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Encapsulation of a Flavonoid-rich *Allium cepa* L. var. *agrogatum* Don Extract in β-Cyclodextrin for Transdermal Drug Delivery

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ABSTRACT: This work aims to evaluate the encapsulation of a flavonoid-rich *Allium cepa* L. var. *agrogatum* Don extract (ACADFE) in β-cyclodextrin (β-CD) by analyzing the percutaneous penetration in vitro and in vivo, leading to an explanation of the physical mechanism during transdermal transport. The optimal inclusion compound containing ACADFE in β-CD was prepared in a 1:3 molar ratio. The physicochemical characterization of the inclusion complex was performed using IR, UV−vis, simultaneous thermogravimetry (TG), and differential thermal analysis (DTA) studies. It was concluded that the inclusion complex could improve the aqueous solubility and bioavailability of ACADFE. The inclusion complex exhibited significant enhancement of percutaneous absorption both in vitro and in vivo. The dorsal skins of hairless mice were photographed using a confocal scanning laser microscope. Confocal scanning laser microscopy shows that penetration of the ACADFE−β-CD inclusion complex proceeds across skin via both follicular and transcellular routes.

KEYWORDS: *ACADFE, quercetin, β-cyclodextrin, inclusion complex, transdermal transport*

INTRODUCTION

In China and other Asian countries, many herbs and their extracts are traditionally used in cooking flavonoid-rich foods (e.g., *Allium*, soybean, Houttuynia, pumpkin flowers, Zanthoxylum); these extracts are used not only as flavoring and coloring agents but also for promoting beneficial health effects due to their antioxidant activities and antimicrobial effects. This paper references *Allium cepa* L. var. *agrogatum* Don (ACAD), a perennial flavonoid-rich plant of the Liliaceae family native to northern China. Flavonoids are most well-known for their antioxidant, anti-inflammatory, and antimicrobial properties[1−3] as well as their health benefits in chronic diseases, such as heart disease, hypertension,[2] and cancer.[1] A flavonoid-rich extract of *A. cepa* L. var. agrogatum Don (ACADFE) contains quercetin and its glycosides, such as quercetin-3,4′-*O*-β-D-glucopyranoside (199.3 ± 27.8 mg g^{-1}), quercetin-4′-*O*-β-D-glucopyranoside (181.7 ± 25.4 mg g^{-1}), and quercetin (157.9 ± 23.1 mg g^{-1}).[4] Previous animal trials have demonstrated that ACADFE treatment may show effective antihyperlipidemic activity because ACADFE acts as an antioxidant, removing free radicals and preventing oxidative damage.[5] However, the quercetin (QT) limited the water solubility of ACADFE, and the inclusion of this compound may be responsible for the extract's limited absorption upon oral administration.[6] This limitation impairs the development of a topical pharmaceutical dosage form.

In pharmaceutical product development, β-cyclodextrin (β-CD, Figure 1) falls within the category of pharmaceutical excipients and has been widely utilized to improve the solubility, chemical stability, and bioavailability of a number of poorly soluble and oxidizing compounds. Srinivasan et al.[7] investigated the photophysical, electrochemical, and photoprototropic behaviors of diphenylamine (DPA) in aqueous β-CD solutions using absorption spectroscopy and cyclic voltametric techniques. Absorption of the neutral and cationic forms of DPA is enhanced due to the formation of a 1:1 inclusion complex with β-CD. Kakran et al.[8] demonstrated that quercetin complexes with β-CD displayed enhanced solubility. Yan et al.[9] investigated the inclusion process of β-CD and quercetin by using the PM3 quantum-mechanical semiempirical method. Dias et al.[10] synthesized Al(III) quercetin/β-CD inclusion compounds and characterized them using IR, UV−vis, ^{1}H and ^{13}C NMR, and TG and DTA analyses. Kalogeropoulos et al.[11] prepared the inclusion complex of *Hypericum perforatum* (HP) with β-CD by mixing 1:4 mass ratios of its components in aqueous media and subsequently freeze-drying the complex. In recent years, β-CD has gained appreciable acceptance among the various types of cyclodextrins and thus has been recorded into pharmacopoeia of the United States and China.

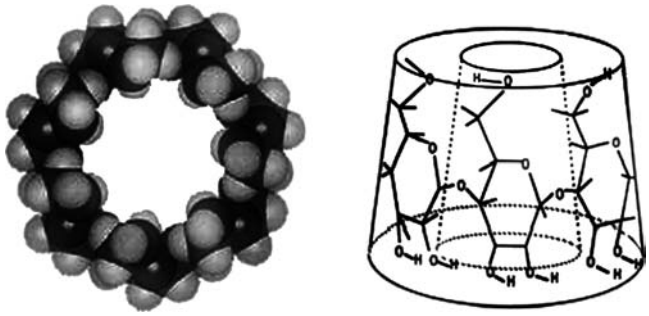

Figure 1. Structure of β-CD.

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Transdermal drug delivery is a convenient method of drug administration that enables physicians to provide the controlled delivery of drugs to patients with minimal discomfort.[12] This method offers many benefits over delivery via oral,[13,14] intravenous, or injection routes,[15,16] which often result in adverse clinical side effects or fail to deliver the active ingredient to the intended organ quickly enough or in sufficient concentrations to treat a variety of systemic diseases.[17]

In this paper, we report a novel ACADFE/β-CD inclusion complex formulation for use in transdermal drug delivery. To our knowledge, no previous study has reported the encapsulation of ACADFE in β-CD, yet the association of ACADFE with β-CD seems to be a promising strategy. A 95% ethanol extract from ACAD, which contains mainly quercetin and quercetin glucosides, was encapsulated in β-CD by coprecipitation and subsequent freeze-drying. Verification of encapsulation was accomplished by UV−vis spectroscopy and Fourier transform infrared spectroscopy, and the thermal stability of the inclusion complex was examined by analyzing TG and DTA curves after thermal oxidation. Furthermore, the quercetin encapsulation efficiency of the ACADFE/β-CD inclusion complex was determined by HPLC. Confocal scanning laser microscopy demonstrated how the penetration of the inclusion complex proceeds across the skin. Currently, to our knowledge, no previous studies have proposed a mechanism to explain how the ACADFE/β-CD inclusion complex passes through the stratum corneum transport system.

MATERIALS AND METHODS

Chemicals. β-CD was obtained from Shanghai Huishi Biological Chemistry, Inc. (20080308, Shanghai, China). All solvents used for chromatographic purposes were of HPLC grade. All other reagents and solvents used were of analytical grade.

Plant Material. ACAD collected from Huaide of Jilin (China) were purchased from a Changchun franchiser.

Extraction and Purification of ACADFE. ACADFE specimens were donated by the Department of Pharmaceutical Chemistry, School of Pharmacy, Jilin University (Changchun, China). First, the solvent method was selected to extract ACADF, and four important extraction factors were inspected through monofactor and orthogonal experiment. The optimal extraction technology of total flavonoids was found when ACAD was circulated and extracted at ambient temperature with a 3-fold volume of 95% ethanol for 2 h. This procedure was repeated three times. The extract was dried by evaporation under reduced pressure to $^1/_{10}$ of the initial volume. The extract of $^1/_{10}$ of the initial volume was abstracted by acetic ether, precipitated out by Ca^{2+}, filtered by vacuum filtration, and purified by macroporous resin AB-8. (We have compared the results of different purification methods: the optimal method was macroporous resin AB-8.) The purified extract was redissolved in 95% ethanol, and the solvent was evaporated and dried under reduced pressure. The dark red powder thus obtained was sealed under nitrogen in Teflon-coated screw-capped vials and stored in deep freeze at −40 °C until use. The quercetin content of ACADFE was analyzed by HPLC. The total quercetin content of ACAD after extraction and purification was found to be 62.7 ± 2.95%.

HPLC of Quercetin Content in ACADFE. The quercetin concentrations of the samples were determined by UV-HPLC analysis using a Shimadzu (Kyoto, Japan) liquid chromatograph equipped with an LC-10 AT VP solvent pump unit and an SPD-10A VP UV−visible detector. The chromatographic column used was an Agilent C18 column (5 μm, 250 mm × 4.6 mm). A mixture of methanol and 0.4% phosphoric acid solution (51:49) was used as the mobile phase, with a flow rate of 1.0 mL min^{-1} and an injection volume of 10 μL. UV detection was performed at a wavelength of 365 nm, and the retention time was 8.9 min. No interface from other formulation components was observed. All samples were filtered through a 0.2 μm pore size membrane filter (0.2 μm nylon, Millipore Millex-GN) before injection. The obtained data were expressed as the average of triplicate determinations. Statistical analysis was performed using DAS software.

Complexation with β-CD. In the encapsulation experiment, ACADFE was first obtained from dried ACAD plant material. Three inclusion complexes with different ACADFE/β-CD molar ratios (1:1, 1:2, and 1:3 based on quercetin's M_r) were prepared by coprecipitation followed by subsequent freeze-drying. First, three dry ACADFE powders (0.5 g each) were dissolved in three solutions of 95% v/v ethanol, 20 mL each, and were subsequently suspended in 20, 40, and 50 mL of aqueous solution containing β-CD (1.2, 2.4, and 3.6 g; molar ratio based on M_r of quercetin and β-CD ≈ 1:1, 1:2, and 1:3; mass ratios 1:2.4, 1:4.8, and 1:7.2, M_r of quercetin and β-CD ≈ 1:3 was used for the experiments) at 60 °C. Next, these aqueous solutions were highly sheared for 2 min (FA 25 high cuts dispersible mulser, Germany) and stirred for 5 h in the dark at room temperature. After this process, these aqueous solutions were left in the dark for 12 h, and were filtered through a 0.45 μm PVDF filter (Chromafil P-45/25, Macherey-Nagel, Duren, Germany). The precipitate was collected and washed with 95% ethanol (to wipe off the ACADFE on the surface of inclusion) and dried under reduced pressure at 60 °C until the weight remained constant. The powder was stored under nitrogen in a gastight glass container at −40 °C until use. The aqueous soluble filtrate was freeze-dried (Telstar, Cryodos, Terrassa, Spain) at −40 °C. The freeze-dried inclusion powders were washed with 95% v/v ethanol followed by evaporation of the ethanol. The inclusion powder was stored under nitrogen in a gastight glass container at −40 °C until use.

Preparation of the Physical Mixture of ACADFE and β-CD. Dried ACADFE powder and β-CD were pulverized using a pestle and mixed together with a spatula until a homogeneous mixture was obtained.

HPLC of the Quercetin Content in the Inclusion Complexes and the Physical Mixture. The inclusion complexes were accurately weighed and dissolved by the addition of water (5 mL), and the quercetin content was subsequently extracted with methanol (5 mL) by sonication for 40 min. After centrifugation, the upper organic layer was filtered through 0.45 mm membrane filters, and 0.5 mL aliquots of the filtrate were transferred to HPLC.[11]

The quercetin encapsulation efficiencies of the ACADFE inclusion complexes were determined by HPLC and calculated as

$$\mathrm{EE}\,\% = \frac{W_{q(\mathrm{inclusion})}}{W_{q(\mathrm{ACADFE})}} \times 100$$

where EE% is the encapsulation efficiency, $W_{q(\mathrm{inclusion})}$ is the weight of quercetin in the inclusion complex, and $W_{q(\mathrm{ACADFE})}$ is the weight of quercetin in the dry ACADFE that was used for the preparation of the complex. Because the higher EE% was obtained for the complex prepared with a molar ratio of ACADFE/β-CD of 1:3 (based on the M_r of quercetin), that complex was used for the transdermal delivery experiments.

UV−Visible Spectroscopy. The UV−vis spectra of ACADFE solutions at pH 7.0 in the presence or absence of β-CD were recorded in the 200−400 nm wavelength range at 25 ± 1 °C (UV-8453 spectrophotometer, Agilent, USA). The concentration of ACADFE, which is dependent on quercetin content, was constant at 50 μM, whereas β-CD concentrations varied from 0 to 8 mM.[18] (Quercetin, quercetin-4′-*O*-β-D-glucopyranoside, and quercetin-3,4′-*O*-β-D-glucopyranoside dissolved completely in water or partially precipitated in the presence of β-CD.) In the spectra, the UV−vis absorption of ACADFE with β-CD from 0 to 8 mM, the β-CD physical mixture, and the inclusion complex were monitored.

Fourier Transform Infrared Spectroscopy (FT-IR). The FT-IR spectra of ACADFE, β-CD, the physical mixture, and the inclusion complex (prepared with a molar ratio of ACADFE/β-CD of 1:3, based on the M_r of quercetin) were collected between 4000 and 500 cm^{-1} on a FT-IR NEXUS spectrometer (Drook, Germany) with 254 nm scans at a resolution of 4 cm^{-1}. Each sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into a 1 mm pellet (2 mg of sample per 200 mg dry KBr). A blank KBr disk was

used as background. FT-IR spectra were smoothed, and the baseline was corrected automatically using the spectrophotometer's built-in software.

Thermogravimetric and Differential Thermal Analyses (TG and DTA). The thermal annealing process of the inclusion complex may be studied by methods of thermal analysis, specifically using simultaneous TG and DTA. DTA curves were obtained by using a TG/DTA analyzer. The measurements were performed under a dynamic atmosphere of dry nitrogen at a flow rate of 150 mL min^{-1} over a temperature interval of 0−425 °C at a heating rate of 10 °C min^{-1}. Aluminum crucibles were used to hold 5.0 ± 0.5 mg samples for analysis.[19]

In Vitro Skin Permeation Study. In vitro skin permeation studies were performed using a modified Franz-type diffusion cell (TP-5, China). The dorsal skins of excised hairless mice were chosen as models. The skin was mounted on the receptor compartment with the stratum corneum side facing upward into the donor compartment and the dermal side facing downward into the receptor compartment. Inclusion complex (prepared with a molar ratio of ACADFE/β-CD of 1:3, based on the M_r of quercetin, 2 mL, 0.012 mol L^{-1}) ACADFE (2 mL) and the physical mixture of ACADFE and β-CD (2 mL) were placed in the donor compartment (quercetin content was equivalent to inclusion complex), and 17 mL of physiological saline solution was placed in the receptor compartment. The contents of the receptor compartment were agitated at 300 rpm and placed over a multimagnetic stirrer (TP-5, China). The study was conducted at 37 ± 0.5 °C, and 2 mL samples were collected at predetermined time points (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, and 48 h) and replenished with physiological saline solution. The samples withdrawn from the receptor compartment were then analyzed by HPLC. Each data point represents the average of three separate determinations. The equation that accounts for the cumulating flux of the drug is

$$Q_n = \frac{1}{S}\left[C_n \times 17.0 + \sum_{i=1}^{n-1} C_i \times 2.0\right]$$

where n is the number of times samples were drawn, Q_n is the cumulative flux of the drug, C_n is the concentration of drug in the receptor chamber, and S is the effective surface area.

Transdermal Drug Delivery in Vivo. Hairless mice were obtained from the Experimental Animal Centre of Medical Sciences of Jilin University and maintained at 25 °C for the study. Necessary approval was obtained to conduct this study. Animals were fasted for 24 h prior to drug formulation administration but had free access to water. Quercetin's pharmacokinetic parameters from the ACADFE inclusion complex (prepared with a molar ratio of ACADFE/β-CD of 1:3, based on the M_r of quercetin) were compared with ACADFE alone and the mixture of ACADFE and β-CD. These solutions were prepared by suspending each sample in water. The animals were divided into three groups, each consisting of 15 mice. To one group was applied 2 mL of the inclusion complex formulation (0.012 mol L^{-1}) to a limited area (2 cm^2) on the dorsal skin of each mouse. ACADFE (2 mL) and the physical mixture of ACADFE and β-CD (2 mL, quercetin contents were equivalent to inclusion complex) were administered to another two groups. Blood samples (1.5 mL) from the eyeball vein were collected at preset intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 30, and 48 h after the application of inclusion complex, ACADFE, and the ACADFE/β-CD mixture. Blood samples were not allowed to clot and were centrifuged for 10 min at 4000 rpm. The serum was separated and transferred into clean microcentrifuge tubes. The serum was stored at −20 °C until HPLC analysis, where the amount of quercetin in the samples was estimated. Conjugated quercetin metabolites were analyzed following the enzymatic hydrolysis of quercetin conjugates in plasma using β-glucuronidase and sulfatase.[20] Concurrently, the mice were sacrificed. The formulation-exposed skin areas were removed from the animals for confocal scanning laser microscopy analysis. These samples were frozen in liquid nitrogen, and cryosections were prepared perpendicular to the skin's surface using a cryomicrotome (Microm D-6900, Germany). The skin slices were then analyzed using a confocal scanning laser microscope (Leica TCS-SP2 SE, Manheim, Germany). Fluorescence signals were detected using the spectral detector set in the 400−600 nm range. The detector settings were kept constant for all images.

RESULTS AND DISCUSSION

Preparation of the Inclusion Complex and Encapsulation Efficiency. In recent years, inclusion complexes of β-CD have been successfully utilized to improve the solubility, chemical stability, and bioavailability of a number of poorly soluble compounds.[21]

The coprecipitation method is the most efficient and versatile technique that is used to prepare inclusion complexes. Tsai et al.[22] prepared the PAE/β-CD complex (PAE, paeonol, 2′-hydroxy-4′-methoxyacetophenone, the main active compound of the *Paeonia lactiflora* Pallas) by coprecipitation. Kalogeropoulos et al.[11] prepared the inclusion complex of *Hypericum perforatum* (HP) with β-CD by mixing its components in aqueous media and subsequently freeze-drying the solution. Purified methanolic extracts of HP are very flavonoid-rich and mainly consist of quercetin glucosides, catechins, and quercetin. Complexation of quercetin with β-CD by freeze-drying has been shown by recent studies[23] to increase significantly quercetin's water solubility. The inclusion complexes of quercetin with CDs also retain their antioxidant potential,[6] indicating that the inclusion complexes of quercetin with cyclodextrins retained their antioxidant potential.

In this study three inclusion complexes of ACADFE/β-CD were prepared (with molar ratios of 1:1, 1:2, and 1:3, based on the M_r of quercetin) by pulverizing β-CD and ACADFE (coprecipitation), followed by freeze-drying of the solution. Thus, the quercetin molecule had two opportunities to enter the β-CD cavity. In addition, in the case of the complex prepared with an ACADFE/β-CD 1:3 molar ratio, more cavities of β-CD were offered for quercetin. The EE% of this complex was measured to be 71.05 ± 3.16%. We used two methods in the experiment: the first is the coprecipitation method, the subsequent method is freeze-drying. We used both methods because the encapsulation efficiency of the quercetin inclusion was higher than with the coprecipitation method or freeze-drying method alone. For instance, the measured EE% of a HP/β-CD inclusion complex prepared with suspension in aqueous media and subsequent freeze-drying was determined to be 35%.[11] However, it is worth pointing out that, in that study, a mass ratio of HP:β-CD 1:4 (molar ratio based on the M_r of quercetin ≈1:1) was used for the preparation of the complex, whereas in our study, the complex was prepared in a mass ratio of ACADFE/β-CD 9:5 (molar ratio based on the M_r of quercetin ≈1:3). The EE% of the complex that was prepared with an ACADFE/β-CD 1:1 molar ratio (based on the M_r of quercetin) was measured to be 31.81 ± 2.58% and that of the ACADFE/β-CD 1:2 complex higher (47.05 ± 4.25%).

UV−Visible Spectroscopic Behavior. In this paper, the inclusion phenomena of ACADFE within the hydrophobic cavity of β-CD were studied using UV−vis spectroscopy by monitoring the variation of absorbance peak intensities at λ = 254 and 359 nm. The spectral curves (Figures 2 and 3) showed that in the presence of increasing β-CD concentrations, the intensity of the absorbance peaks of 50 μM ACADFE solution at λ = 254 and 359 nm decreases. In addition, the increasing molar ratios of β-CD in solution led to the attenuation of ACADFE absorption peaks at 254 and 356 nm, which suggests that the chromophore electrons of quercetin, quercetin-4′-*O*-β-

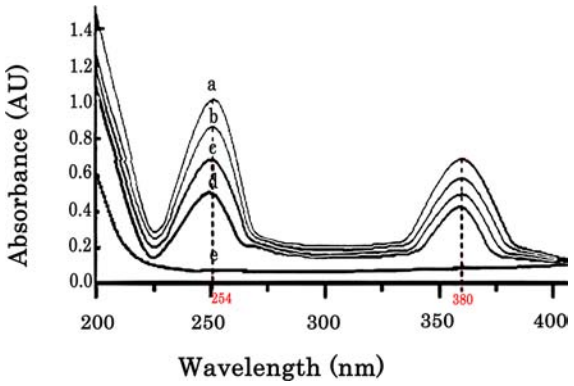

Figure 2. UV−vis spectra of 50 μM ACADFE solution in the presence of increasing concentrations of β-CD from 0 to 8 mM: (a) ACADFE; (b) ACADFE + 2 mM β-CD; (c) ACADFE + 4 mM β-CD; (d) ACADFE + 8 mM β-CD; (e) β-CD saturated solution.

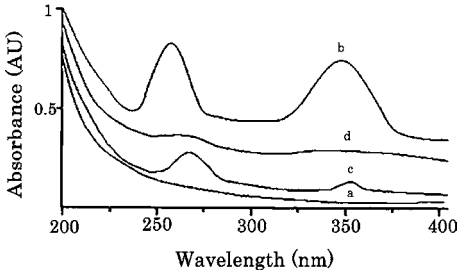

Figure 3. UV−vis spectra of β-CD saturated solution (a), ACADFE (b), physical mixture (c), and inclusion complex (d).

D-glucopyranoside, and quercetin-3,4′-*O*-β-D-glucopyranoside are covered by the β-CD cavities following inclusion complex formation. This observation was reinforced by the decay curve of a maximum absorption peak at 254 nm, which is another strong indication of inclusion complex formation. In addition, as depicted in Figure 3, in the inclusion complex the characteristic absorption peaks of ACDFE (quercetin) at 254 and 359 nm disappear, contrary to the physical mixture of β-CD and ACADFE.

FT-IR Analysis. Figure 4 shows the IR spectrum of the ACADFE/β-CD inclusion compound compared with the physical mixture, the ACADFE alonem and β-CD alone. The IR spectrum of β-CD (Figure 4a) displays the β-CD characteristic bands and is in agreement with literature data.[10] The intense band at 1652 cm^{-1} corresponds to the HOH bending of water molecules attached to β-CD; the 1419 cm^{-1} band corresponds to the CCH and OCH bending; the 1330 cm^{-1} band to the CCH, COH, and HCH bending; the 1250 cm^{-1} band to the OCH, COH, and CCH bending; the 1162 cm^{-1} band to the CO and CC stretching and to the COH bending; the 1028 cm^{-1} band to the CC stretching and to the CO and COH bending; the 947 cm^{-1} band to the skeletal vibration involving the α1,4 linkage; the 857 cm^{-1} band to CCH bending and CO and CC stretching; and the 574 cm^{-1} band to the skeletal vibration.

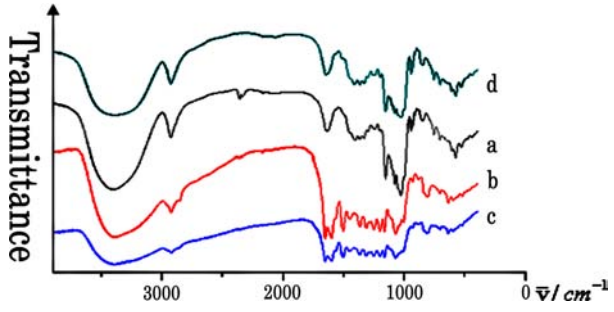

Figure 4. FT-IR spectra in the region from 800 to 4000 cm^{-1} of pure β-CD (a), ACADFE (b), physical mixture (c), and inclusion complex (d).

ACADFE (Figure 4b) has two characteristic bands at 1691 cm^{-1} corresponding to the C═O conjugated stretching. Specifically, for the free quercetin, this band appears at 1668 cm^{-1}; for ACADFE it was shifted to the higher 1691 cm^{-1} frequency. This 57 cm^{-1} displacement suggests that the coordination of quercetin to a broad range of flavonoids involves the oxygen atom of the carbonyl group. The 1620 cm^{-1} band corresponds to the aromatic nucleus. The 1595 cm^{-1} band corresponds to the stretching of the C═C double-bond of the ring conjugated with the carbonyl group (C═O); the 1515−1431 cm^{-1} bands are due to the C═C stretching of the ring; the 1328 cm^{-1} band corresponds to the O−H in-plane bending; the 1272 cm^{-1} band is attributed to the C═O stretching due to conjugation of the oxygen with the ring; and the band at 1172 cm^{-1} is characteristic of the carbonyl group. The sharp bands at 804 and 850 cm^{-1} are due to two adjacent hydrogen atoms on the phenyl ring of ACADFE.

The FT-IR spectrum of the physical mixture (Figure 4c) displays well-defined vibrational bands and is essentially the superposition of the main bands of ACADFE with those of β-CD.

In the IR spectrum of the ACADFE/β-CD inclusion complex (Figure 4d), the stretching vibration of quercetin aromatic ring of ACADFE appears at 1661 cm^{-1}, whereas the vibration of "free" β-CD appears at 1652 cm^{-1}. The result of the vibration showed the effect of placing the aromatic ring into the constrained environment of β-CD. There may also be a hydrogen-bonding interaction of ACADFE with β-CD hydroxyl groups. In the case of the characteristic C═O stretching band of the ACADFE within the inclusion compound, this band appears at the same frequency (1668 cm^{-1}), and its intensity shows little reduction when compared to the ACADFE complex alone (1634 cm^{-1}). The C═C aromatic stretching vibration appears in the same position as that observed for the nonincluded guest (1438 cm^{-1}), which is an indication that the structural integrity of the guest molecule is maintained. This result likely corresponds to an overlap of the band that is characteristic of quercetin within ACADFE stretching with the intense band corresponding to the β-CD skeletal vibration, located in the same region. This is most likely due to quercetin's orientation in the β-CD cavity for the ACADFE/β-CD inclusion complex. Moreover, according to these changes, we propose that quercetin's phenyl ring from ACADFE is involved in the inclusion process. It has been reported by NMR analysis and molecular modeling that quercetin's aromatic ring and ethylene side chain are embedded inside the cavity of β-CD, leaving more polar groups exposed outside the cavity.[24]

TG and DTA and Curve Analysis. Thermogravimetric curves were used to examine the thermal behavior of the inclusion complex. The thermal stability of a compound may differ depending on whether it is defined as a pure material, in solution, or in the form of an inclusion compound.[25] Figure 5a shows the TG/DTA curves of pure β-CD for comparison purposes. The decomposition of pure β-CD is well-

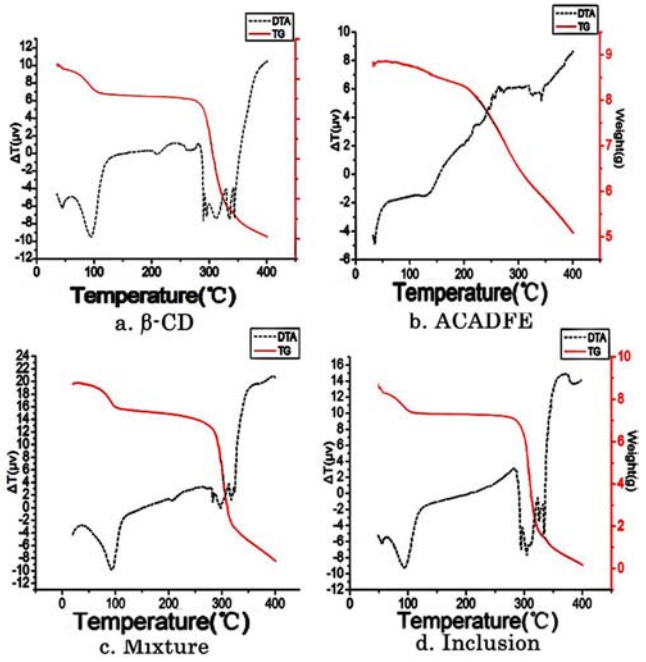

Figure 5. TG and DTA curves of β-CD (a), ACADFE (b), physical mixture (c), and inclusion complex (d).

documented, and the data obtained in this study are in good agreement with the reported literature.[11] Dehydration occurs in two steps for free β-CD, with a loss of approximately 8 mol of hydration water molecules (11.79%, 55−119 °C). The thermal decomposition of β-CD also occurs in one stage, with a rapid mass loss of 61.63%. Five endothermic peaks are observed in the thermal decomposition of β-CD. The first and second peaks (T_p = 41 °C, T_p = 93 °C) correspond to the dehydration of β-CD. Yilmaz et al.[26] claimed that due to the presence of a small endoderm, some of the water molecules included in the β-CD cavity escape below 64 °C, whereas the rest are released between 64 and 100 °C. The third and fourth peaks are two small, endothermic peaks without any weight loss between 200 and 260 °C that clearly represent a physical process, specifically being attributed to the molecular rearrangement of β-CD. The fifth peak is related to the degradation of the β-CD structure; decomposition begins at 272 °C, and rapid weight loss continues up to 359 °C, yielding approximately 61.63% total weight loss. However, Yilmaz et al.[26] reported that decomposition starts at 248 °C and that rapid weight loss continues up to 345 °C. The melting of β-CD also occurs in the fifth stage and, thus, the temperature range of 272−310 °C also corresponds to the endothermic changes of melting. From the measured curves (Figure 5b−d) and thermoanalytical results for ACADFE, the mixture, and the inclusion complex of ACADFE with β-CD, some general features can be recognized. Zhang et al.[19] reported that quercetin displayed two-stage mass loss, with the first mass loss corresponding to the loss of water at approximately 76−117 °C and the second mass loss corresponding to its decomposition at the range of 295−405 °C, with the total mass loss of 33.95%. By comparison with this work on ACADFE, the curve showed two-stage mass loss; the first dehydration occurred at a temperature range of 54−167 °C (24.52%), and the second mass loss occurred at a temperature range of 167−398 °C (11.73%), corresponding to the decomposition of ACADFE. Dias et al.[10] reported that the dehydration process occurs in two steps for quercetin-β-CD: first, 2 mol of water are lost (9%), and second, 1 mol of water is lost (4%). The split dehydration process of the inclusion compound shows that the water molecules occupy different positions within the structure. The endothermic peaks observed in the DTA curve of the inclusion compound are consistent with the TG curve, although the compound's thermal stability is slightly decreased in comparison with that of the host molecule (114−240 °C). However, in this study, the dehydration of the inclusion complex is completed at much lower temperatures (12.19%, 29−121 °C) than the dehydration of pure β-CD (11.79%, 55−119 °C). During the inclusion process, most of the water molecules in the β-CD cavity are replaced by ACADFE, which indicates that the inclusion of ACADFE into the β-CD cavity results in bond weakening between the remaining water molecules and β-CD so that their liberation requires much less energy, thus corresponding to lower temperatures. After the dehydration stage, the decomposition of the inclusion complex occurs in one stage, with a rapid mass loss of 61.63%. DTA and TG curves of the inclusion complex showed that thermal stability is greatly increased in comparison to ACADFE alone.

In Vitro Skin Permeation Study. The cumulative amounts of quercetin from a physiological saline solution in the receptor medium are shown at different time points in Figure 6. The

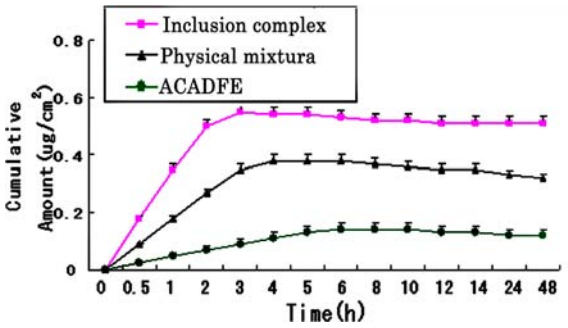

Figure 6. In vitro permeation−time profiles of quercetin from inclusion complex, physical mixture, and ACADFE (mean ± SD; n = 5).

inclusion complex and the physical mixture exhibited faster penetration accumulation in the receptor compared to ACADFE alone. This phenomenon indicates that the percutaneous penetration enhancement of ACADFE may be achieved by using β-CD. A rapid drug permeation rate was observed for solutions containing the drug complex and physical mixture, where >50% of the drug permeated the skin within the first 1 h. Klang et al.[27] reported that a 10-fold increase in applied dose from 5 to 50 mg cm^{-2} and from 50 to 500 mg cm^{-2} was clearly reflected in the permeated drug amounts and fluxes (p < 0.05) in both systems containing additional β-CD. Concurrently, the addition of CD magnified the increase in permeation with increasing doses. Therefore, β-CD enhances penetration by increasing drug solubility and reducing skin barrier function, presumably by interacting with stratumcorneum lipids.[28] Furthermore, the inclusion complex displayed controlled release during transdermal transport. These findings could be exploited to modulate the ACADFE release rate, depending on the need either for rapid drug release (e.g., for acute treatments) or for more prolonged drug release (e.g., for a long-term therapy), with the final aim to improve clinical efficacy.[29]

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Pharmacokinetic Studies. Mean quercetin concentration−time curves in plasma from the inclusion complex,

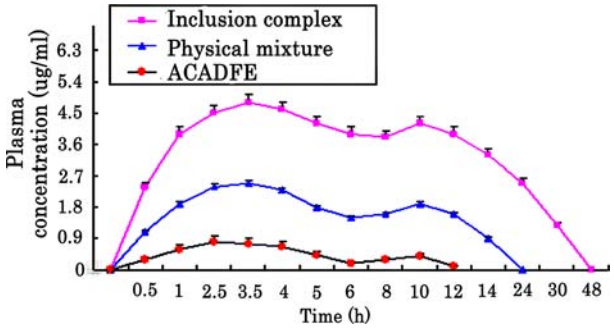

Figure 7. In vivo plasma concentration−time profiles of quercetin from inclusion complex, physical mixture, and ACADFE (mean ± SD; n = 5).

ACADFE alone, and the physical mixture are shown in Figure 7 and were calculated as follows:

$$\overline{C} = \frac{\sum_i^n C_1 + C_2 + \ldots + C_n}{n}$$

i = 1,2, ..., n;n = 5; and $\overline{C}$ = mean quercetin concentration in plasma. The peak concentrations of quercetin in plasma from ACADFE occurred at 5.1 h, whereas those from the inclusion complex and from the physical mixture occurred at 3.5 h. These results indicate that the inclusion complex and the physical mixture were absorbed more rapidly than ACADFE due to the penetration enhancement by β-CD. Interestingly, a weak double peak was observed in the quercetin concentration−time (C−T) curve. It should be noted that all three quercetin C−T curves exhibited the double-peak phenomenon in this study, suggesting strong enterohepatic recirculation.[30] Using DAS software, a two-compartment model was selected for pharmacokinetic modeling, as this model fits the data obtained from mice. The pharmacokinetic parameters of quercetin from the inclusion complex, ACADFE alone, and the physical mixture in mice are presented in Table 1. These results also suggest that the β-CD inclusion technique could be considered as a novel method to enhance the transdermal delivery of ACADFE.

Transdermal Transport Mechanism. The penetration pathway of ACADFE within skin samples was studied using confocal laser scanning microscopy. Representative fluorescence images of ACADFE in skin samples are shown in Figure 8, and the pathway of ACADFE penetration into the skin was examined. In the first 0.5 h, the fluorescent signal of ACADFE

Table 1. Pharmacokinetic Parameters of Quercetin from Inclusion Complex, ACADFE, and Physical Mixture

parameter	inclusion complex	ACADFE	ACADFE + β-CD
$t_{1/2(\beta)}$ (h)	2.67 ± 1.11	1.98 ± 0.78	2.16 ± 1.08
$t_{1/2(\alpha)}$ (h)	0.27 ± 0.09	0.33 ± 0.12	0.31 ± 0.05
T_{max} (h)	3.56 ± 0.97	5.1 ± 1.23	3.41 ± 1.08
C_{max} (μg mL^{-1})	0.23 ± 0.02	0.09 ± 0.01	0.31 ± 0.07
$AUC_{0\to t}$ (μg h mL^{-1})	0.78 ± 0.24	0.41 ± 0.08	0.65 ± 0.13
$AUC_{0\to\infty}$ (μg h mL^{-1})	0.79 ± 0.42	0.42 ± 0.07	0.65 ± 0.22
MRT (h)	230.7 ± 5.19	5.98 ± 1.21	7.59 ± 2.34

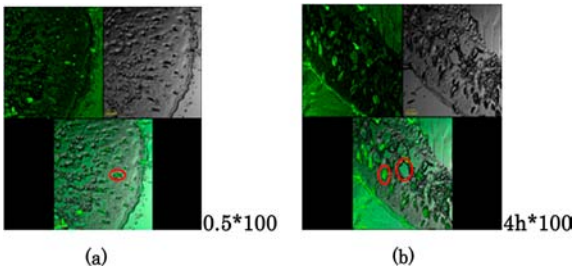

Figure 8. Fluorescent images of the skin penetration after 0.5 h (a) and 4 h (b).

was predominantly located around the follicular region (Figure 8a). At 4 h (Figure 8b), the fluorescent signal of ACADFE indicated that penetration into the skin proceeded via two pathways: follicular and transcellular. In cellular membranes, flavonoids can influence the appearance and development of rafts and raft-like membrane domains and thus influence the lateral diffusion of lipid molecules.[31] On the basis of these results, we conclude that inclusion complex penetration proceeds into the skin via follicular and transcellular routes.

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Notes

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