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Transdermal permeation enhancement of *N*-trimethyl chitosan for testosterone

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Abstract

The aim of this study was to evaluate the transdermal permeation enhancement of *N*-trimethyl chitosan (TMC) with different degrees of quaternization (DQ). TMCs with DQ of 40 and 60% (TMC40 and TMC60) were synthesized and characterized by ^{1}H NMR. Testosterone (TS) used as an effective drug, four different gels were prepared without enhancer, with 5% TMC40, 5% TMC60 or 2% Azone, respectively as enhancer. The effect of TMC60 on the stratum corneum was studied by Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) combined with the technique of deconvolution. The results showed that TMC60 could significantly affect the secondary structure of keratin in stratum corneum. *In vitro* permeation studies were carried out using Franz-diffusion cells and *in vivo* studies were performed in rabbits. Both *in vitro* and *in vivo* permeation studies suggested the transdermal permeation enhancement of TMCs. Compared to the TS gel without enhancer, TS gels with enhancers all showed significant enhancing effect on transdermal permeation of TS ($P < 0.05$). Meanwhile, compared to 2% Azone, 5% TMC60 had a stronger enhancement ($P < 0.05$) while 5% TMC40 had a similar effect ($P > 0.05$). The results suggested that the enhancement of TMCs increased with the increase of DQ.
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Keywords: *N*-Trimethyl chitosan; Transdermal permeation; Enhancement; Testosterone; Gels

1. Introduction

Chitosan (CS) is the only polycationic polysaccharide in nature with promising biocompatibility, good biodegradability and low toxicity. It has attracted much interest as a multifunctional excipient in pharmaceutical applications. Recently, much attention has been focused on mucosa permeability enhancement of CS (Thanou et al., 2001). It can widen the tight junction between mucosa epithelial cells reversibly by interaction of protonated CS with anionic components of glycoprotein on the surface of the epithelial cells and with fixed negative charges in the interior of the tight junction, which leads to absorption enhancement of drugs (Junginger and Verhoef, 1998). However, CS can only be dissolved in acidic solvents of pH below 6.5, which limits its potential application.

N-Trimethyl chitosan (TMC), one of CS water-soluble derivatives, has been investigated for permeation-enhancing properties using Caco-2 cells as a model for intestinal epithelium (Kotze et al., 1997a, 1999; Thanou et al., 2000a). The results showed that TMC could considerably increase the permeation of model drugs across Caco-2 intestinal epithelia with the same mechanism shown by CS with high safety. Therefore, the charge of TMC, as determined by the degree of quaternization (DQ), is an important factor determining its potential use to obtain both mucoadhesion and penetration enhancement towards intestinal, nasal and corneal epithelia and buccal mucosa (Hamman et al., 2002; Jonker et al., 2002; Sinswat and Tengamnuay, 2003; Di Colo et al., 2004; Giuseppina et al., 2005). In general, the higher the DQ, the better the effect of TMC (Thanou et al., 2000b). Furthermore, TMC is also active at neutral and basic pH, which can lead to broader pharmaceutical applications.

Stratum corneum of skin is the main barrier against drug transdermal penetration. Composed of dead keratinized cells and fibrosis protein, it is very different from epithelial cells. However, stratum corneum has fixed negative charges in the tight junction between cells similar to those found in epithelial cells (Hamman et al., 2002). It is therefore reasonable to speculate that CS and its derivatives could be promising transdermal pen-

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etration enhancers with the ability to improve the transdermal absorption of drugs with the similar mechanism to the one above. Until now such research has not been performed.

In this study, TMC with DQ of 40 and 60% (TMC40 and TMC60) were synthesized and characterized by ^{1}H NMR. Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR), combined with the deconvolution, was employed to study the protein conformation change of stratum corneum under the effect of TMC60. In addition, the enhancements of TMC40 and TMC60 on transdermal penetration of testosterone (TS) gel *in vitro* and *in vivo* were performed to determine the influence of DQ of TMC. Those effects were also compared to that of Azone (one of transdermal enhancers frequently used in transdermal delivery systems).

2. Materials and animals

2.1. Materials

CS (MW 210 kDa, DD > 95%) from a shrimp shell was purchased from Haipu Biotechnology Co. Ltd. (Qingdao, China). Chp 2005 grade testosterone was purchased from Quanxin Medi-chemical Co. (Shanxi, China). Azone was supplied by Xingqi Chemical Plant. (Shanxi, China). All the other chemicals were of analytical grade and used without further purification.

Healthy male Kunming species mice weighing 20 ± 2 g and healthy New Zealand male rabbits weighing 2.5 ± 0.2 kg were supplied by the Experimental Animal Breading Center of Medical College of Wuhan University. All animal experiments complied with the rules set forth in the NHI Guide for the Care and Use of Laboratory Animals.

2.2. Synthesis and characterization of TMCs with different DQ

TMCs with DQ of 38.8% (TMC40) and 67.2% (TMC60) were synthesized according to the reported methods (Sieval et al., 1998; Thanou et al., 2000b). Briefly, sieved chitosan (<500 μm) was mixed with methyl-iodide in a basic solution of *N*-methylpyrrolidinone at 60 °C for 75 min. The product was isolated by ethanol precipitation and subsequent centrifugation. After this first step, the product underwent a second step of reductive methylation for 60 and 90 min, yielding the final products TMC iodide having DQ of 38.8 and 67.2%, respectively. Both products were precipitated by ethanol and isolated by centrifugation. Purification of the final products also included exchange of the counterion iodide with chloride. The products were then dissolved in 10% NaCl containing aqueous solutions, re-precipitated by ethanol, isolated by centrifugation, thoroughly washed with ethanol and ether, and then dried in vacuo at 40 °C.

Both TMC40 and TMC60 were characterized by ^{1}H NMR [Fig. 1(A) and (B)]. The products were measured in D_2O at 80 °C, using a 300-MHz spectrometer (Mercury Vx-300 Varian). The DQ of the synthesized TMC polymers were calculated with the following equation (Hamman and Kotze, 2001): DQ (%) = $[(\int TM/\int H) \times (1/9)] \times 100$, where $\int TM$ is the integral of the trimethyl amino group (quaternary amino) peak at 3.7 ppm and $\int H$ is the integral of the ^{1}H peaks from 4.7 to 5.7 ppm.

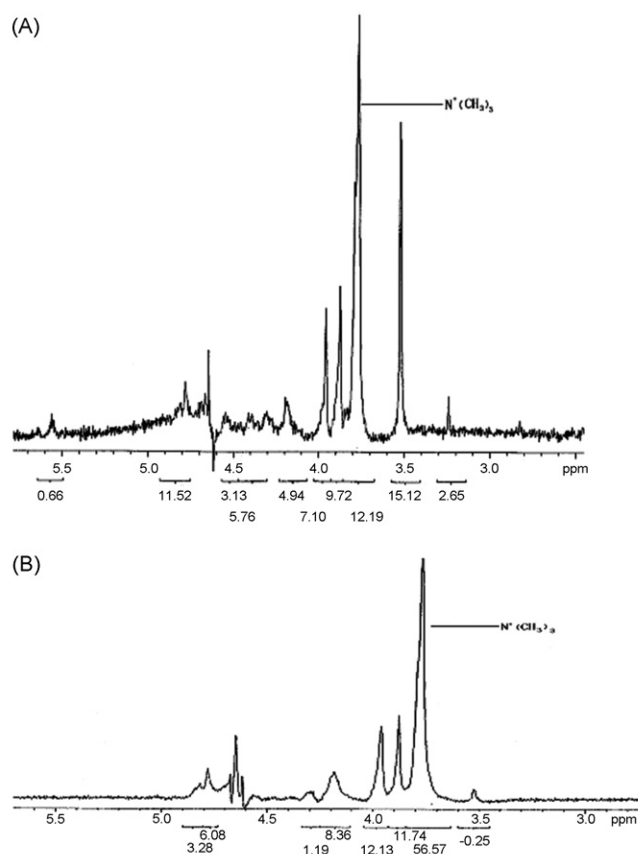

Fig. 1. ^{1}H NMR spectra of (A) TMC40 (38.8% quaternized) and (B) TMC60 (67.2% quaternized), respectively. Peak assignment: $–N^{+}(CH_3)_3$: 3.7 ppm; –H proton: 4.7–5.7 ppm.

2.3. Change of stratum corneum under the effect of TMC60 by ATR-FTIR determination

Stratum corneum was obtained from the male mice. After hair was carefully removed with a razor, a 2.5 cm × 2.5 cm patch of skin (thickness of ca. 350 μm) was excised from the abdomen region from each sacrificed mouse and the subcutaneous fat and other extraneous tissue were trimmed. After immersion in protease solution (composed of 1.0 mg/l trypsin and 0.04 mol/l Na_2CO_3) at 37 °C for 12 h, the free stratum corneum of the skin was isolated, then rinsed with cool hexane (−4 °C) for 30 s in order to remove surface lipids. Then the stratum corneum was immersed in TMC60 solution (50 g/l) for 24 h followed by rinsing with pure water to remove TMC60 from the surface. After removing residual water of the surface with filter paper, the samples of stratum corneum were lyophilized and stored in a vacuum desiccator for determination.

Normal stratum corneum was obtained using the same methods as described above, but without the treatment by TMC60 solution.

ATR-FTIR was performed on an FTIR8300 Infrared Spectrophotometer (Shimadzu, Japan) under the following conditions: scanning temperature range of 18–20 °C, number of

Table 1
Compositions of four different TS gels

No.	TS (%)	P407 (%)	TMC40 (%)	TMC60 (%)	Azone (%)
A	1	25	5	–	–
B	1	25	–	5	–
C	1	25	–	–	2
D	1	25	–	–	–

scanning times of 64, resolution of 4 cm^{-1}, and scanning range of 800–4000 cm^{-1}. The curves of normal stratum corneum and the stratum corneum treated by TMC60 were recorded.

2.4. Preparation of TS gels

Four kinds of TS gels were prepared according to the compositions listed in Table 1. TS, TMC40, TMC60 and Azone were dissolved in 67% ethanol at room temperature and then cooled down to 4 °C. Poloxamer 407 (P407) was then slowly added to the solution with continuous agitation. The gels were left at 4 °C until clear solutions were obtained.

2.5. In vitro skin permeation

Full-thickness skins were obtained from the testing mice. After hair was removed carefully with a razor, a 2.5 cm × 2.5 cm patch of skin (thickness of ca. 350 μm) was excised from the abdomen region from each sacrificed mouse and the subcutaneous fat and other extraneous tissue were trimmed. And then the skins were washed and examined for integrity. The skins were stored at 4 °C and used for experiments within 24 h.

In vitro skin permeation across mouse skin was conducted with Franz-diffusion cells (Shanghai Kaikai Instrumental Co., China) at 37 ± 0.2 °C. The freshly excised skin was mounted between the donor and receptor cell (stratum corneum side facing the donor). The area of diffusion for all *in vitro* studies was 2.8 cm^2. The donor cells respectively contained the four TS gels 0.5 g each, and were occluded with Parafilm. The receptor cells were filled with normal saline solution containing 40% (v/v) PEG400 to maintain skin condition (6.5 ml) (Kim et al., 2001). At predetermined time intervals, 0.2 ml of the receptor solution was withdrawn and refilled with the same volume of fresh receptor solution. Samples were kept in a freezer (−20 °C) until analysis by high performance liquid chromatography (HPLC, Agilent 1100-series, USA) under the following conditions: column of ZORBA × SB C_{18} (4.6 mm × 250 mm, 5 μm), mobile phase of methnol:water (75:25), detective wavelength of 243 nm, flow rate of 1.0 ml/min, sensitivity of 0.01AUFS, and injection volume of 20 μl.

2.6. In vivo permeation studies and skin irritation evaluation

The testing rabbits were divided into four groups randomly with eight rabbits in each group and housed in stainless steel cages at 25 °C for the study. The groups were TMC40 group, TMC60 group, Azone group and control group, respectively, which would be treated by TS gels with Number A, B, C and D, respectively. Animals were fasted 24 h prior to the administration of drug formulations but had free access to water. One day prior to the experiment, hair on the backside area was clipped by a razor (area of 5 cm × 10 cm) and washed with distilled water. The four different TS gels containing TS of 1% were applied on the skin surface with the dosage of 3 g each (Slater et al., 2001; Pierre, 2005). Blood samples (1.5 ml) were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after administration through auricular vein into heparinized glass tubes. The plasma was separated immediately and frozen at −20 °C until analysis. The test sites were observed for signs of irritation for 3 days following the experiment. Skin irritation was visually assessed according to the following scale: (+?)doubtful reaction, (+)erythema with infiltration, (++)erythema, infiltration, papules, (+++)the same with the formation of vesicles, (++++)strong positive reaction with marked edema and confluent vesicles/bullae (De Groot et al., 1994).

Plasma levels of TS were estimated by HPLC. 0.1 ml (1.0 mg/l) of hydrocortisone and 0.01 ml (2 mol/l) of NaOH solution were added to 0.3 ml plasma, and then extracted with 5 ml of extractant (NG and Yuen, 2003) (dichlormethane:2,2,4-trimethylpentane = 4:1) for 1 min. Organic layer was separated and evaporated at 35 °C under a stream of nitrogen. The residue

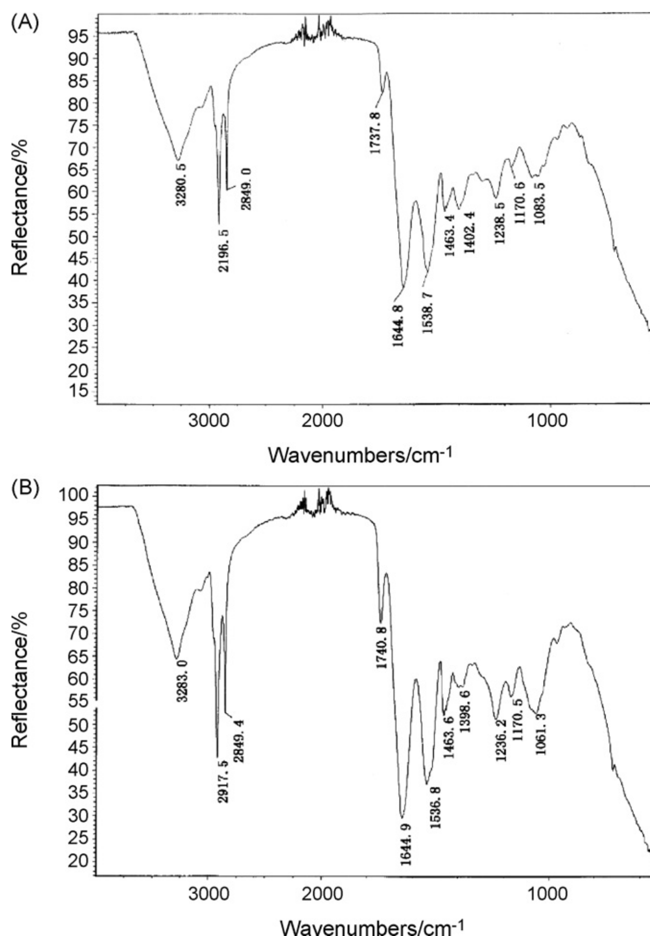

Fig. 2. ATR-FTIR spectra of stratum corneum of mouse. (A) Normal; (B) treated by 5% TMC60.

was reconstituted with 0.1 ml of mobile phase, and then TS was determined using HPLC by UV detector at 241 nm. The lowest detection limit by the method was 1.8 μg/l. The method recovery rate was 101.10 ± 2.62%, and the extraction recovery rate was 78.73 ± 2.73%. The R.S.D. for intra- and inter-day estimations were 2.03 and 4.57%, respectively, demonstrating good reproducibility.

PK parameters such as peak plasma concentration (C_{max}) and time of its occurrence (t_{max}) were read directly from the individual plasma concentration–time profiles. The other PK parameters, e.g. area under the curve ($AUC_{0\to t}$), absorption rate constant (K_a), elimination rate constant (K_e) and initial lag time ($t_{lag\ time}$) were calculated using a statistics software.

2.7. Statistical analysis

All skin permeation experiments *in vitro* were repeated four times, and experiments *in vivo* were using eight rabbits. Data were expressed as the mean value ± S.D. Statistical data were analyzed by one-way analysis of variance (ANOVA). A multiple comparison test was used to compare different TS gels, and a *P*-value of 0.05 was considered to be significant.

3. Results and discussion

3.1. Effect of TMC60 on the stratum corneum

Amide I and II absorption peaks are the main indices to evaluate the secondary structure of keratin in stratum corneum. Using Amide I absorption peak of keratin as a benchmark (1644.8 cm^{-1}), the displacement of amide II absorption peak of keratin was moved from 1538.7 to 1536.8 cm^{-1} after treatment with 5% TMC60 [Fig. 2(A) and (B)], which suggested the amount of α-spiral structure of keratin was decreased along with the increase of β-folding structure and non-regulative structure (Zhou et al., 2004). Deconvolution of amide II absorption peak of keratin also proved the significant increase of intensity of β-folding structure and non-regulative structure of keratin after treatment with 5% TMC60 (Fig. 3). It sug-

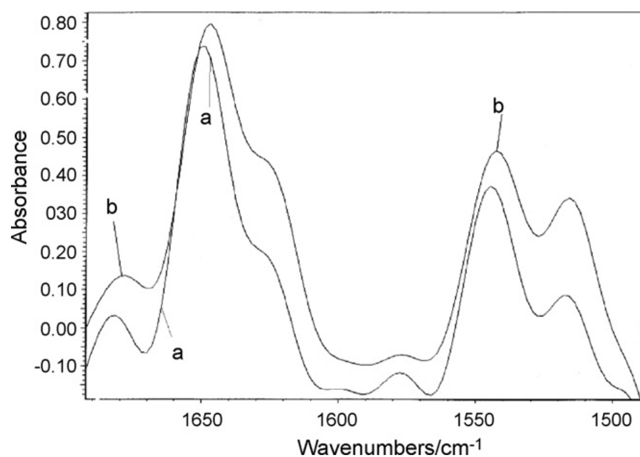

Fig. 3. Deconvolution of amide II absorption peak of keratin. (a) Normal; (b) treated by 5%TMC60.

Table 2
The accumulative penetration and steady penetration rate of TS gels ($n = 5$, $\bar{X} \pm S$)

No.	The cumulative penetration (μg·cm^{-2})							Equation	R^2	J (μg/cm^2 h)
	1 h	2 h	4 h	6 h	8 h	10 h	12 h			
A	370.27 ± 15.36[a]	555.58 ± 17.42[a]	754.55 ± 13.97[a]	881.08 ± 19.82[a]	997.55 ± 16.31[a]	1155.27 ± 15.29[a]	1330.60 ± 11.03[a]	$Q = 95.567t + 241.98$	0.92	95.567
B	580.21 ± 19.11[a,b]	861.23 ± 17.26[a,b]	1189.70 ± 20.31[a,b]	1400.09 ± 16.23[a,b]	1528.76 ± 18.79[a,b]	1639.33 ± 17.45[a,b]	1719.43 ± 20.16[a,b]	$Q = 123.96t + 452.9$	0.87	123.96
C	290.52 ± 18.45[a]	440.37 ± 12.11[a]	6033.65 ± 15.14[a]	723.41 ± 19.17[a]	840.41 ± 18.42[a]	997.73 ± 16.26[a]	1225.20 ± 20.76[a]	$Q = 88.505t + 165.7$	0.96	88.505
D	59.01 ± 11.13	135.58 ± 10.86	254.60 ± 12.44	387.63 ± 10.28	456.40 ± 19.11	543.66 ± 20.79	638.03 ± 21.92	$Q = 53.153t + 23.663$	0.98	53.153

[a] Compared to No. D, $P < 0.05$.
[b] Compared to No. C, $P < 0.05$.

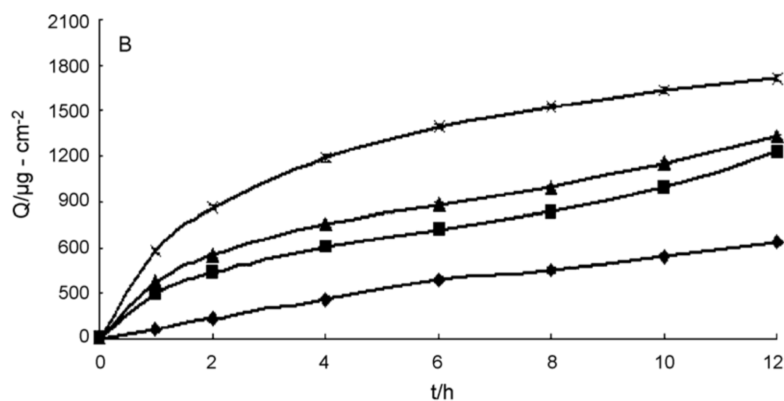

Fig. 4. Cumulative precutaneous amount vs. time profiles of TS gels *in vitro*. (✱) TMC60 group; (▲) TMC40 group; (■) Azone group; (◆) control group.

gested that TMC60 could change the secondary structure of keratin in stratum corneum leading to more loose accumulative structure of keratin and a larger degree of freedom for carbon movement, which could enhance the transdermal permeation of drugs.

3.2. In vitro permeation of four different TS gels

The accumulative percutaneous amount (Q, µg·cm^{-2}) and steady-state permeation rate [J, µg/(cm^2 h)] of TS gels were presented in Table 2. The skin permeation profiles were presented in Fig. 4. The effects of 5% TMC40 and TMC60 on the skin permeation of TS were evaluated and compared to those of 2% Azone.

The results showed that Q and J of TS gels with 5% TMC60 as enhancer both had significant difference compared to those of TS gels without enhancer, with 5% TMC40 and 2% Azone as enhancer ($P<0.05$). Meanwhile, compared to those of TS gels without enhancer, Q and J of TMC40 contained TS gels also increased significantly ($P<0.05$), however, no significant difference was seen when compared to TS gels with 2% Azone ($P>0.05$). Therefore, both 5% TMC60 and TMC40 could increase transdermal absorption of TS considerably, and the effect of TMC60 was more significant than that of TMC40 at the same concentration. The results were likely due to the different charge density of TMCs, as determined by DQ, and the influence of methyl on the stereochemical structure of TMCs (Kotze et al., 1997b; Hamman et al., 2002). The increase in DQ of TMCs leads to an increase in the penetration enhancement properties of TMCs when used as transdermal absorption enhancers.

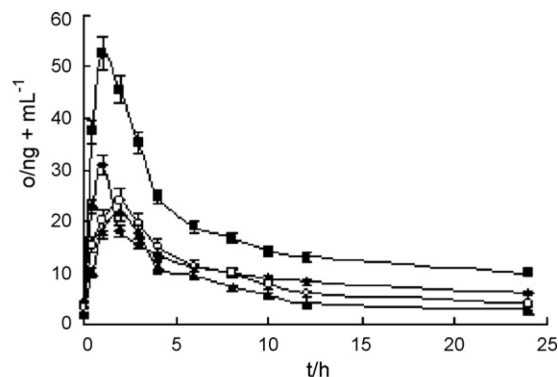

Fig. 5. Drug concentrations in plasma-time profiles of TS gels in rabbits. (✱) TMC40 group; (■) TMC60 group; (▲) control group; (○) Azone group.

3.3. In vivo permeation studies

The mean plasma concentration–time profiles of TS after administration of the four different TS gels were shown in Fig. 5. The calculated PK parameters were given in Table 3.

The results indicated that 5% TMC60 could significantly enhance the transdermal absorption of TS with a C_{max} of 58.34 ± 1.54 µg/l at a t_{max} of 0.84 ± 0.49 h and $AUC_{0–24}$ of 206.37 ± 1.26 µg/l/h, which were significant different from those of 5% TMC40 group, 2% Azone group and blank group ($P<0.05$). In addition, TMC60 group had the shortest t_{max} and t_{lag} (0.56 ± 0.13 h), suggesting the fastest effect of TMC60 on the transdermal permeation of TS.

Compared to those of control group, the PK parameters of TMC40 group also showed significant difference ($P<0.05$),

Table 3
Pharmacokinetic parameters of TS after administration of four different TS gels in rabbits ($n=8$, $\bar{X} \pm S$)

Parameters	Blank group	TMC40 group	TMC60 group	Azone group
C_{max} (ng·ml^{-1})	17.81 ± 1.17	30.71 ± 1.36[a]	58.34 ± 1.54[a,b]	23.90 ± 0.49
t_{max} (h)	3.42 ± 1.02	1.16 ± 0.23[a]	0.84 ± 0.49[a,b]	2.10 ± 0.67
t_{lag} time (h)	1.12 ± 0.33	0.72 ± 0.27	0.56 ± 0.13	0.85 ± 0.34
AUC (ng·h·ml^{-1})	98.46 ± 7.91	151.98 ± 2.08[a]	206.37 ± 1.26[a,b]	113.51 ± 3.42
k_a (h^{-1})	0.44 ± 0.14	1.81 ± 0.09[a,b]	2.32 ± 0.16[a,b]	0.62 ± 0.05
k_e (h^{-1})	0.31 ± 0.20	0.76 ± 0.10[a]	0.92 ± 0.17[a]	0.54 ± 0.07

[a] Compared to control, $P<0.05$.
[b] Compared to Azone group, $P<0.05$.

which proved the enhancing effect of TMC40. However, compared to 2% Azone, TMC40 provided no significant enhancement ($P > 0.05$). The results were in agreement with those of *in vitro* studies, which further proved that the transdermal enhancement of TMCs was close related to DQ of TMCs.

Ideally, transdermal enhancers should be pharmacologically inert and quick but reversible effect on the skin. Azone is one kind of percutaneous penetration enhancers commonly used in transdermal drug delivery systems with promising enhancing effect and low toxicity. The primary skin toxicity testing suggested that at the concentration used in TS gels (5%), TMCs showed no toxicity and no irritancy on the skin just like Azone (2%). No clearly positive reactions of all the four testing groups were observed, showing the preliminary safety of TMCs used as transdermal permeation enhancers.

4. Conclusions

TMCs with different DQ, i.e., TMC40 and TMC60 were synthesized and used as transdermal enhancers in TS gels. TMCs could change the secondary structure of keratin in stratum corneum to enhance the transcutaneous permeation of drugs. *In vitro* and *in vivo* permeation studies suggested the transdermal enhancement of 5% TMCs when compared to the control and 2% Azone. Furthermore, the charge density determined by DQ of TMCs was a key factor in obtaining the enhancement effect. The enhancing effect of TMCs was increased along with the increase of DQ. The mechanism and biosafety at cellular level for transdermal enhancement of TMCs is being further studied in our lab.

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