A Novel Self-Micromulsifying System for the Simultaneous Delivery and Enhanced Oral Absorption of Curcumin and Resveratrol

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Key words
self-micromulsifying drug delivery system, curcumin, resveratrol, oral absorption, co-delivery

Introduction
Diferuloylmethane, commonly called curcumin, is a yellow pigment present in the rhizomes of turmeric (Curcuma longa L., Zingiberaceae). Curcumin exhibits antioxidative, anti-inflammatory, anticarcinogenic, and chemopreventive properties. In clinical studies, curcumin has been shown to prevent or treat various cancers in humans [1]. Resveratrol (3,5,4′-trihydroxystilbene) is a major component of grapes, wine, peanuts, and Polygonum cuspidatum Sieb. & Zucc. (Polygonaceae). It also has anticancer, antioxidant, and anti-inflammatory activities [2]. Recently, curcumin and resveratrol, two particularly important polyphenolic compounds, have been found to exhibit a synergistic anticancer effect against various cancer types, including colon cancer and hepatocellular carcinoma, and also in the treatment of diseases associated with oxidative stress [3]. However, both curcumin and resveratrol have low aqueous solubility and are rapidly metabolized. These problems result in poor oral bioavailability [4], which is an important restriction on their therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness. Many studies have indicated the importance of using self-micromulsifying drug delivery systems (SMEDDS) to improve solubility, absorption, and to increase the therapeutic usefulness.

ABSTRACT
The use of curcumin and resveratrol in combination has now become increasingly of interest because of their synergistic effects as therapeutic agents for various diseases, especially cancer. To overcome the poor oral bioavailability of both compounds and improve patient compliance, a novel self-micromulsifying formulation containing curcumin together with resveratrol was developed. Capryol 90, Cremophor EL, and Labrasol were selected as the oil, surfactant, and co-surfactant in the formulation, respectively, based on the solubility study of both compounds. More than 70% and 80% of curcumin and resveratrol, respectively, were released in 20 min. The formulation formed a fine oil in water microemulsion with droplet sizes in aqueous media of 15–20 nm. In addition, the formulation containing curcumin and resveratrol showed greater antioxidant activity than that of the formulations with individual compounds, while the cytotoxic activity against HT-29 of the co-formulation (IC50 = 18.25 µM; curcumin and resveratrol in the ratio 1:1) was less than the formulation with only curcumin (IC50 = 30.1 µM) and only resveratrol (IC50 = 25.4 µM). After oral administration to rabbits, the self-micromulsifying formulation containing curcumin together with resveratrol increased the total plasma concentrations of curcumin and resveratrol by 10-fold and 6-fold, respectively, compared to the unformulated combination. This study clearly demonstrated the potential use of the self-micromulsifying formulation for co-delivery, and enhanced oral absorption of poorly water-soluble natural compounds. In addition, the combination was found to produce synergistic antioxidant activity and cytotoxicity against HT-29 cells.
In order to enhance the solubility and oral absorption of both compounds, a new self-microemulsifying formulation containing curcumin in combination with resveratrol (CR-SME) was developed. The synergistic antioxidant activity and cytotoxicity of the new formulation against HT-29 cells were also evaluated. It is expected to be a promising approach to improve the performance of medicines and functional foods used to prevent and treat some important diseases in the future.

Results and Discussion

Suitable excipients in a self-microemulsifying system should have good solubilizing properties for the drug combination and form a monophasic liquid at an ambient temperature. The solubility in various vehicles of curcumin and resveratrol in combination is presented in Table 1. The combination had a higher solubility in Cremophor EL than in other vehicles with 85.98 ± 0.67 and 110.84 ± 0.54 mg/mL of curcumin and resveratrol, respectively, therefore, Cremophor EL was selected as a surfactant. The result might be due to the ability of the two polyethenols to form hydrogen bonds with the polyethylene oxide (PEO) groups [8]. In the same manner, Labrasol as a co-surfactant, composed of PEO groups, also exhibited a high solubilization capacity for the curcumin/resveratrol combination. Polyethylene glycol (PEG) 400 had a high solubilizing capacity for both compounds. These probably involved hydrophobic interactions between the ethylene units of PEG and the aromatic rings of the compounds [9], but the formulation could not form a translucent microemulsion upon dilution. In the case of Transcutol HP, it was not chosen to be a co-surfactant due to the color change of the produced solution. Among the oily phase compounds, Capryol 90 provided the highest solubility for both curcumin and resveratrol at 6.71 ± 0.07 mg/mL and 17.35 ± 0.02 mg/mL, respectively.

Ternary phase diagrams were constructed to study the proportion of components that can produce the best microemulsion. The mixture of Cremophor EL, Capryol 90, and Labrasol at the ratio 85:10:5 by weight was chosen as the best system for the curcumin/resveratrol combination. Polyethylene glycol (PEG) 400 had a high solubilizing capacity for both compounds. These probably involved hydrophobic interactions between the ethylene units of PEG and the aromatic rings of the compounds [9], but the formulation could not form a translucent microemulsion upon dilution. In the case of Transcutol HP, it was not chosen to be a co-surfactant due to the color change of the produced solution. Among the oily phase compounds, Capryol 90 provided the highest solubility for both curcumin and resveratrol at 6.71 ± 0.07 mg/mL and 17.35 ± 0.02 mg/mL, respectively.

Emulsion droplet size plays a vital role in the oral delivery of SME. A uniform and small particle size has an influence on the transport of a drug for delivery to a specific target [10]. The average droplet size of microemulsion from the formulation without compound (blank-SME) and CR-SME after dilution with water was 13.10 ± 0.40 nm and 15.90 ± 0.10 nm, respectively. In this study, deionized (DI) water, simulated gastric fluids (SGF), and simulated intestinal fluids (SIF) were used as a medium for the dispersion of a microemulsion from the CR-SME formulation. The average particle sizes in the DI water, SGF, and SIF were 15.85 ± 0.07 nm, 18.29 ± 0.04 nm, and 20.15 ± 0.37 nm, respectively (Fig. 1). The polydispersity index (PDI) was in the range of 0.075–0.125. This result indicated that the types of media had some influence on the particle size and PDI. Generally, the small oil droplet size was observed in the presence of high amounts of surfactant in the formulation. There is a possibility that Cremophor EL, a nonionic surfactant was able to solubilize a hydrophobic drug (HLB = 12–14), promoted the small-sized particles and augmented the entrapment property of the combination for delivery [11]. Transmission electron microscopy observations were performed with a volume ratio of the CR-SME/water at 1/75. This demonstrated the spherical shape of the particles with no signs of coalescence, even after 24 h of dilution. The image is shown in Fig. 2 in which the average diameter of this formulation was less than 30 nm, which agreed with the data obtained by the dynamic light scattering technique.

Table 1 The solubility of curcumin in combination with resveratrol in various vehicles.

<table>
<thead>
<tr>
<th>Vehicles</th>
<th>Curcumin solubility (mg/mL) mean ± S.D. (n = 5)</th>
<th>Resveratrol solubility (mg/mL) mean ± S.D. (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>0.39 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Capryol 90</td>
<td>6.71 ± 0.07</td>
<td>17.35 ± 0.02</td>
</tr>
<tr>
<td>Labrafil PGMC</td>
<td>13.93 ± 0.07</td>
<td>16.83 ± 0.05</td>
</tr>
<tr>
<td>Cremophor EL</td>
<td>85.98 ± 0.67</td>
<td>110.84 ± 0.54</td>
</tr>
<tr>
<td>Cremophor RH 40</td>
<td>3.11 ± 0.04</td>
<td>2.46 ± 0.14</td>
</tr>
<tr>
<td>Labrafil M 2125 CS</td>
<td>0.60 ± 0.01</td>
<td>2.38 ± 0.07</td>
</tr>
<tr>
<td>Lauroglycol 90</td>
<td>3.94 ± 0.09</td>
<td>10.67 ± 0.02</td>
</tr>
<tr>
<td>Co-surfactants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Labrasol</td>
<td>62.99 ± 0.38</td>
<td>69.25 ± 0.17</td>
</tr>
<tr>
<td>Lauroglycol FCC</td>
<td>0.08 ± 0.01</td>
<td>1.20 ± 0.08</td>
</tr>
<tr>
<td>PEG 400</td>
<td>74.49 ± 0.20</td>
<td>66.68 ± 0.15</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>12.57 ± 0.53</td>
<td>18.25 ± 0.02</td>
</tr>
<tr>
<td>Transcutol HP</td>
<td>70.13 ± 0.36</td>
<td>76.50 ± 0.23</td>
</tr>
</tbody>
</table>
The in vitro release profiles of curcumin and resveratrol from the SME formulation in SGF, pH 1.2, are shown in Figs. 3 and 4. This CR-SME formulation exhibited an immediate release of curcumin, with over 70% of curcumin released within 20 min, which was similar to that from the self-microemulsifying formulation with individual curcumin (C-SME; Fig. 3). In contrast, only 5% of the curcumin was released from the non-formulated combination. A rapid release of resveratrol from the CR-SME formulation was also found with over 80% within 20 min, and in a similar manner to that from the self-microemulsifying formulation with individual resveratrol (R-SME; Fig. 4), whereas only 18% of the dose was released from the non-formulated combination. These results demonstrated that the release profile of each polyphenol, either curcumin or resveratrol, was not affected by the presence of the other ingredients in the formulation. It should be noted, however, that resveratrol exhibited a greater percentage release than curcumin, because of its greater number of hydroxyl groups that led to an increased association of interactions with water. The hydrogen bonding of both the polyphenol compounds seemed to be a vital variable during the solubilization process [12]. The stability of the CR-SME formulation was evaluated for its physicochemical properties in the intermediate condition (30 ± 2°C/65 ± 5% RH) compared to the accelerated stress condition of 45°C/75% RH for 0, 1, and 3 months. A brown glass or container with light protection is recommended for storage of the CR-SME due to the resveratrol undergoing photodegradation, and curcumin is also sensitive when exposed to light [13]. From the results of Table 2, the CR-SME formulation showed no significant change in appearance after 3 months of storage in both conditions. The emulsion droplet size was in the range of 19.4–21.5 nm. The liquid formulation was found to be stable, and there was no change in the content of curcumin (99–101%) and resveratrol (101–102%).

Among the several types of cells, HT-29 (human colon adenocarcinoma cell lines) is frequently used as an in vitro cancer model. In order to test the cytotoxicity of each compound and the CR-SME formulations against HT-29 cells, MTT assays were carried out. The results in Fig. 5 clearly showed that the CR-SME (the combination ratio for curcumin/resveratrol was 1:1) had a lower IC50 than each separate polyphenol used in the SME (18.25 µM for CR-SME, 25.4 µM for R-SME, and 30.1 µM for C-SME). However, the blank SME had an IC50 value of 100 µg/mL. As Cremophor EL was the main component in this formulation, it might further increase the therapeutic effect of some anticancer agents to produce oxidative stress [14]. In a similar previous study from Majumdar et al. [3], they reported that curcumin in combination with resveratrol was a more effective chemopreventive agent than each polyphenol alone. In this study, it has been demonstrated that the co-delivery treatment of polyphenols prepared in the SME formulation caused greater inhibition of colon cancer cells than using each compound in the same system.

To test the combined antioxidant effect of curcumin and resveratrol in the SME, the ferric reducing antioxidant power (FRAP) assay was performed. The CR-SME formulation produced a higher absorbance at 590 nm than both agents alone in the formulation (C-SME and R-SME) and the blank SME (Fig. 6). The number and position of the hydroxyl groups in phenolic acids play an important role in their antioxidant activity. The antioxidant activity of CR-SME could be related to the functional groups of resveratrol. It has three hydroxyl groups and a conjugation between both the
aromatic rings [15]. Similarly, the phenolic and the methoxy group on the phenyl ring and the 1,3-diketone system are important for contributing to the antioxidant activity of curcumin. When both compounds were present, there was a potential for synergy that one antioxidant helped regenerate the other. In a previous study, the decay kinetics of a curcumin/resveratrol combination was also found to be lower than each individual compound [16].

Finally, in order to evaluate whether the combination administration of curcumin and resveratrol affected the oral absorption, studies were performed on CR-SME, C-SME plus R-SME, and curcumin/resveratrol suspensions. The oral absorption of the combination or individual compound in SME was much greater than that of the combination in suspension form, as shown in Figs. 7 and 8 for curcumin and resveratrol, respectively. The plasma concentration time profiles were similar between the administration of CR-SME and C-SME following with R-SME. The pharmacokinetic parameters in rabbits are summarized in Table 3. The total plasma concentrations of each compound from CR-SME were significantly higher when compared with the curcumin/resveratrol suspension (p < 0.05). The AUC0–6h of CR-SME increased by about 13.7-fold for resveratrol and 34.5-fold for curcumin than that of the suspension. The CR-SME administration gave a significantly higher AUC0–6h of curcumin than did C-SME following with R-SME (p < 0.05). However, there was no significant difference in the AUC0–6h of resveratrol between these two administration methods. In addition, all of the treatments have similar Tmax values at 60 min for curcumin and 90 min for resveratrol.

Materials and Methods

Chemicals

Curcumin (purity ≥ 70%) was from Sigma-Aldrich. Trans-resveratrol (purity ≥ 98%) (P. cuspidatum root extract resveratrol powder) was from Pioneer Herb. Capryol 90, Labrafac CC, Labrasol, Lauroglycol FCC, Labrafil M 2125 CS, and Lauroglycol 90 were from Gattefosse. Cremophor EL and Cremophor RH 40 were from BASF. PEG 400 and propylene glycol (PG) were from the PC Drug Center Co., Ltd. Corn oil was from the Thai Vegetable Oil Public Company limited. Trichloroacetic acid, sodium phosphate dibasic, sodium phosphate monobasic, acetonitrile, and methanol (HPLC grade) were from RCI Labscan. Potassium ferricyanide was from Ajax Finechem Pty Ltd. Ferric chloride was from Sigma-Aldrich. Ascorbic acid was from Chem-Supply Pty Ltd. Hard gelatin capsules (size 00) were from Capsugel. All other chemicals used were of analytical grade.
Cell culture

Human colon adenocarcinoma cell lines (HT-29 cells; HTB-38) were from ATCC. McCoy’s 5 a, FBS, and penicillin (100 IU/mL)-streptomycin (100 mg/mL) (pen-strep) were from Gibco, Invitrogen. Trypsin-EDTA 0.25% was from Gibco, Invitrogen. MTT was from Molecular Probes, Invitrogen. PBS (pH 7.4), 2-(N-Morpholino) ethanesulfonic acid (MES) sodium salt, was from Sigma. DMSO was from Amresco.

Solubility measurement

The shake flask method was utilized to study the equilibrium solubility of the curcumin/resveratrol combinations in different oils (corn oil, Capryol PGMC, Cremophor EL, Cremophor RH40, Labrafal M 2125 CS, Lauroglycol 90) and co-surfactants (Labrasol, Lauroglycol FCC, PEG 400, propylene glycol, Transcutol HP). An excess amount of curcumin and resveratrol in the ratio of 1:1 was added to each Eppendorf tube containing 1 g of the vehicle. The sample was vortexed at a maximum speed for 10 min using a mixer (Vortex-gene 2, Becthai Bangkok Equipment & Chemical) and allowed to equilibrate in a water bath shaker (Heto Lab, Scientific Promotion) (37°C at 100 rpm); the equilibration time was set at 48 h. Solid-phase separation was achieved using centrifugation (30 min, 1000 rpm at 37°C) and filtration [0.2 µm polyvinylidenedifluoride (PVDF) filter]. The supernatants were collected and diluted with the mobile phase [acetonitrile and 1% citric acid (v/v) (55:45)] for quantification of curcumin and resveratrol by the HPLC method. All solubility experiments were performed in triplicate.

Ternary phase assay

The compositions of oil, surfactant, and co-surfactant from the solubility study were selected to construct ternary phase diagrams. The ternary phase diagrams were plotted to identify the self-microemulsifying regions and to find the optimal concentrations of components. The mixture series of oil, surfactant, and co-surfactant were prepared. The vehicles were weighed into glass test tubes and mixed using a vortex mixer. The concentration range of each component was 10–50% oil, 25–90% surfactant, and 0–25% co-surfactant. One gram of each mixture was dispersed in 20 mL of distilled water. The efficiency of the self-microemulsification was observed visually and scored according to the grading system described by Singh et al. [17].

Preparation of curcumin/resveratrol in self-microemulsifying formulations

According to the ternary phase diagram studies, the SME formulation at the optimal component ratios was selected for the incorporation of curcumin and resveratrol. Thirty mg of each compound was added to 1 g of the self-microemulsifying mixture and the dispersion was stirred continuously until a homogenous solution was formed. The formulations were left for 48 h at room temperature. Hard gelatin capsules (size 00) were manually filled with the CR-SME formulation and stored in a tightly sealed glass bottle at room temperature until examined.

Emulsion droplet size and size distribution

One gram of each formulation was diluted with DI water, SGF, and SIF (20-fold dilution). The content was gently stirred by a magnetic stirrer for 5 min. The droplet size and the polydispersity index of the resultant microemulsion were determined by the dynamic light scattering technique using ZetaPALS, Zeta potential, and a particle size analyzer (Brookhaven Instruments Corporation). The light scattering was performed at a fixed angle of 90 at a temperature of 25°C. The measurement time was 1 min, and each run comprised 10 subruns.

Morphological characterization

The morphology of microemulsion formed was observed by transmission electron microscopy (TEM; JEOL). The CR-SME formul-
tion was diluted with distilled water at a ratio of 1:75 and mixed by gentle shaking. A drop of the sample obtained after the dilution was placed on copper grids. Any excess liquid was drawn off with filter paper. The grid surface was then air-dried at room temperature.

Release of curcumin and resveratrol from the self-microemulsifying formulation
The release profiles from the capsules filled with CR-SME containing 30 mg of each curcumin and resveratrol, C-SME containing 30 mg of curcumin, and R-SME containing 30 mg of resveratrol were compared to the unformulated combination. This study was carried out using the USP 30 rotating paddle apparatus with 900 mL of simulated gastric fluid (SGF, pH 1.2) at 37.0 ± 0.5°C and 75 rpm. The prepared formulations were subjected to the release studies for 2 h. Samples were withdrawn and replaced with the fresh medium at 5, 10, 15, 30, 45, 60, 90, and 120 min. The concentrations of curcumin and resveratrol were assayed by HPLC. The test was repeated six times, and the data were reported as the mean ± SD. A plot of the cumulative % release of curcumin and resveratrol against time was constructed to illustrate the drug release profiles.

HPLC analysis of curcumin in combination with resveratrol
The analysis of curcumin in combination with resveratrol in the drug release samples was performed on an Agilent separation module with a photodiode array detector (HP 1100, Agilent). A C18 column (VertiSep™ UPS C18 column 4.6 x 250 mm, 5 µm, Ligand Scientific) was used. The mobile phase consisted of a mixture of acetonitrile and 1% citric acid (v/v) (55:45) [18] with an isocratic solvent system. The injection volume was 20 µL. The flow rate of the mobile phase was 1 mL/min, and the detector wavelength was kept at 325 nm and 425 for curcumin and resveratrol detection, respectively.

Stability studies
The stability testing was carried out according to the ICH guidelines (2003) on the topic of Q1 A (R2): stability testing of the new drug substances and products. The optimized CR-SME formulation was subjected to stability studies in order to evaluate its physical and chemical stability. Samples were kept in a stability chamber (Patron AH-80) under intermediate conditions (30 ± 2°C, 65 ± 5% relative humidity (RH)), and evaluated under accelerated conditions (45 ± 2°C, 75 ± 5% RH), with the humidity and temperature control taken at 0, 1, and, 3 months for both conditions. Samples were prepared for the assay in the mobile phase and injected directly onto the HPLC column three separate times (n = 3).

Ferric reducing antioxidant power assay
The FRAP assay was used to evaluate the antioxidant capacity in order to determine the ferric reducing activity of the polyphenol co-delivery in the SME formulation. PBS 2.5 mL was mixed with the same amount of potassium ferricyanide and the sample solution (CR-SME, R-SME, and C-SME), standard ascorbic acid, or blank formulation was added into the tube. After incubation for 20 min at 50°C, 2.5 mL of trichloroacetic acid were added and centrifuged for 10 min at 3000 rpm to separate the layers. The supernatant was transferred for mixing with 2.5 mL of DI water and 0.5 mL of ferric chloride. Finally, the absorbance of all samples, standards, and blank were measured at 590 nm.

Cell culture studies
The HT-29 cells were grown in McCoy’s 5 A (modified) medium supplemented with 10% v/v FBS, and 1% v/v pen-strep. The cells were maintained at 37 ± 0.5°C in an atmosphere with 5% CO2 and 90% RH, and passed every 2 days. When the cell monolayer reached 80–100% confluency, the cells were removed from the culture flask using a 0.25% trypsin-EDTA solution. Viable cell numbers were counted prior to use by a standard hemocytometer. The cells were then used in cell cytotoxicity studies.

The cytotoxicity test
To evaluate the cytotoxicity of CR-SME at different concentrations, each polyphenol in the formulation was mixed at ratios of 5:5, 10:10, 15:15, 30:30, and 50:50 µM to get the final concentrations of the combination at 10, 20, 30, 60, and 100 µM, respectively. Each sample was dispersed in DI water and then diluted with the complete medium. The HT-29 cells were seeded in 96-well cell culture plates at a density of 8 x 10^3 cells/well and incubated for 24 h. After overnight incubation, the culture medium was removed, and the cells were washed with 100 µL of PBS. One hundred microliters of the samples at different concentrations were added to each well. Complete medium and 1% sodium lauryl sulfate were used as the negative control and positive control, respectively. After 24 h treatment, the samples were removed and the cells were washed with PBS. Fifty microliters of 0.5 mg/mL MTT solution were added to each well and the cells were incubated for another 4 h. After removing the MTT solution carefully, 100 µL of DMSO were added to dissolve the formazan crystals formed by the living cells. The absorbance of the samples was
measured by a microplate reader (DTX 880 Multimode Detector, Beckman Coulter Inc.) at a wavelength of 570 nm. Duplications were performed. The percentage of cell viability was calculated relative to the measured absorbance of the negative control that exhibited 100% cell viability.

**In vivo absorption studies**

The male New Zealand white rabbits with a mean body weight of 2.5 ± 0.2 kg were supplied by the Animal House, Faculty of Science, Prince of Songkla University. The animal protocol was approved under the guidelines of the Animal Care and the Committee of Prince of Songkla University (MOE 0521.11/061). The overnight fasted rabbits were divided into three groups with three rabbits per each group. The compounds 1) CR-SME formulation (curcumin 50 mg/kg and resveratrol 50 mg/kg) 2) curcumin/resveratrol in aqueous suspension (curcumin 50 mg/kg and resveratrol 50 mg/kg), or 3) C-SME formulation (50 mg/kg) followed by R-SME formulation (50 mg/kg) were orally administered as a single dose. Blood samples (1 mL) were collected from the auricular artery [19] 0, 15, 30, 45, 60, 90, 120, 180, 210, 240, 300, and 360 min after oral administration and were immediately transferred to a heparinized microcentrifuge tube and centrifuged at 4000 g for 20 min at 4°C. The plasma samples were separated. Acetonitrile was added to each plasma sample (acetonitrile:plasma = 1:1 v/v), vortexed, sonicated, and allowed to stand for 5 min for deproteinization. The protein precipitate was removed by centrifugation. The supernatant was pipetted into a tube. Samples were then diluted with methanol in a ratio of 1:0.5. The solutions were filtered using a 0.2-µm membrane filter and subjected to HPLC method. The pharmacokinetic parameters including the maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), and the area under the concentration-time curve (AUC_{0–6h}) were determined.

**Statistical analysis**

All results are expressed as the mean ± SD. Differences between two related parameters were assessed by Student’s t-test or one-way ANOVA. Differences were considered significant at p < 0.05.

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**Conflict of Interest**

There is no conflict of interest.

**References**


