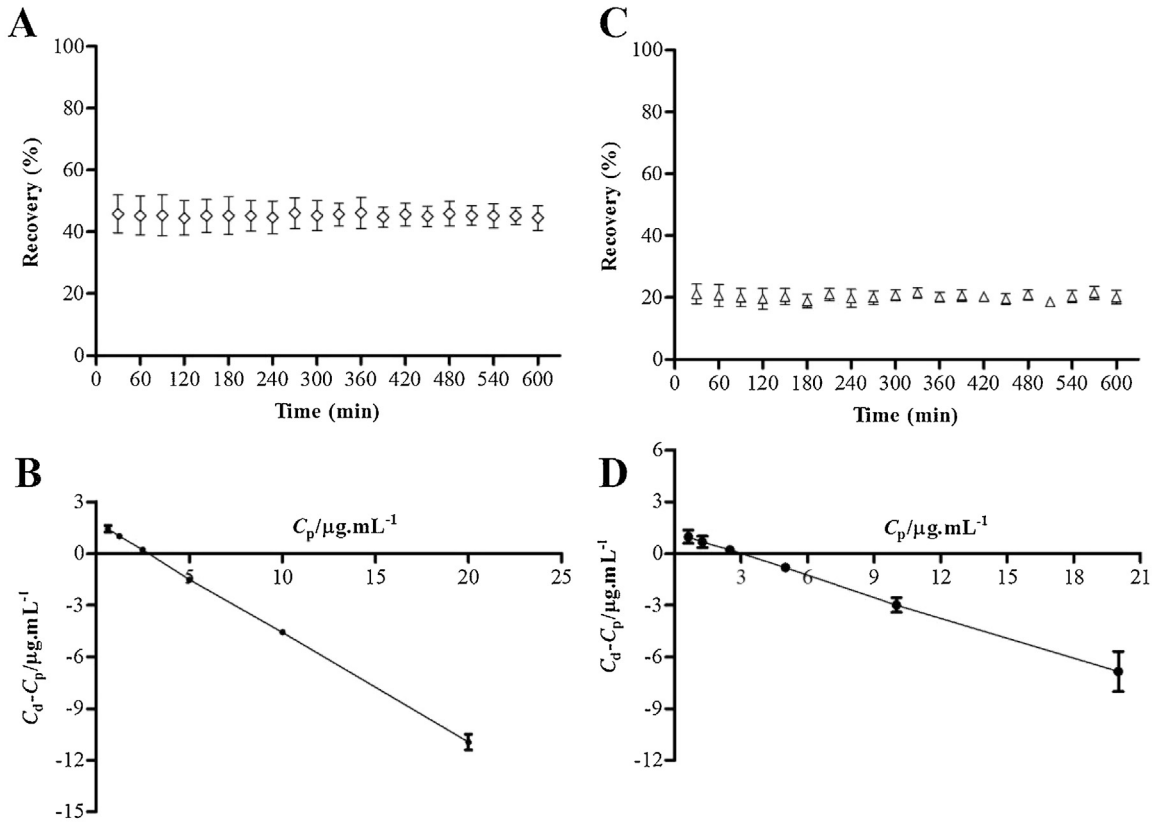

Fig. 6. *In vitro* and in vivo recovery validation of microdialysis probe (n = 5).

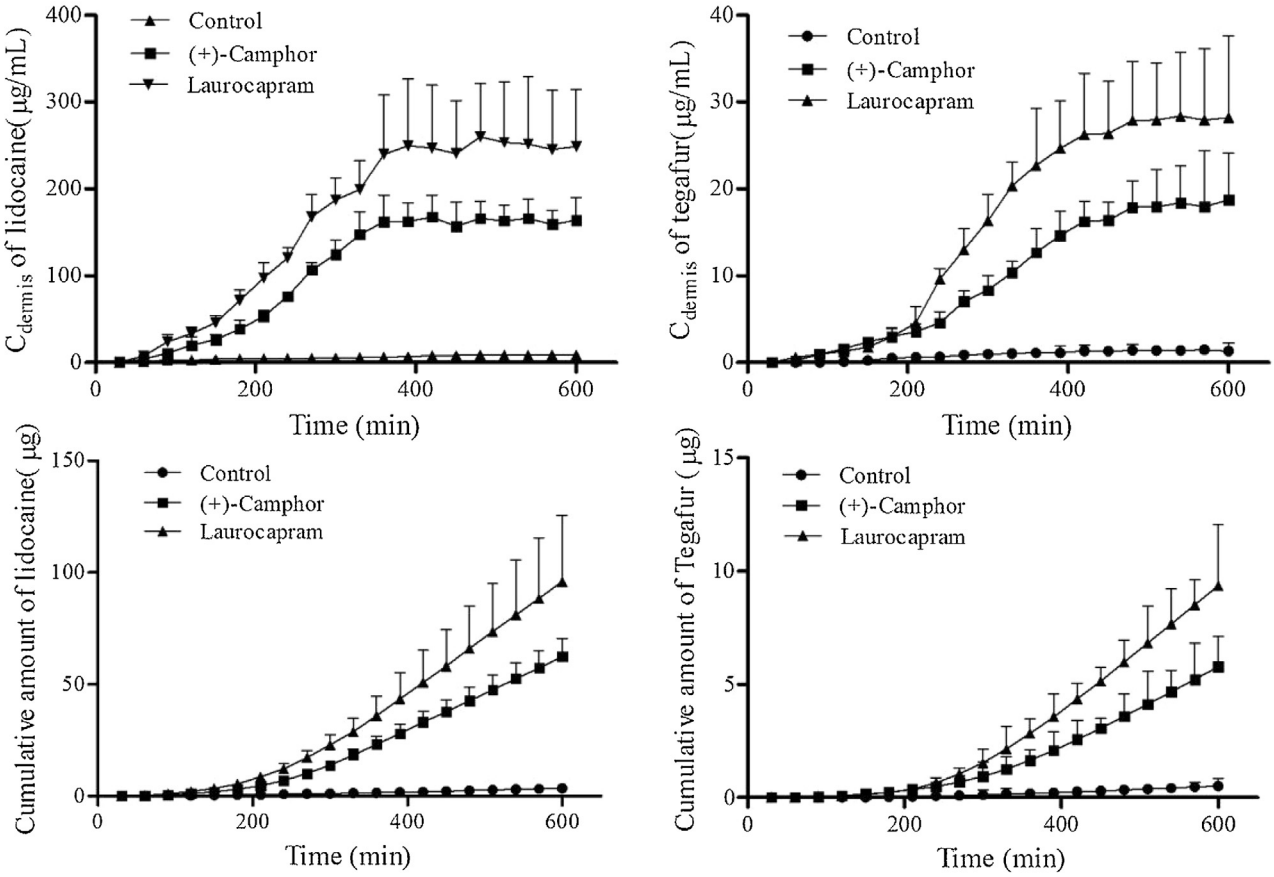

Fig. 7. *In vivo* transdermal permeation profiles of lidocaine and tegafur after treated with differing enhancer samples (n = 6).

Table 7
In vitro transdermal permeation parameters and partition coefficients of indometacin and lidocaine after treated with different enhancer samples.

Model drugs	Samples	Penetration parameters			
		J_{ss} (μg/cm^2/h)	Q_{24} (μg/cm^2)	T_{lag} (h)	ER_{Jss}
Lidocaine	Control	0.0088 ± 0.0012	3.43 ± 0.58	3.45 ± 0.98	–
	Camphor	0.1630 ± 0.0329[a]	62.26 ± 9.06[a]	3.63 ± 1.04	18.52
	Laurocapram	0.2495 ± 0.0572[a]	95.89 ± 11.24[a]	3.59 ± 1.31	28.35
Tegafur	Control	0.0014 ± 0.0008	0.51 ± 0.21	3.93 ± 1.09	–
	Camphor	0.0182 ± 0.0059[a]	5.79 ± 0.93[a]	4.70 ± 0.89	13.00
	Laurocapram	0.0281 ± 0.0091[a]	9.36 ± 2.68[a]	4.46 ± 1.29	20.07

Values are expressed as the means with S.D. ($n = 6$).
[a] Indicates statistically significant difference in comparison to the control at $p < 0.05$.

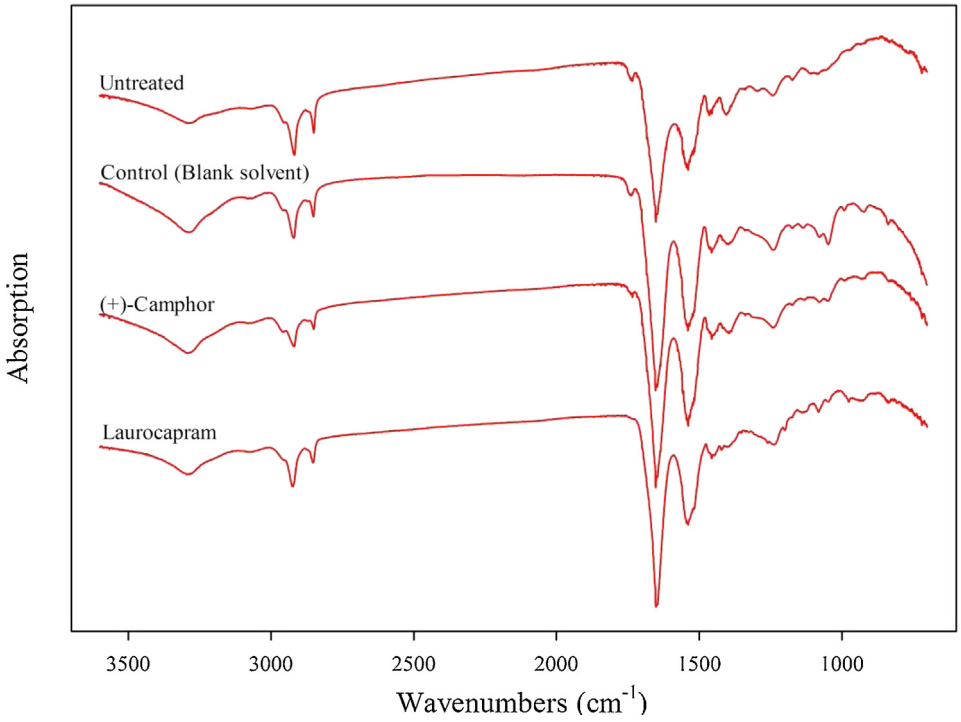

Fig. 8. ATR-FITR spectra of rat SC after treatment with differing enhancer samples.

After treatment with different enhancers, the FTIR spectra of rat SC are displayed in Fig. 8. Treatment of rat SC with the solvent alone produced no significant change in these absorption peaks (as shown in Table 8), which further confirmed that the solvent mixture of 70/30 (v/v) PG/water had a negligible impact on the molecular organization of the normal skin. However, the presence of camphor and laurocapram resulted in the shift of two CH_2 vibrations to higher wavenumbers, and the peak shifts by camphor in $\nu_{as}CH_2$ and ν_sCH_2 were 0.52 and 3.13 cm^{-1}, respectively. Meanwhile, the treatment of SC with the standard enhancer laurocapram also produced a higher shift in CH_2 vibrations, and the peak shift in $\nu_{as}CH_2$ and ν_sCH_2 were greater than that of camphor, which also in roughly accordance with the results of the *in vitro* transdermal permeation tests. In addition, the use of camphor or

Table 8
Peak position and area of the absorption bands in ATR-FTIR after treated different agents.

Samples	ν_sCH_2		$\nu_{as}CH_2$		Amide I		Amide II	
	Wavenumber (cm^{-1})	Peak area	Wavenumber (cm^{-1})	Peak area	Wavenumber (cm^{-1})	Peak area	Wavenumber (cm^{-1})	Peak area
Untreated	2850.13	136.47 ± 21.65	2918.84	517.61 ± 72.95	1651.79	2296.46 ± 527.89	1538.15	1176.63 ± 341.49
Control	2850.79	132.68 ± 37.74	2918.43	503.97 ± 58.04	1651.83	2718.78 ± 469.43	1538.80	1352.96 ± 462.81
Camphor	2851.31	49.63 ± 14.89[a]	2921.56	306.35 ± 45.44[a]	1651.93	2539.79 ± 540.63	1538.67	1452.25 ± 394.65
Laurocapram	2852.13	57.18 ± 19.57[a]	2922.07	351.84 ± 83.27[a]	1651.82	2481.17 ± 536.36	1538.93	1594.89 ± 409.35

Values are expressed as the means with S.D. ($n = 3$).
[a] Indicates statistically significant difference in comparison to the control at $p < 0.05$.

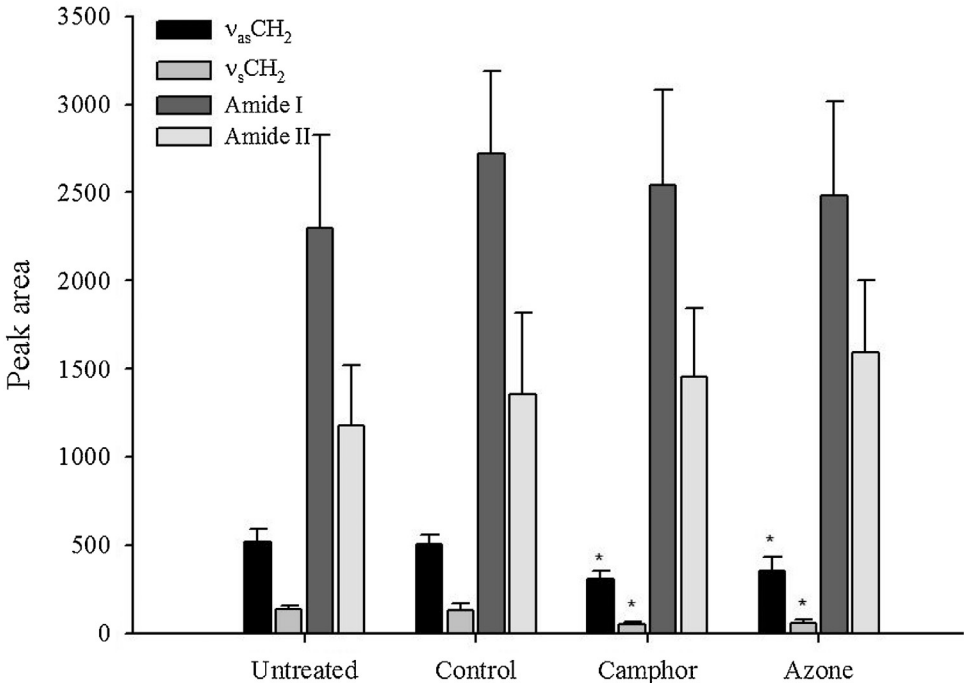

Fig. 9. Peak areas of the absorption bands in ATR-FTIR after treated different agents ($n = 3$).

laurocapram induced significant reductions in the peak areas of $\nu_{as}CH_2$ and ν_sCH_2 (as shown in Table 8 and Fig. 9), implying that the enhancers could extract the SC lipids to some extent. However, camphor and laurocapram appeared to produce a negligible change in peak position or peak area of amide I and amide II. These results indicated that camphor reduced the skin permeability mainly by perturbing the molecular organization of SC lipids or directly extracting part of SC lipids.

4. Conclusions

Based on the results of skin irritation studies, camphor exhibited lower toxicities to epidermal keratinocytes and dermal fibroblasts, and had a weaker impact on the TEWL of rat skin, compared with the frequently-used and standard penetration enhancer laurocapram, indicating that camphor could be used as a safer natural penetration enhancer. The *in vitro* skin permeation studies showed that camphor could markedly enhance the transdermal permeation of model drugs with differing lipophilicity, though below the penetration-enhancing efficacy of laurocapram. Meanwhile, camphor displayed greater efficiency for the transdermal permeation of weak lipophilic or hydrophilic drugs (an estimated log P value of 0), and the relationship between the enhancement ratio (ER_{Jss}) and drug log P values exhibited a parabola curve going downwards. *In vivo* skin microdialysis also showed that camphor had a similar penetration behavior on transdermal absorption of model drugs. Results of SC/vehicle partition coefficient measurements indicated that camphor could improve the partition of lipophilic drugs into SC to certain extent, which partly contributed to increase the skin permeability of these drugs. ATR-FTIR studies showed that camphor disturbed the orderly-arranged molecular organization primarily by affecting the intercellular lipid of SC, resulting in the alteration of skin permeability. In conclusion, camphor could effectively promote the transdermal permeation of drugs with various lipophilicities, and might be an excellent natural penetration enhancer.

Acknowledgements

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