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Assay of 6-gingerol in CO_2 supercritical fluid extracts of ginger and evaluation of its sustained release from a transdermal delivery system across rat skin

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Ginger has been widely used as healthy food condiment as well as traditional Chinese medicine since antiquity. Multiple potentials of ginger for treatment of various ailments have been revealed. However, the biological half-life of 6-gingerol (a principal pungent ingredient of ginger) is only 7.23 minutes while taken orally. Delivery of ginger compositions by routes other than oral have scarcely been reported. Therefore, we studied a noninvasive transdermal drug delivery system (TDDS) of ginger to bypass hepatic first pass metabolism, avoid gastrointestinal degradation and achieve long persistent release of effective compositions. After establishment of a HPLC analysis method of 6-gingerol, assays of 6-gingerol were performed to compare two kinds of ginger extracts. Then, the characteristics of transdermal delivery of 6-gingerol in TDDS were exhibited. The results showed that the contents of 6-gingerol in two kinds of ginger extracts were significantly different. The maximal delivery percentage of 6-gingerol across rat skin at 20 h was more than 40% in different TDDS formulations. TDDS may provide long-lasting delivery of ginger compounds.

1. Introduction

Ginger (*Zingiber officinale* Roscoe) has been used worldwide as natural beneficial spicy food for thousands of years, but also as a dietary supplement for safely treating various diseases. 6-Gingerol as a principal pungent ingredient present in ginger has been experimentally evidenced to play important roles in prevention and amelioration of diseases, such as antioxidant and anti-apoptosis bioactives, reduction of toxin activity, attenuation of proinflammatory responses, prevention of adipogenesis, modulation of cytochrome P450 enzymes, inhibition of cancer cell growth and invasion, treatment of cardiovascular disease, remission of nausea and vomiting and intervention of development of hair follicle (Schwertner and Rios 2007; Kim et al. 2012).

However, there are only few studies on effective delivery systems of ginger compounds. The intravenous administration of phytochemical compositions is usually not recommended. Oral administration of ginger compositions traditionally will result in transient biological effects because of the brief half-lives of ginger compounds. For example, the biological half-life of 6-gingerol is 7.23 min after oral administration.

In this study, a transdermal drug delivery system (TDDS) has been introduced as a safe and noninvasive alternative for the delivery of ginger ingredients. It could circumvent "first-pass" inactivation by the liver, reduce gastrointestinal irritation, provide a steady absorption of the medication over long periods of time, reduce the frequency of dosing and improve patient compliance (Durand et al. 2012). Thus, TDDS products can offer advantages over the possibilities of the poor bioavailability and brief pulse of oral medication. Meanwhile, CO_2 supercritical fluid extraction (SFE-CO_2) which is highly efficient serve as a preferential method for ginger extraction. In this study, we addressed the SFE-CO_2 technology for ginger extraction, compared with traditional ethanol extraction, by the quantitative analysis of 6-gingerol and emphasized on the characteristics of percutaneous permeation of 6-gingerol in TDDS and the evaluation of the TDDS formula by determining the permeability of 6-gingerol. Optimized TDDS may provide an excellent delivery access for ginger compounds to develop their therapeutic effectiveness.

2. Investigations and results

2.1. Validation of HPLC methodology

The 6-gingerol standard was qualitatively determined by ^{1}H-NMR, ^{13}C-NMR, IR, UV, MS (Fig. 1), and quantitatively

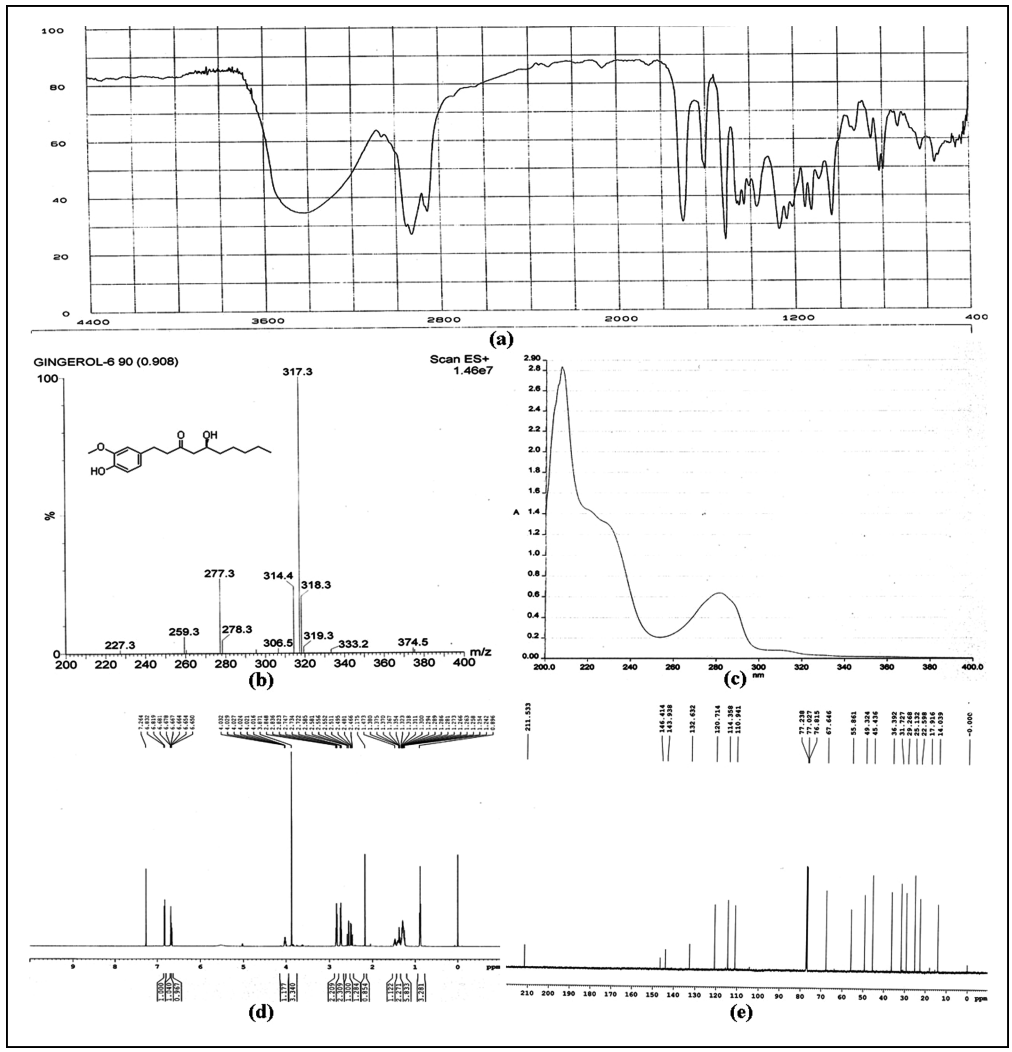

Fig. 1: Determination of the 6-gingerol standard. (a) IR spectrum of the 6-gingerol standard, (b) ESI-MS spectrum of the 6-gingerol standard, (c) UV spectrum of the 6-gingerol standard, (d) ^{1}H-NMR spectrum of the 6-gingerol standard, (e) ^{13}C-NMR spectrum of the 6-gingerol standard. The 6-gingerol standard obtained from Zhongxin was validated to be 6-gingerol by serial qualitative analysis.

determined by HPLC, chemical purity > 99%). The HPLC methodology proposed showed a good separation of 6-gingerol in the ginger samples. The linear regression equation between the content of 6-gingerol and the peak area was y = 0.3267x - 5.0920 (x is the content of 6-gingerol(ng), and y is the peak area). The correlation coefficient (R^2) value is 1, showing good linearity. The LOD and LOQ were in the range 0.01–0.03 μg/mL.

The RSD values of the intra-day and inter-day tests were found to be 1.80, 0.88, 0.72 and 2.29, 1.16, 0.95 (n = 5) for 100, 200, 300 μg/mL of 6-gingerol standard solutions, respectively. The RSD values were lower than 5%, which demonstrated good reproducibility of the analytical method.

The average recovery percentage of 6-gingerol ranged from 97.99 to 102.03%, and RSD values were 0.93%, 0.26%, 0.61% for 8, 10, 12 mg added 6-gingerol standard. The results were summarized in Table 1, which shows that the established method was reliable and accurate.

2.2. *Analysis of ginger extracts*

HPLC chromatograms of 6-gingerol standard, ginger SFE-CO_2 extracts (SFEE) and ethanol percolation extracts (EPE) are shown in Fig. 2. We did not notice any substance that might interfere with the analysis of 6-gingerol in the ginger extracts. The yields of the ginger extracts and the contents of 6-gingerol are shown in Table 2. The yields of ginger oil by SFE-CO_2 were lower than that by ethanol percolation (EP) significantly, but the contents of 6-gingerol in SFEE were found to be significantly higher than that in EPE.

2.3. *Transdermal permeation studies on 6-gingerol in TDDS*

The formulations designated as numbers 1, 2, 3, 4, 5, 6, 7 and 8 in Table 3 are related to the patches prepared with SFEE (EPE), 1% Azone (no Azone, as a penetration enhancer), and polyacrylate pressure sensitive adhesive (from Eudragit Roma Corp. or Beijing Chemical Corp., abbreviated to RM and BC, respectively) with the different composition. The cumulative amount (Q, $\mu g \cdot cm^{-2}$), the steady state flux (J_{SS}, $\mu g \cdot cm^{-2} \cdot h^{-1}$), the transdermal permeation percentage (P %) of 6-gingerol permeated through skin was calculated by the following equation (Yang et al. 2013).

$$Q = \frac{VC_n + V_iC_{n-1} + \ldots + V_iC_1}{S}$$

$$J_{SS} = \frac{dQ}{dt}$$

$$P\% = \frac{Q \times 10^{-3}}{C_0}$$

Table 1: Recovery test of 6-gingerol (n = 9)

No.	Amount of sample (mg/50ml)	Spiked (mg/50ml)	Measured (mg/50ml)	Recovery (%)	X (%)	*RSD* (%)
1	10.259	8	18.174	98.94		
2	10.259	8	18.099	97.99	98.92	0.93
3	10.259	8	18.247	99.84		
4	10.259	10	20.316	100.57		
5	10.259	10	20.272	100.13	100.43	0.26
6	10.259	10	20.317	100.58		
7	10.259	12	22.379	101.00		
8	10.259	12	22.372	100.94	101.32	0.61
9	10.259	12	22.503	102.03		

Table 2: Comparison of extraction methods for ginger: SFE-CO_2 and EP

Extraction method	SFE-CO_2 No.1	SFE-CO_2 No.2	SFE-CO_2 No.3	EP No.1	EP No.2	EP No.3	P
Weight of dried ginger (g)	85.6	89.0	81.4	90.5	89.6	91.0	
Yield (%)	2.37	2.12	2.41	3.24	2.96	3.37	$P < 0.01$
Content of 6-gingerol (%)	19.02	23.16	18.92	10.82	11.59	10.60	$P < 0.01$

where C_n is the 6-gingerol concentration at time point 'n'($\mu g \cdot mL^{-1}$), V is the volume of acceptor cell (7.0 mL), V_i is the volume of each sample (1.5 mL), S is the effective permeation area (1.767 cm^2), C_0 is the initial drug concentration in donor side ($mg \cdot cm^{-2}$).

The cumulative amount and the permeation percentage of 6-gingerol in each formulation at each time point is shown in Table 3. Each data was the mean ± SD of three determinations. The steady-state permeation equation. were calculated (Table 4). J_{SS} represents the best-fit slopes of the apparent linear portions, usually several data points in the beginning were removed during fitting. Statistical analysis of group differences between SFEE and EPE, 1% Azone and no Azone, RM and BC was performed using paired T-test. The results showed that there was no significant difference in Q_{10h} and Q_{20h} between the groups (Table 5).

3. Discussion

Ginger is recorded in the Chinese Pharmacopoeia as a traditional Chinese medicine that could be effectively used to treat various diseases. The potentials and mechanisms of the effect of 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol contained in ginger have been revealed recently, but minutes of short duration of their action has not been considered in previous preclinical and clinical studies, which might represent a major limitation for the therapeutic application of ginger compositions. In this study, we explored SFE-CO_2 methods (Shi et al. 1999) for extracting

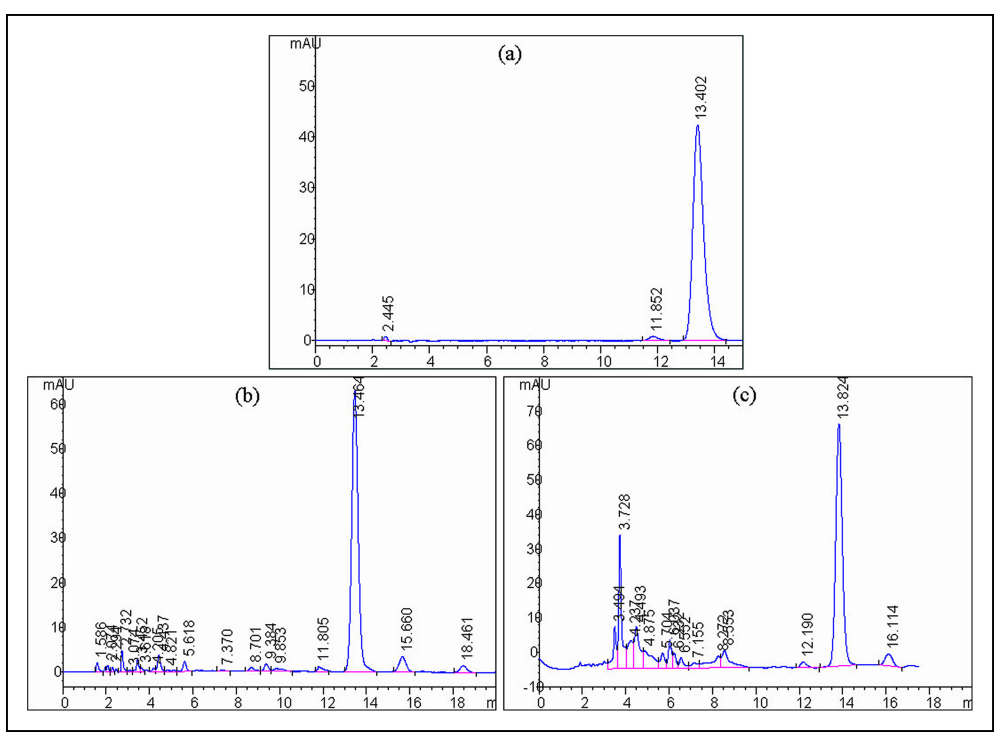

Fig. 2: HPLC analysis of the ginger extracts. (a) HPLC chromatogram of 6-gingerol standard. (b) HPLC chromatogram of the ginger SFEE sample, the retention time of 6-gingerol was 13.464. (c) HPLC chromatogram of the ginger EPE sample, the retention time of 6-gingerol was 13.824.

Table 3: Data of 6-gingerol permeation through skin in different TDDS formulations

Formulation	The Q(P%) values at each time point ($\mu g \cdot cm^{-2}$)						C_0 ($mg \cdot cm^{-2}$)
	0.5	2	6	10	16	20	
1. EPE + BC	21.287 ± 1.726(1.65%)	62.599 ± 2.751(4.84%)	130.719 ± 5.843(10.11%)	251.967 ± 7.618(19.49%)	372.329 ± 6.073(28.81%)	471.515 ± 9.283(36.48%)	1.2925
2. SFEE + 1% Azone + BC	26.770 ± 2.356(1.90%)	55.443 ± 2.306(3.94%)	143.466 ± 6.542(10.19%)	233.331 ± 5.691(16.57%)	433.510 ± 9.801(30.79%)	515.908 ± 11.873(36.64%)	1.4080
3. SFEE + 1% Azone + RM	62.443 ± 2.698(4.50%)	289.140 ± 8.016(20.9%)	298.318 ± 7.046(21.51%)	425.353 ± 10.548(30.68%)	534.553 ± 11.371(38.55%)	585.898 ± 12.043(42.25%)	1.3866
4. EPE + 1% Azone + BC	23.480 ± 1.591(1.52%)	83.048 ± 3.183(5.39%)	203.764 ± 6.943(13.22%)	297.833 ± 8.713(19.32%)	454.490 ± 9.307(29.48%)	559.823 ± 15.827(36.32%)	1.5415
5. EPE + 1% Azone + RM	59.085 ± 2.850(4.52%)	115.090 ± 4.215(8.81%)	248.629 ± 12.548(19.02%)	355.345 ± 11.817(27.19%)	468.421 ± 10.817(35.84%)	532.125 ± 14.176(40.72%)	1.3069
6. EPE + RM	20.137 ± 0.981(1.34%)	55.930 ± 2.482(3.73%)	155.034 ± 8.257(10.33%)	249.443 ± 6.547(16.62%)	388.994 ± 5.386(25.92%)	474.122 ± 10.365(31.59%)	1.5008
7. SFEE + BC	24.751 ± 2.392(1.72%)	56.086 ± 3.628(3.91%)	134.541 ± 6.543(9.37%)	234.405 ± 8.572(16.33%)	417.482 ± 9.716(29.08%)	516.515 ± 11.523(35.97%)	1.4358
8. SFEE + RM	35.232 ± 1.526(2.59%)	89.371 ± 5.916(6.56%)	219.907 ± 9.517(16.14%)	338.429 ± 10.257(24.85%)	451.517 ± 12.627(33.15%)	523.572 ± 13.358(38.44%)	1.3621

ginger and developed a TDDS of ginger extracts as a prolonged-action preparation of ginger (Alexander et al. 2012).

The results of determination showed that SFE-CO_2 extracts contained about two folds of 6-gingerol in EP extracts. HPLC chromatograms showed that there were fewer amounts of farragoes in SFE-CO_2 extracts than that in EP extracts, because of selectively extracting low polarity substances by SFE-CO_2, which might be the reason for the lower yields of SFE-CO_2 extracts. In a word, ginger could be refined by SFE-CO_2 with higher content of gingerol and less foreign matter, which might help gingerol to develop its biological function more effectively, and the volatile aromatic components contained in SFE-CO_2 extract were also beneficial to our health.

Gingerol is the characteristic component of ginger and 6-gingerol is the major ingredient in gingerol (about 60% of gingerol in ginger SFE-CO_2 extract is 6-gingerol). Thus, in this study, 6-gingerol was measured to evaluate the transdermal permeation properties of ginger effective component. The results in Table 5 show that the cumulative permeation amounts of 6-gingerol for 20 h in patches prepared with SFEE, 1% Azone and RM were higher than that with EPE, no Azone and BC, respectively, but there was no significant difference between them. Meanwhile, the results of percutaneous permeation in Table 3 indicated that formulation 3 (SFEE + 1% Azone + RM) produced the highest permeation rate of 6-gingerol at each time point (except 0.5 h) as compared to other systems. The cumulative amount of 6-gingerol at 20 h could reach 42.25%, which might imply that the release of 6-gingerol in optimized reservoir-type TDDS of ginger extracts might maintain more than one day. Therefore, the results of this investigation might provide a scientific evidence for the exploration of ginger TDDS. The problem of transient effect of gingerol resulting from short half lives of gingerols might be surmounted by using optimized TDDS of ginger ingredients to achieve long-term effectiveness.

4. Experimental

4.1. Apparatus

The quantitative study of 6-gingerol was performed by a HPLC System (Agilent ChemStation, 1050, USA). The HPLC System consisted of a low-pressure quaternary pump (model Agilent 1050), an autosampler (model Agilent 1050) and an UV visible detector (G79853A). The transdermal tests were performed by Automatic Transdermal Diffusion Cell System (Hanson Research, USA, USP Standard). The patches were prepared by a film applicator (Sidart Corp., Beijing, China). The SFE-CO_2 experiments were performed using a CL-10-J-3 SFE-CO_2 device (He Si Corp., Beijing, China).

4.2. Chemicals and reagents

6-Gingerol standard was obtained from Pharmacy Institution of Zhongxin (Tianjin, China). Dried ginger was obtained from Pharmacy of Beijing Military General Hospital (Beijing, China) and authenticated by Prof. Zhang-mei (The director of Pharmacy of Beijing Military General Hospital, China). Polyacrylate pressure sensitive adhesives were purchased from Eudragit Roma Corp., German and Beijing Chemical Corp., China). Azone was purchased from FTS Reagent Corp. (Hebei, China). Ethanol was purchased from Beijing Chemical Corp. (China). HPLC grade methanol was purchased from Sigma (USA), and water was double distilled for HPLC. Other chemicals used were all of analytical grade.

4.3. Extraction of ginger

4.3.1. Extraction of ginger by SFE-CO_2

The dried ginger was extracted by SFE-CO_2 for three times. The conditions were as follows (Li et al. 2011). No.1: Extraction temperature and pressure were 40 °C and 20 MPa. Extraction time was 2.0 h. No.2: Extraction temperature and pressure were 40 °C and 20 MPa. Extraction time was 3.0 h. No.3: Extraction temperature and pressure were 45 °C and 25 MPa. Extraction time was 3.0 h. Separation temperature was 42 °C for No.1 and No.2, 47 °C for No.3. Separation pressure was 6 MPa. CO_2 flow rate was 0.3-0.9 L/min.

Table 4: The steady-state permeation equation and permeation characteristics of 6-gingerol in different TDDS formulations

Formulation	Q-t equation	r	Jss ($\mu g \cdot cm^{-2} \cdot h^{-1}$)	Q_{10h}	Q_{20h}
1. EPE + BC	Q = 17.787t + 7.4172	0.9984	17.787	251.967	471.515
2. SFEE + 1%Azone + BC	Q = 18.343t + 0.0976	0.9966	18.343	233.331	515.908
3. SFEE + 1%Azone + RM	Q = 16.711t + 112.13	0.9403	16.711	425.353	585.898
4. EPE + 1%Azone + BC	Q = 17.466t + 16.763	0.9987	17.466	297.833	559.823
5. EPE + 1%Azone + RM	Q = 18.508t + 58.718	0.9892	18.508	355.345	532.125
6. EPE + RM	Q = 15.591t + 7.5971	1.0000	15.591	249.443	474.122
7. SFEE + BC	Q = 17.843t-1.4429	0.9973	17.843	234.405	516.515
8. SFEE + RM	Q = 18.367t + 36.046	0.9910	18.367	338.429	523.572

Finally, the extraction vessel was depressurized and the oil was collected from the separation vessel and the extract was stored at − 4 °C.

4.3.2. Extraction of ginger by ethanolic percolation

The dried ginger which had the same batch number than that by SFE-CO_2 was mixed with 60% ethanol (250 mL) and the mixture was allowed to stand overnight. The mixture was transferred to a percolator and 60% ethanol was added. The extraction was done at room temperature until the percolate was transparent and colourless. The extract was concentrated on a vacuum rotary evaporator until there was no residual ethanol, and extracted with ethylacetate for three times. Finally, the collection of ethylacetate layers was concentrated in a vacuum rotary evaporator and stored at − 4 °C. The EP extraction was repeated for three times (EP No.1, No.2, No.3).

4.4. HPLC chromatographic analysis for 6-gingerol

4.4.1. Preparation of standard solutions and sample solution

600 μg/mL of 6-gingerol were prepared by dissolving a weighed amount in mobile phase (methanol–water, 62:38, v/v). Serial dilutions of the 600 μg/mL standard were made to produce the 300, 200, 100, 40, and 10 μg/mL working standards. These working solutions were used for calibration curves and method validation. All the stock and working solutions were stored at 4 °C. 100-200 mg SFEE and EPE were weighed and added to 50 mL volumetric flasks containing 40 mL of mobile phase. The samples were sonicated for 30 min in an ultrasound bath, and the 50 mL volume was completed with mobile phase. After homogenization, samples were filtered through a 0.45 μm cellulose filter.

4.4.2. HPLC analytical conditions for 6-gingerol

Separations were carried out on an Agilent ZORBAX Eclipse DB C18 column (250 mm × 4.6 mm, 5 μm). The chromatographic conditions were the following: mobile phase, methanol–water (62:38, v/v); column flow rate, 1.0 mL/min; column temperature, room temperature; chromatographic run time, 13.0 min; injection volumes, 10 μL; wavelengths, 280 nm.

Table 5: Statistical analysis of group differences between SFEE and EPE, 1% Azone and no Azone, RM and BC

Groups	Mean of Q_{20h}	SD of Q_{20h}	N	P, Q_{10h}	P, Q_{20h}
SFEE	38.3250	2.81686	4	P > 0.05	P > 0.05
EPE	36.2775	3.73056	4		
1%Azone	38.9825	2.95926	4	P > 0.05	P > 0.05
no Azone	35.6200	2.88995	4		
RM	38.2500	4.70789	4	P > 0.05	P > 0.05
BC	36.3525	0.28652	4		

4.4.3. Method validation

The described method was validated according to the International Conference on Harmonization guidelines by determination of the linearity, detection and quantitation limits, precision and recovery test (ICH 2005; Weon et al. 2012).

Linearity: To establish the calibration curve, the six different concentrations of 6-gingerol standards were analyzed three times. The calibration curve was plotted by using the value of the peak areas versus concentration of each analyte. Linearity was indicated by the correlation coefficient (R^2) values of the linear regression line of the calibration curves.

Detection and quantitation limits: The detection limit (LODs) and quantitation limit (LOQs) were measured at a signal-to-noise ratio (S/N) of 3 and 10, respectively, in order to establish the minimum concentration at which the analytes can be reliably detected or quantified.

Precision: The precision of the method was evaluated by repetitive testing, inter-day and intra-day test. The inter-day test was determined by analyzing 100, 200, 300 μg/mL of standard solutions five times within five successive days. For the intra-day test, the same solutions were analyzed five times within one day. The relative standard deviation (RSD) was taken as the measurement of precision [RSD = (standard deviation/mean measured amount) × 100].

Accuracy: The recovery test was applied to investigate accuracy. Three different amounts of 6-gingerol standards were added to the ginger sample solution (10.259 mg/50 ml) respectively for three times. Recovery (%) was calculated according to the equation (amount found – original amount)/amount spiked × 100%).

4.5. Assay of 6-gingerol in ginger extracts

Each ginger extract was prepared according to the previous preparation method of sample solutions and analyzed by the previously developed HPLC method. The contents of 6-gingerol were calculated from the calibration curve of standards.

4.6. In vitro percutaneous permeation studies of 6-gingerol from TDDS

4.6.1. Preparation of reservoir-type TDDS

As presented in Table 3, SFEE (or EPE), 1% Azone (or no Azone), polyacrylate pressure sensitive adhesive (RM or BC), and some solvent were mixed together to obtain a solution which could be evenly applied on the backing layer to form a film by using a film applicator. The film was allowed to stand at room temperature and then further dried in an oven at 50 °C for 1 h.

4.6.2. Preparation of rat skin

Male Wister rats (200 ± 20 g) obtained from Laboratory Animals Center of China Science Institution were killed by cervical dislocation (Taghizadeh et al. 2010). Hair of the abdominal region was carefully removed, and a 5 × 5 cm full-thickness skin was excised from this region of each killed rat. The dermis side was wiped with isopropyl alcohol to remove the residual adhering fat. The skin was dipped and soaked in normal saline solution. It was then washed with distilled water, wrapped in aluminum foil, and stored

at − 20 °C for further use. One hour prior to the experiments, the samples were thawed.

4.6.3. In vitro transdermal release studies

Rat skin was mounted between the donor and receptor compartments of the Hanson diffusion cells with the stratum corneum facing the donor compartment (for each cell, effective diffusion area was 1.767 cm^2, acceptor volume was 7.0 mL) (Kezutyte et al. 2011). Hundred milliliters of acceptor medium, composed of 70/30 normal saline solution/ethanol, provided sink conditions. After driving air bubbles out of the system, accurately measured round patches (1.50 cm diameter) were pasted on the skin surface. The acceptor medium was stirred at 200 rpm, using a magnetic stirrer. The whole assembly was kept in a water bath (37 °C). After aliquots of 0.5 mL were wasted, aliquots of 1 mL were collected at 0.5, 2, 6, 10, 16 and 20 h and replaced automatically with an equal volume of acceptor medium to maintain a constant volume. The quantitative analysis of samples was performed using HPLC under the previous condition.

4.7. Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis of group differences was performed using T-test and eans analysis (spss 17.0). A value of $p < 0.05$ was considered statistically significant.
Competing interest statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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