

## Fabrication of Compound K-loaded Polymeric Micelle System and its Characterization *in vitro* and Oral Absorption Enhancement *in vivo*

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Received June 13, 2014, Accepted July 5, 2014

Compound K (CK) was formulated as polymeric micelles (PM) using Pluronic<sup>®</sup> F-127 to enhance the oral absorption of CK, an intestinal bacterial metabolite of ginseng protopanaxadiol saponin. The physicochemical properties of CK-loaded PM were characterized and an *in vitro* transport study using the Caco-2 cell system as well as an *in vivo* pharmacokinetic study using SD rats was carried out. The hydrodynamic mean particle size of CK-loaded PM (CK-PM) was  $254 \pm 23.45$  nm after rehydration and the drug loading efficiency was ca. 99.9%. The FT-IR spectroscopy, X-ray diffraction, differential scanning calorimetry and scanning electron microscopy data supported the presence of a new solid phase in the PM. The  $P_{app}$  value of *in vitro* Caco-2 cell permeation of CK-PM and the oral absorption of CK was enhanced about 1.2-fold and 2.6-fold compared to CK suspension, respectively, showing that the present PM formulation enabled an enhancement of oral CK absorption.

**Key Words :** Absorption enhancement, Compound K, Ginseng saponins, Pharmacokinetics, Polymeric micelle

### Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been orally taken as an important traditional medicine worldwide. To date, the biological activities of ginseng saponin metabolites biotransformed by human intestinal bacteria have been reported occasionally. One of the major metabolites of ginseng is 20-*O*-( $\beta$ -D-glucopyranosyl)-20(*S*)-protopanaxadiol, which is known as Compound K (herein named as CK) (Figure 1).

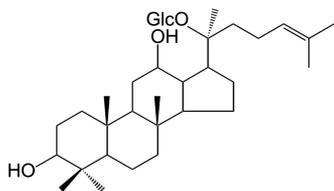
Taking ginseng radix orally, the majority of saponins are changed to CK, a final active form of ginsenoside which is biotransformed by anaerobic bacteria of intestine such as *Prevotella oris* and *Eubacterium* species.<sup>1-3</sup> CK has been known for its various pharmacological activities *in vitro* as well as *in vivo*,<sup>4</sup> such as anti-metastatic, antitumor activities<sup>2,3,5-7</sup> and its diabetic<sup>8</sup> and antipruritic effect.<sup>9</sup> However, in spite of the potentials of CK as drug candidate few studies have been reported regarding the oral absorption

enhancement of CK.

Solubilization technique,<sup>10</sup> solubilization in self-emulsifying or self-micro-emulsifying drug delivery systems,<sup>11,12</sup> microemulsions or nanoemulsions,<sup>13</sup> formation of inclusion complexes with cyclodextrins,<sup>14</sup> entrapment in nanoparticles<sup>15</sup> and polymer micelles<sup>16</sup> have been used to enhance the oral absorption of poorly water soluble drugs. Of them, polymeric micelles (PM) has been considered as potential drug delivery system for poorly water soluble drugs because it enhances the dissolution rate and oral absorption of drug and consequently advances undesired pharmacokinetic characteristics.<sup>17,18</sup> Usually PM was formulated with synthetic or natural hydrophilic polymers and/or nonionic amphiphilic copolymers, of which the hydrophilic segments form outer shell while the hydrophobic segments solubilize drug molecules.<sup>17,19-21</sup>

The core-shell structure of PM incorporates poorly soluble drugs, while the outer shell or the hydrophilic part of the polymer protects incorporated drug molecules from direct inactivation due to the gastro-intestinal environment.<sup>22</sup> The small particle size (< 200 nm) promotes drug absorption *via* endocytosis through enterocyte cell membrane.<sup>23</sup> In addition, PM enables high drug loading of poorly water soluble drugs comparing to low-molecular weight surfactants due to the lower critical micelle concentration (CMC) values of PM.<sup>24</sup>

One representative polymer used for PM preparation is pluronic block copolymer, which is an amphiphilic synthetic



**Figure 1.** Chemical structure of Compound K. Glc is  $\beta$ -D-glucopyranose.

polymer containing hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks consisting the triblock structure of PEO-PPO-PEO. These copolymers are very useful because they are low-priced, easily available, biocompatible and low toxic.<sup>25</sup> Pluronic<sup>®</sup> F127 (Poloxamer 407) is the representative diblock co-polymer of PEO and PPO which molecular weight ranges from 9,840 to 14,600 (g/mol), and they are choice of excipients of the pharmaceutical industry field.<sup>26</sup> All these issues related to PM enabled them as ideal carriers of poorly water soluble drugs for an oral drug delivery.

In this study, CK-loaded PM was prepared and characterized using physicochemical property-indicating methods such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), particle size analyzer (PSA), Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC) and X-ray diffractometry (XRD). The *in vitro* transport study using Caco-2 cell and *in vivo* pharmacokinetic study were also performed to evaluate the enhancement of oral CK absorption.

## Experimental

**Materials.** Purified CK (M.W. 622.878) with > 98.0% purity was purchased from Fleton Natural Products Co, Ltd. (Chengdu, China). Pluronic<sup>®</sup> F-127 was purchased from BASF (Ludwigshafen, Germany). Acetonitrile, ethanol and ethyl acetate of HPLC grade were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and other chemicals were of HPLC grade or the highest quality available.

**Preparation of Compound K-loaded polymeric micelles (CK-PM).** CK was incorporated into PM by a solid dispersion method.<sup>26</sup> Briefly, 10 mg of CK and 290 mg of Pluronic<sup>®</sup> F-127 were dissolved in 6 mL of ethanol. After 30 minutes of stirring at 37 °C, ethanol was evaporated under reduced pressure at 45 °C until a clear gel-like matrix was formed, which was hydrated with 10 mL of water at 50 °C to obtain a micellar solution. Then, the solution was frozen at -80 °C and lyophilized to obtain a product in powder state.

**Measurement of Drug Loading Efficiency.** The drug loading efficiency (%) of CK in PM was determined by reverse-phase HPLC after dissolving the CK-PM with ethanol. The drug loading efficiency was calculated based on the amount of CK encapsulated into the PM versus the amount of CK initially added to the PM using the following Eq. (1):

$$\text{Drug loading efficiency (\%)} = \frac{\text{amount of CK in PM}}{\text{initial amount of CK}} \times 100 \quad (1)$$

**Scanning Electron Microscopy (SEM).** Shapes and morphology of freeze dried CK-PM were investigated using a scanning electron microscopy (S4300-SE, Hitachi, Japan). CK-PM powder was dipped on the carbon tape and sputter coated with gold-palladium for 20 minutes to minimize a surface charging. After that, the sputter coated samples were observed for surface morphology with 500 fold magnifi-

cation under a scanning electron microscope.

**Transmission Electron Microscopy (TEM).** The water rehydrated morphology of CK-PM was observed by TEM (JEM 1200 EX II, JEOL, Japan). CK-PM suspension diluted in water was placed onto carbon-coated copper grid and dried. One drop of uranyl acetate was immediately added and the excess fluid was removed from the grid surface with a filter paper. The morphology of aqueous dispersion of particles was observed by TEM.

**Particle Size Analysis (PSA).** The particle size of CK-PM was determined using the dynamic light scattering method by a particle size analyzer (BI-90 Plus, NY, USA). The aqueous dispersion of PM was 1,000-fold diluted with distilled water and the intensity of 90°-scattered light was measured. For each specimen, 10 autocorrelation functions were analyzed using the scattered intensity and the mean diameter of the particles was calculated.

**Fourier Transform Infrared Spectroscopy (FT-IR).** CK, Pluronic<sup>®</sup> F-127 and CK-PM were mixed with KBr powder and the pellets were prepared under conditions of 20 °C and 30% relative humidity. The pellet was prepared under 7.54 ton/cm<sup>2</sup> of pressure. The FT-IR spectra of each sample were measured in a range of 4,000 to 400 cm<sup>-1</sup>. The final spectrum was obtained from the average of 32 scans.

**Differential Scanning Calorimetry (DSC).** The DSC experiment was carried out using a differential scanning calorimeter (DSC 2910, TA Instruments Co., UK) to measure the melting temperature (T<sub>m</sub>) and thermal decomposition temperature (T<sub>d</sub>). The measurements were carried out from 25 to 200 °C under nitrogen gas at a scanning rate of 10 °C/min.

**X-ray Powder Diffraction (XRD).** X-ray patterns of CK, Poloxamer<sup>®</sup> F-127, and CK-PM were analyzed by wide-angle X-ray scattering with a general area detector diffraction (Philips X'pert MPD System, Philips, Eindhoven, Netherlands) using Cu K radiation. The X-ray source was operated at 35 kV and 20 mA. Diffraction intensity was measured in reflection mode at a scanning rate of 2°/min in a range of 5 to 50°.

**HPLC Analysis Condition.** The drug contents and drug loading efficiency of formulation were measured by a validated reverse-phase HPLC method. CK was analyzed with ZORBAX<sup>®</sup> ODS C<sub>18</sub> column (5 m, 4.6 × 250 mm, Agilent Technologies, Palo Alto, CA, USA) at 30 °C. The composition of mobile phase was a mixture of acetonitrile and distilled water (60:40, v/v) and the flow rate was 1 mL/min. 10 µL of samples were injected into a HPLC system (Agilent Technologies, Palo Alto, CA, USA) and the eluent was monitored with an UV/VIS detector at 203 nm. The standard curve was prepared over a concentration range of 10 to 100 g/mL.<sup>27</sup>

**LC/MS Analysis Condition.** The Caco-2 cell permeation samples and blood specimens were analyzed by a high sensitive LC/MS system equipped with degasser, pump and auto sampler. The analysis was performed with ZORBAX<sup>®</sup> ODS C<sub>18</sub> column using isocratic mobile phase composed of acetonitrile and distilled water (80:20, v/v) at a flow rate of

1.5 mL/min. The temperature of column oven was 30 °C and of autosampler tray was 4 °C. The eluent was directly introduced into the tandem quadrupole mass spectrometer through the negative ionization electrospray interface. The optimum capillary voltage for ionization was 2,500 V and for fragmentor was 150V. Selected ion monitoring (SIM) mode was used for the quantitation of CK at  $m/z$  622.<sup>28</sup> The standard curve was prepared over the concentration range of 10 to 500 ng/mL.

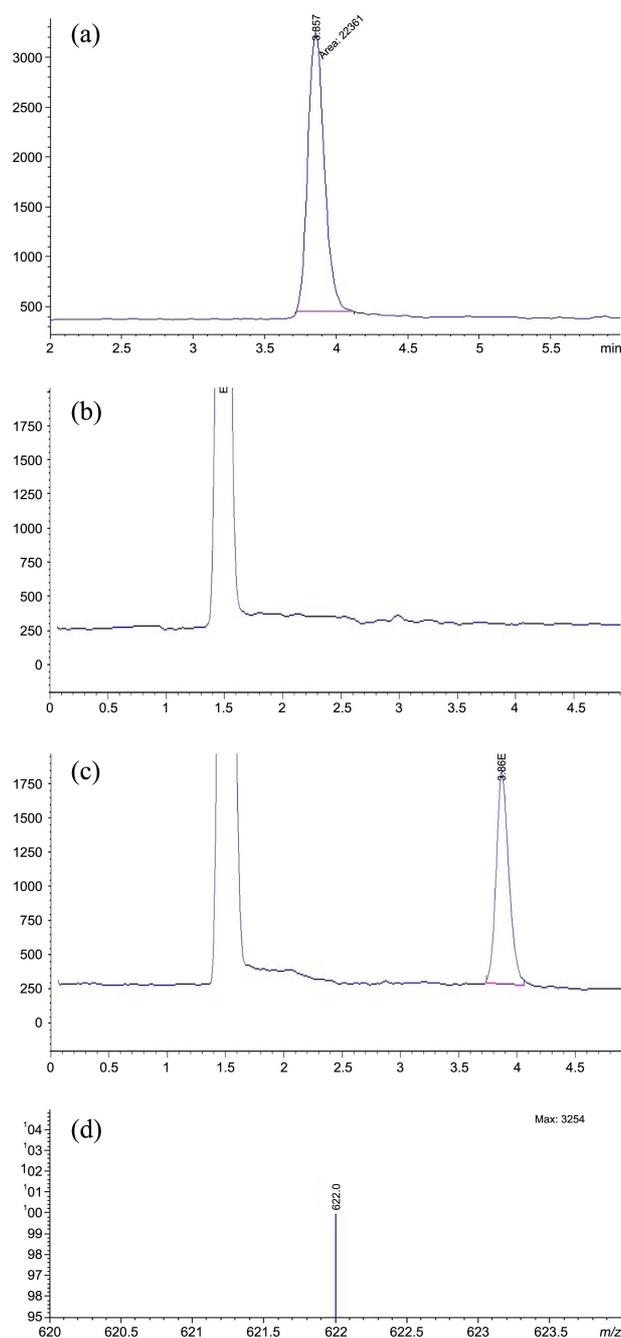
**Compound K Transport Study via Caco-2 Cell Monolayers.** Caco-2 cells (Korean cell line bank, Seoul, Korea) were grown in minimum essential medium (WelGENE Inc, Korea) supplemented with 10% fetal bovine serum (WelGENE, Inc, Korea) containing 1% penicillin-streptomycin (Gibco, UK) in a humidified 37 °C incubator with 5% CO<sub>2</sub> atmosphere. Caco-2 cells (passage number 31) were seeded onto polyester membranes of Transwells<sup>®</sup> (12 mm i.d., 0.4 μm pore size, Costar, Corning Inc., USA) at a density of  $1 \times 10^6$  cells/well. The medium was added to both, the apical and basolateral compartment. After a culture period of 21 days, integrity of cell monolayer was carefully evaluated by determining transepithelial electrical resistance (TEER) values using Millcell<sup>®</sup> ERS-2 (Millipore, Bedford, MA). Caco-2 cells with TEER values  $\geq 300 \Omega \times \text{cm}^2$  were used for the transport experiment. Prior to the experiment, cell monolayers were washed with transport medium (pH 7.4) twice and pre-incubated for 30 minutes at 37 °C. 0.5 mL of CK and CK-PM suspension (50 μM as CK) was added to the apical side and 1.5 mL of drug-free transport medium was added to basolateral side. Membrane inserts were moved to other wells containing the same volume of fresh medium every 15 minutes for 1 h. An aliquot (1 mL) of each sample was collected from the basolateral side and stored at -80 °C until LC/MS analysis.<sup>4</sup> The apparent permeability coefficients ( $P_{app}$ ) were calculated according to the following Eq. (2):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \cdot C_0} \quad (2)$$

where,  $P_{app}$  is the apparent permeability coefficient in cm/s,  $dQ/dt$  is the permeability rate,  $A$  is the diffusion area of the monolayer (cm<sup>2</sup>) and  $C_0$  is the initial concentration of the CK.

**In vivo Pharmacokinetic Study.** Male Sprague-Dawley (SD) rats (7-8 weeks, 220-240 g, Hyochang Science, Korea) were used for the pharmacokinetic studies. The animals had free access to food and water before the experiments. Rats were anesthetized with 30% urethane *via* i.p. injection and the jugular vein was cannulated for blood sampling. CK suspension and CK-PM at a dose of 10 mg/kg as CK were orally administered by a tube. Blood samples (400 μL) were collected into heparinized tube at predetermined times (0, 5, 15, 30, 45 minutes, 1, 2, 4, 8 and 12 h). Collected blood samples were centrifuged at 4 °C, 10,000 rpm for 5 minutes (5417R, Eppendorf AG, Germany). The aliquots of plasma samples (200 μL) were stored at -80 °C until LC/MS analysis. The plasma samples (100 μL) were extracted with

900 μL of ethyl acetate for 5 minutes and centrifuged at 10,000 rpm for 5 minutes at room temperature. The organic layer was transferred with pipette and evaporated to dryness at 35 °C. The residues were dissolved in 100 μL of 70% acetonitrile in water by vortex-mixing for 2 minutes, transferred to injection vials and 40 μL was injected into the LC/MS system. Pharmacokinetic parameters such as  $C_{max}$ ,  $T_{max}$  and AUC were determined. All experimental procedures were designed according to the 3R (Replacement, Refinement and Reduction) principle, and approved by the Institutional Animal Care and Use Committee (Protocol No. IJ-2009-70).



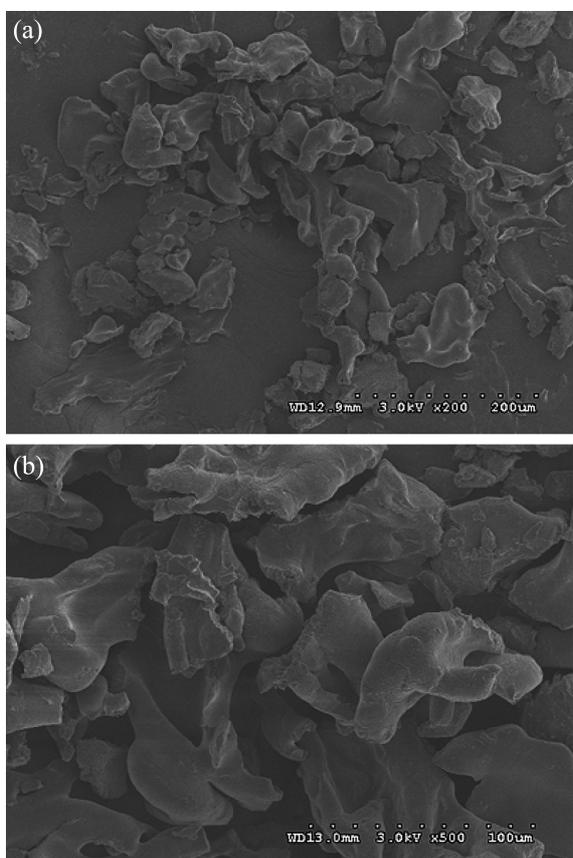
**Figure 2.** Representative LC/MS chromatograms of Compound K. Compound K (a), blank plasma (b), spiked Compound K (c) and negatively ionized mass spectrum of Compound K (d).

**Data Analysis.** Results are indicated as mean  $\pm$  standard deviation ( $n=3$ ). Statistically significant differences were determined using the student's t-test with  $p < 0.05$  as a minimal level of significance.

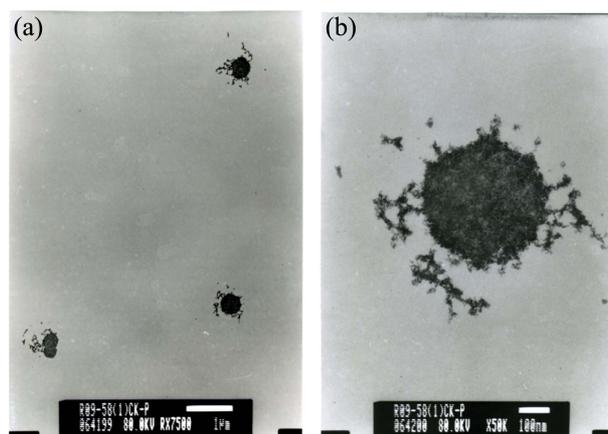
### Results and Discussion

**Analysis of Compound K by HPLC and LC/MS.** The standard curve for HPLC analysis showed good linearity over the concentration range of 10 to 100  $\mu\text{g/mL}$  ( $y = 7.7544x + 53.918$ ,  $R^2 = 0.9967$ , data not shown). In LC/MS analysis, the electrospray ionization of CK with negative ion mode produced molecular ions at  $m/z$  622. The retention time of CK was 3.8 minutes and confirmed the specificity of the present method. The standard curve was linear over the concentration range of 10 to 500  $\text{ng/mL}$  ( $y = 54.792x + 213.93$ ,  $R^2 = 0.9996$ , data not shown). The representative chromatograms of CK in LC/MS are shown in Figure 2.

**Physico-chemical Characteristics of Compound K-loaded Polymeric Micelle.** The drug content reflects the potential of PM for an incorporation of CK into PM. The analysis revealed that the drug content reached over 99.9% supporting the present solid dispersion-based PM preparation method to incorporate CK into PM with a high loading efficiency. SEM images of freeze dried CK-PM are shown in Figure 3. The morphology of freeze dried CK-PM was



**Figure 3.** Scanning electron microscopy of Compound K-loaded polymeric micelle  $\times 200$  (a),  $\times 500$  (b).

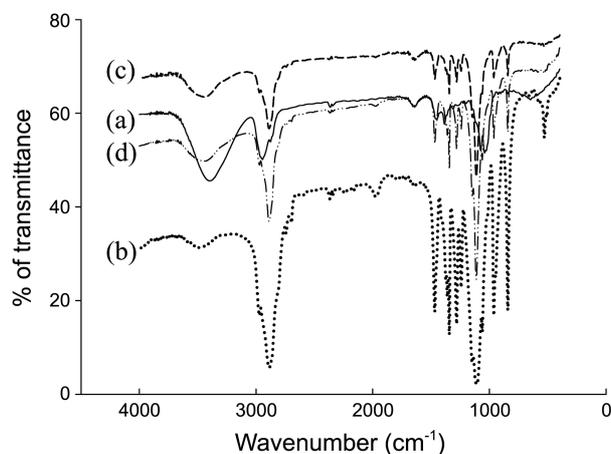


**Figure 4.** Transmission electron microscopy of Compound K-loaded polymeric micelle. (a);  $\times 7,500$ , (b);  $\times 50,000$ .

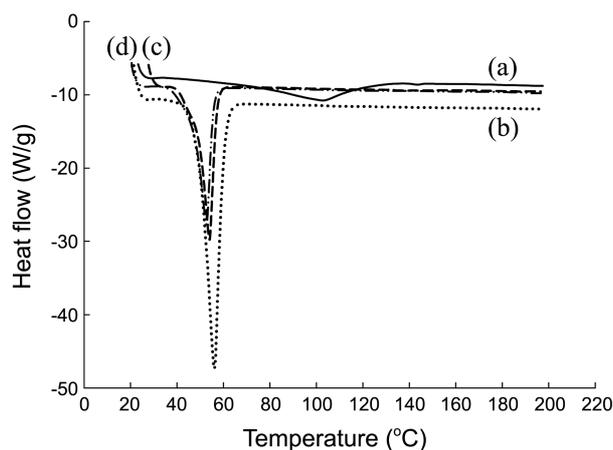
irregular and of amorphous shapes. However, dissolving it into water media, the CK-PM dispersed spontaneously and showed spherical particle shapes (Figure 4). The optically measured approximate diameter of PM by TEM was 200 to 300 nm and the average particle size of CK-PM measured with particle size analyzer was  $254 \pm 23.46$  nm, the optically observed data coincident well with TEM image data.

The FT-IR spectra of CK, Pluronic<sup>®</sup> F-127, drug free PM and CK-PM are shown in Figure 5. CK shows an -OH stretching vibration near  $3,400 \text{ cm}^{-1}$  and C=C stretching vibration near  $1,640 \text{ cm}^{-1}$ . Comparing each spectrum, we found that the absorption band of -OH at  $3,397 \text{ cm}^{-1}$  slightly shifted to  $3,451 \text{ cm}^{-1}$  and weakened after encapsulation of CK into PM. In addition, the C=C stretching vibration was weakened at  $1,639.9 \text{ cm}^{-1}$ . These results support the possibility of interactions between -OH, C=C of CK and the functional group of Pluronic<sup>®</sup> F-127 at the molecular level within PM.

A thermogram of CK (Figure 6(a)) exhibited a broad endothermic peak at  $103 \text{ }^\circ\text{C}$ , corresponding to the melting



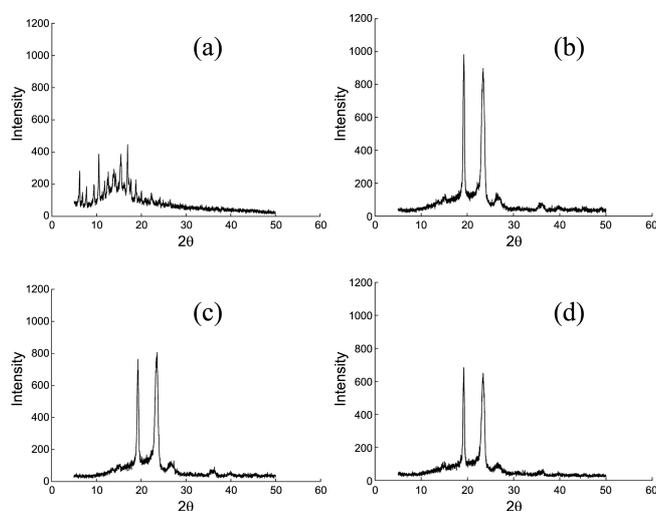
**Figure 5.** FT-IR spectra of Compound K (a), Pluronic<sup>®</sup> F-127 (b), Compound K free polymeric micelle (c) and Compound K-loaded polymeric micelle (d).



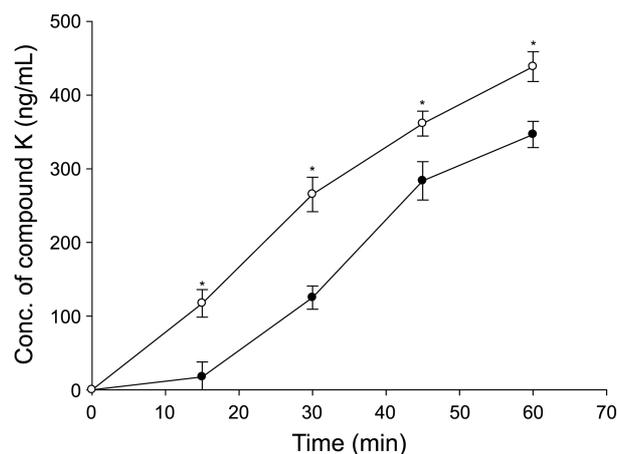
**Figure 6.** DSC thermograms of Compound K (a), Pluronic® F-127 (b), Compound K free polymeric micelle (c) and Compound K-loaded polymeric micelle (d).

point ( $T_m$ ) of CK. Pluronic® F-127 showed  $T_m$  near 56 °C (Figure 6(b)). Each endothermic peak was maintained although the  $T_m$  of Pluronic® F-127 have slightly shifted to 52 °C in CK-free PM and CK-PM. However, the endothermic peak of CK was disappeared in CK-PM (Figure 6(d)). The thermogram of CK-PM showed no endothermic peak corresponding to CK. This might be because of an increased solubility of CK as a temperature function in the Pluronic® F-127 carrier.<sup>29</sup> The thermal profile of CK-PM showed a complete disappearance of the CK endothermic peak, supporting the formation of a new solid phase, an amorphous solid dispersion, within the PM matrix.

The X-ray diffraction patterns of CK, Pluronic® F-127, CK-free PM and CK-PM are shown in Figure 7. The diffractogram of CK exhibited a series of intense peaks, which is indicative of their crystalline characteristics. The X-ray diffraction patterns of Pluronic® F-127, drug free PM and CK-PM did not show the distinctive CK peaks which



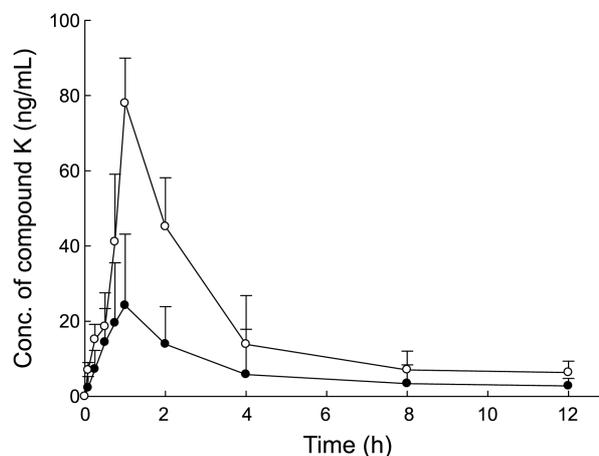
**Figure 7.** X-ray diffractograms of Compound K (a), Pluronic® F-127 (b), Compound K free polymeric micelle (c) and Compound K-loaded polymeric micelle (d).



**Figure 8.** Caco-2 cell permeation profile of Compound K through Caco-2 cell monolayers in apical to basolateral. ●; Compound K, ○; Compound K-loaded polymeric micelle (\* $p < 0.05$ ).

already been shown. Along with these results, the prominent crystalline peak of CK situated between 5–23.4° ( $2\theta$ ) clearly disappeared, indicating the decrease in crystallinity or partial amorphization of the drug in its kneaded form.<sup>30</sup>

**In vitro Caco-2 Cell Permeation and in vivo Pharmacokinetics.** We have quantified the cellular uptake efficiency of CK and CK-PM. The measured TEER values were with  $367.3 \pm 46.6 \Omega\text{-cm}^2$  sufficient for the permeability study. Caco-2 cells were incubated with the CK and CK-PM suspension for 1 h. The permeation profiles of CK and CK-PM across Caco-2 cell monolayers are shown in Figure 8. As depicted, CK-PM enhanced *in vitro* Caco-2 cell permeation of CK across the Caco-2 cell monolayer than that of pure CK suspension. The calculated  $P_{app}$  value of CK and CK-PM was  $3.2 \times 10^{-6}$  and  $2.4 \times 10^{-6}$  cm/s, respectively. The  $P_{app}$  value for CK-PM was significantly 1.2-fold higher than that of CK suspension, which indicates that CK-PM enhanced the permeation of CK through Caco-2 cell monolayer. This result may be due to the increased solubility of



**Figure 9.** Plasma concentration-time profile of Compound K after oral administration at a dose of 10 mg/kg as Compound K in rats. ●; Compound K, ○; Compound K-loaded polymeric micelle.

**Table 1.** Pharmacokinetic parameters after oral administration of Compound K and Compound K-loaded polymeric micelle

Pharmacokinetic parameters	Compound K Suspension	Compound K-loaded polymeric micelle
$C_{\max}$ (ng/mL)	24.8 ± 12.3	77.9 ± 9.8 <sup>a</sup>
$T_{\max}$ (h)	1.0 ± 0.2	1.0 ± 0.3
AUC (ng·mL·h <sup>-1</sup> )	80.8 ± 16.3	208.8 ± 12.7 <sup>a</sup>

<sup>a</sup>Statistically significant difference between CK and CK-PM ( $p < 0.05$ ).

CK by PM formulation.

An *in vivo* PK study with rats was performed to evaluate the oral absorption property of CK-PM in comparison with an aqueous suspension of CK and the plasma CK concentration was determined by LC/MS. The maximum plasma concentration ( $C_{\max}$ ) was observed at 77.9 µg/mL after 1 hour ( $T_{\max}$ , time to reach maximum plasma concentration) (Table 1). On the other hand, the  $C_{\max}$  obtained with CK powder was 24.8 µg/mL. Also, the AUC of CK-PM was approximately 2.6-times greater than that of CK suspension (Figure 9 and Table 1). As a result, CK-PM was found to cause better absorption than CK suspension after oral administration. This increased oral absorption exhibited by the PM may be attributed to an enhanced CK solubility and release in the gastrointestinal tract.<sup>31</sup>

PM has been usually used for intravenous administration whereas the usage for oral delivery is still a rather recent event.<sup>18</sup> Among only a few reports, Lee *et al.* have reported that the oral absorption of CK could be about 1.9-fold higher enhanced by the use of β-cyclodextrin. The BA of CK is very low and known as just 3–4%, which is mainly due to the poor absorption through the intestine. But the fundamental reason for the low BA of CK is mainly due to its low water solubility. As shown in the dissolution study of CK in water and diverse buffers with broad pH ranges between pH 1.2 to 8.0, the dissolution rate (%) was below *ca.* 3%.<sup>1</sup> Taken together Caco-2 cell permeability and PK results, our study clearly revealed that the PM of the present study can be a good delivery system for the oral delivery with CK.

### Conclusion

The oral delivery of poorly water-soluble drugs is a major confronting issue for the pharmaceutical industry. PM are promising drug carriers for poorly water soluble drugs but their potential use as oral drug delivery systems for CK has not been widely demonstrated *in vivo*. In this study, a solid dispersion-based CK-PM was successfully fabricated with a high encapsulation efficiency of *ca.* > 99.9%. The mean particle size of CK-PM was 254 ± 23.46 nm and FT-IR, DSC as well as XRD have shown an incorporation of CK in the PM matrix. They also proved the decrease in crystallinity of CK. Although further detailed studies are required to prove the mechanism by which the PM are able to transport across intestinal membrane, the *in vitro* Caco-2 cell permeability study and also the *in vivo* study have revealed that CK-PM could enhance the cell permeability and oral absorp-

tion of CK. In conclusion, Pluronic® F-127 PM is effective in solubilization and oral delivery of the poorly water soluble drug CK.

**Acknowledgments.** This work was supported by the Postdoctoral Research Program of Inje University, Gimhae, Republic of Korea, 2009. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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