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Article

Improvement of the Solubility and Evaluation of the Physical Properties of an Inclusion Complex Formed by a New Ferulic Acid Derivative and γ-Cyclodextrin

Nao Ikeda, Yutaka Inoue,* Yuka Ogata, Isamu Murata, Xuan Meiyan, Jun Takayama, Takeshi Sakamoto, Mari Okazaki, and Ikuo Kanamoto

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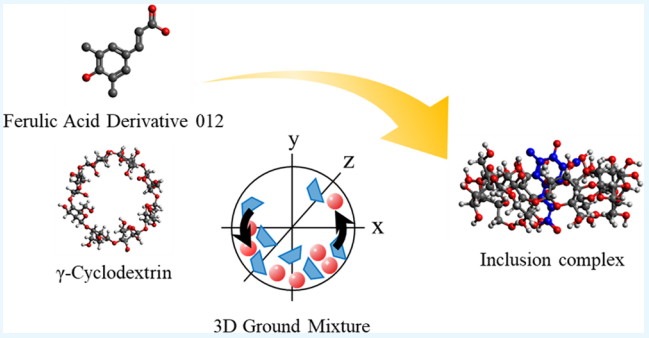
ABSTRACT: Ferulic acid derivative 012 (FAD012) is a ferulic acid (FA) derivative. The current study prepared a solid dispersion of FAD012 and γ-cyclodextrin (γCD) and ground it using a three-dimensional ball mill (3DGM) to prepare an inclusion complex. This study also assessed the physicochemical properties such as solubility of that complex. A Job's plot indicated that FAD012 and γCD formed an inclusion complex at a molar ratio of 1:1. Phase solubility diagrams revealed that FAD012 produced a B_S diagram. According to PXRD, FAD012 produced a diffraction peak at 2θ = 7.0° and γCD produced a diffraction peak at 2θ = 9.1°. Those two peaks were not produced by the 3DGM, but new peaks (2θ = 7.3 and 16.5°) were evident. DSC patterns revealed an endothermic peak due to the melting of FAD012 at 190 °C, but no endothermic peaks were evident with the 3DGM. NIR spectra of the 3DGM indicated that the methyl group of FAD012 produced a higher peak and that the OH groups of γCD produced a higher peak. ^{1}H-^{1}H ROESY NMR spectra (D_2O) revealed cross peaks for protons of the methyl group of FAD012 and a proton (H-3) in the cavity of γCD, so FAD012 presumably interacts with the wide opening of the γCD torus. A solubility test (25 °C) indicated that solubility improved about 5-fold for the 3DGM in comparison to the solubility of FAD012 alone (about 140 μg/mL). Based on these findings, an FAD012/γCD complex was formed by cogrinding, and its solubility improved. These observations are expected to expand the usefulness of cogrinding of FAD012 with γCD using a 3D ball mill.



INTRODUCTION

Cerebrovascular disorders include intracerebral hemorrhage, subarachnoid hemorrhage, and cerebral infarction. Cerebral infarction is the most serious of those conditions because it accounts for most deaths. Drug therapies for cerebral infarction include intravenous thrombolysis (tissue plasminogen activator (t-PA) therapy), neuroprotective therapy, and antiplatelet therapy. However, only certain patients can receive drug therapy because it must be received within 4.5 h of onset, and drugs can cause adverse reactions such as bleeding and kidney damage, so existing drug therapies have disadvantages. A study some years ago examined the neuroprotective action of ferulic acid (FA), a natural antioxidant.[1]

FA is a hydroxycinnamic acid prepared from rice bran. FA has a high antioxidant capacity[2] and ultraviolet-absorbing action, which is why it is used as a sunscreen,[3] as a food additive to prevent lipid peroxidation,[4] and as a supplement with brain-boosting action.[1] However, FA is water-soluble, which is why it has difficulty reaching the brain. For reference, the solubility of FA is reported as 780 μg/mL.[5] Thus, FA was molecularly engineered to produce FA derivative 012 (FAD012) with increased fat solubility and improved ability to traverse the central nervous system.[6] FAD012 has a chemical structure where a methyl group is substituted for the methoxy group in the aromatic ring of FA, and FAD012 is more highly fat-soluble and poorly soluble in water than FA, so FAD012 presumably has drawbacks limiting its full efficacy. Our research group has already succeeded in synthesizing FA derivatives other than FAD012 and is finding pharmacological effects on cerebral circulation.[7] Among them, FAD012 is a compound that is of interest for its pharmacological effects, but in view of its administration to animals, its solubility is poor; then, it is thought that a pharmaceutical approach is necessary. Accordingly, if the solubility of FAD012 could be improved

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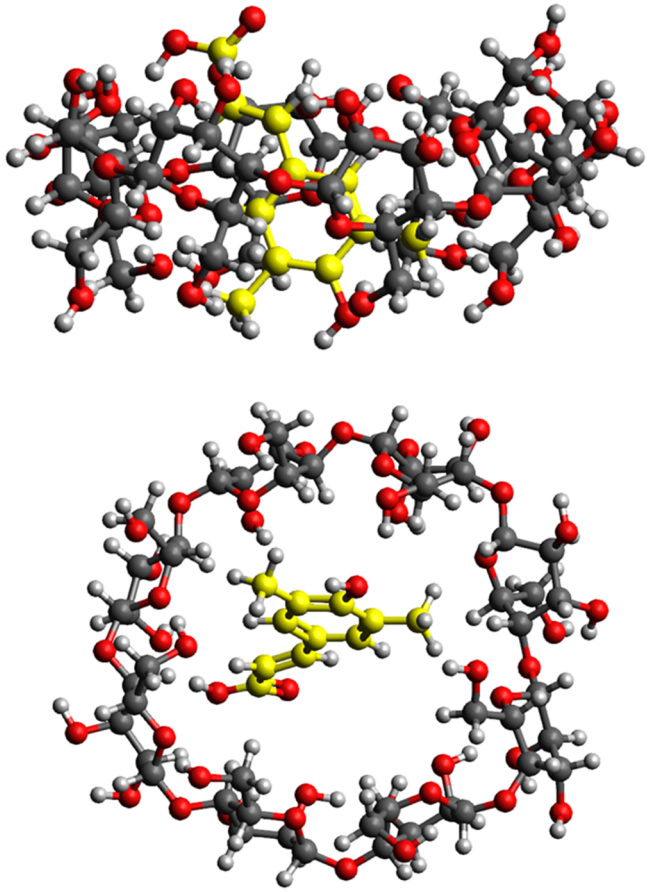
and a preparation retaining the antioxidative action could be devised, then FAD012 might serve as an active pharmaceutical ingredient that would be efficacious in treating cerebral infarction.

A cyclic polysaccharide, cyclodextrin (CD), has glucose pyranose units linked with α-1,4-glucosidic bonds. CD with six glucose pyranose units is known as αCD, CD with seven such units is known as βCD, and CD with eight such units is known as γCD. The exterior of the ring of CD is hydrophilic while the inside of the cavity is hydrophobic, so various hydrophobic guest molecules are included in the cavity as a result of hydrophobic interaction, leading to the formation of inclusion complexes.[8] γCD is an easily digestible water-soluble substance, and its acceptable daily intake is "not specified" according to the Joint FAO/WHO Expert Committee on Food Additives, meaning γCD has very low toxicity. Various methods have been used to prepare inclusion complexes, such as coprecipitation,[9] kneading,[10] freeze-drying,[11] and cogrinding.[12] Cogrinding involves applying mechanical energy in the form of friction, grinding, etc., to a solid substance and changing its physicochemical properties; this mechanochemical technique forms inclusion complexes without needing a solvent.[13] The current authors previously used cogrinding to form caffeic acid (CA) and CD inclusion complexes, improving the elution of CA and retaining its antioxidant capacity.[14] FA is known to form inclusion complexes with CDs.[15,16] The physicochemical evaluation of the complex and the molecular interaction between FA and CDs in the inclusion state have been reported.[17,18] In other words, since FA forms a complex with CD, in this study, we attempted to prepare an inclusion complex with γCD using FAD012.

Cogrinding techniques include the use of a rod mill, ball mill, or jet mill. CA/γCD inclusion complexes have been prepared by grinding with a rod mill,[19] foods and teas have been pulverized with a ball mill,[20] and a jet mill has been used to grind bulk pharmaceuticals.[21] Over the past few years, cogrinding using a three-dimensional (3D) ball mill has been developed as a new grinding technique. A 3D ball mill has a vertical and a horizontal axis of rotation, and it uses the entire inner surface of a spherical container, limiting the heat of friction and uneven mixing, which are drawbacks of a two-dimensional ball mill. A 3D ball mill allows highly uninform grinding and mixing in a short period of time.[22] The current authors previously used a 3D ball mill to grind daidzein, a soybean constituent, to form a daidzein/γCD complex, thus improving the solubility of daidzein.[23] Accordingly, if an FAD012/γCD complex (Scheme 1) could be prepared with a new cogrinding technique using a 3D ball mill, then the functionality of FAD012 should be retained and its solubility should improve. If the FAD012/γCD complex could be prepared by a 3D ball mill, it could be proposed as a novel preparation method. Furthermore, if the physicochemical properties of the FAD012/γCD complex can be elucidated and γCD contributes to solubility improvement as an additive, the dosage form will be changed from an oral formulation to a parenteral formulation such as an injection or an ointment, and it can be considered as a research result that is the basis for expanding the administration routes such as mucosal and transdermal absorption.

Thus, the current study used a 3D ball mill to grind a mixture of FAD012 and γCD in order to prepare an inclusion complex, and this study assessed the physicochemical properties such as solubility of that complex.

Scheme 1. Proposed Structural Images of FAD012/γCD Complexes



RESULTS AND DISCUSSION

Determination of Complexation Stoichiometry. A Job's plot was prepared in order to determine the ratio of FAD012 and γCD in a complex. Job's method (the method of continuous variation)[24] is a method of determining the ratio of components in a complex based on the additive nature of changes in absorbance due to changes in physical properties during complex formation. A plot of the changes in the absorbance of FAD012 when γCD was added is shown in Figure 1. The addition of γCD resulted in the largest changes in absorbance when the ratio of FAD012/γCD was 0.5 (result of the approximate curve equation: $y = -0.0933x^2 + 0.0882x - 0.005$ ($x \approx 0.472$), so in solution, the ratio of FAD012 and γCD in the complex is presumably 1:1.[25] Since the Job's plot indicated that the molar ratio of FAD012/γCD was 1:1, the current study used a 3D ball mill to prepare a mixture at that molar ratio, and this study assessed the physicochemical properties of that complex.

Phase Solubility Studies. Phase solubility diagrams were prepared in order to determine the molar ratio and stability constant for the FAD012/γCD inclusion complex. After shaking for 24 h, the solubility of intact FAD012 was 116 μg/mL. Phase solubility diagrams indicated that the solubility of FAD012 increased as the concentration of γCD increased. At high concentrations of CD, the concentration of FAD012 decreased due to the precipitation of a solid complex, producing a B_S diagram according to the classification of Higuchi and Connors (Figure 2). Rodrigues Sá Couto et al.

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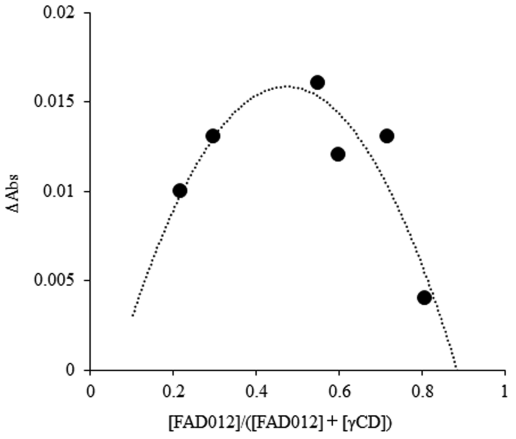

Figure 1. Job's plot of the FAD012/γCD systems. Results were expressed as mean ± SD (n = 3).

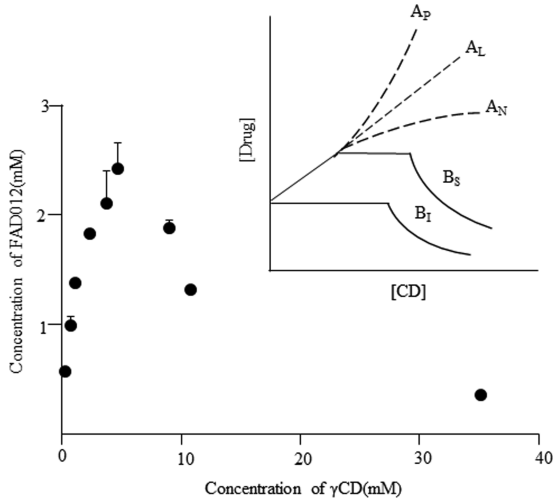

Figure 2. Phase solubility diagrams of FAD012/γCD. Results were expressed as mean ± SD (n = 3).

reported that carbamazepine /γCD produced a B_S diagram.[26] When the stability constant (K_s) was calculated using eq 1, the stability constant for FAD012 was 782.9 M^{-1}. When the complexation efficiency (CE) was calculated using eq 2, it was 0.62. In addition, the slope of the phase solubility diagram was smaller than 1.0, so FAD012 and γCD presumably form an inclusion complex at a molar ratio of 1:1.

PXRD. Job's plot and phase solubility diagrams suggested that in an aqueous solution, FAD012 is included in γCD at a molar ratio of 1:1. Formation of a complex between an inorganic compound and an organic compound or a complex between organic compounds causes a change in the crystal structure of the drug. Therefore, powder X-ray diffraction (PXRD) measurement is useful for confirming changes in the crystal structure in the solid state.[27] Thus, PXRD was performed in order to examine the crystalline state of the 3DGM (FAD012/γCD) prepared by cogrinding using a 3D ball mill. Intact FAD012 produced characteristic diffraction peaks due to FAD012 at 2θ = 7.0 and 11.5°, and γCD produced a characteristic diffraction peak due to γCD at 2θ = 9.1° (Figure 3a, b). The physical mixture (PM) (FAD012/γCD = 1:1) produced a diffraction peak due to FAD012 at 2θ = 7.2° and a diffraction peak due to γCD at 2θ = 9.1° (Figure 3c). With the 3DGM with water (3DGMw) (FAD012/γCD =

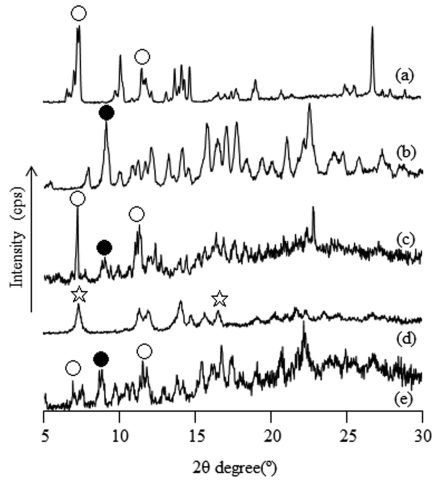

Figure 3. PXRD patterns of FAD012/γCD:(a) intact FAD012, (b) intact γCD, (c) PM (FAD012/γCD = 1:1), (d) 3DGMw (FAD012/γCD = 1:1), and (e) 3DGMnw (FAD012/γCD = 1:1).

1:1), the diffraction peak due to FAD012 disappeared and a new diffraction peak was produced at 2θ = 16.5° (Figure 3d). The 3DGM with no water (3DGMnw) produced diffraction peaks due to FAD012 at 2θ = 6.9 and 11.5° and a diffraction peak due to γCD at 2θ = 8.8° (Figure 3e).

These findings suggest that an FAD012/γCD complex was formed in the 3DGMw, which was prepared using a 3D ball mill and which had water added. A study used a 3D ball mill to prepare a ground mixture of daidzein/γCD, and it reported the usefulness of that 3D ball mill.[23] When a substance is ground using a 3D ball mill without adding distilled water, it is not sufficiently dispersed and mixed, so it is not readily included. When a substance is ground using a 3D ball mill and distilled water is added, however, moisture is removed from γCD, so FAD012 is presumably able to readily enter the cavity of γCD to form an inclusion complex.

DSC. PXRD patterns suggested that an inclusion complex was formed in the 3DGMw (FAD012/γCD). A study has reported that changes in the thermal behavior are evident when formation of an inclusion complex causes a disappearance or shifting of the melting point of the guest molecule.[28] Thus, DSC was performed in order to examine the thermal behavior of the 3DGMw (FAD012/γCD) prepared using a 3D ball mill. DSC patterns indicated that intact FAD012 produced an endothermic peak due to melting at around 200 °C (Figure 4a). The PM (FAD012/γCD) produced an endothermic peak due to the melting of FAD012 at around 190 °C, so crystals of FAD012 are presumably present (Figure 4c). With the 3DGMw (FAD012/γCD), the endothermic peak due to FAD012 disappeared (Figure 4d). These findings indicate that an inclusion complex is formed in the 3DGMw (FAD012/γCD), causing FAD012 to be included in the cavity of γCD. This presumably explains the disappearance of the endothermic peak due to FAD012.

FT-IR Spectroscopy. DSC and PXRD patterns suggested that an inclusion complex may be formed in the 3DGMw (FAD012/γCD). IR measurement is a useful technique for collecting information for understanding intramolecular interactions and intermolecular interactions of drugs. In particular, when a hydrogen bond or the like contributes as an intermolecular interaction between drugs, a peak shift of a functional group such as a hydroxyl group or a carbonyl group

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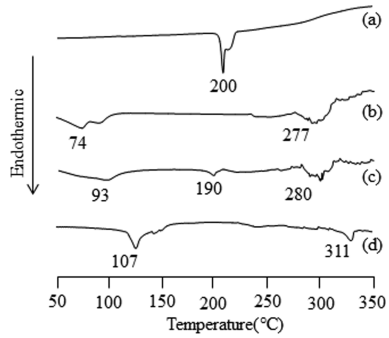

Figure 4. DSC curves of FAD012/γCD systems: (a) intact FAD012, (b) intact γCD, (c) PM (FAD012/γCD = 1:1), and (d) 3DGMw (FAD012/γCD = 1:1).

occurs.[29,30] Thus, FT-IR spectroscopy was performed in order to examine the molecular state of the complex in the solid state. Intact FAD012 produced a peak due to the aromatic ring (benzene ring) at 1600 cm^{-1}, a peak due to the carbonyl group at 1653 cm^{-1}, a peak due to the alkyl groups at 2566−3023 cm^{-1}, and a peak due to hydroxyl groups at 3378 cm^{-1} (Figure 5a). Intact γCD produced a peak due to hydroxyl groups at

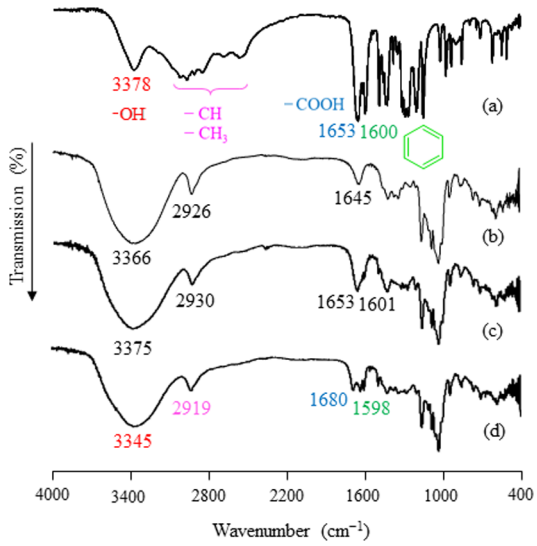

Figure 5. FT-IR spectra of FAD012/γCD systems: (a) intact FAD012, (b) intact γCD, (c) PM (FAD012/γCD = 1:1), and (d) 3DGMw (FAD012/γCD = 1:1).

3366 cm^{-1} (Figure 5b). With the 3DGMw (FAD012/γCD), the aromatic ring (benzene ring) in FAD012 produced a lower peak at 1598 cm^{-1}, the carbonyl group produced a higher peak at 1680 cm^{-1}, alkyl groups produced a lower peak at 2919 cm^{-1}, and hydroxyl groups produced a lower peak at 3345 cm^{-1} (Figure 5d) in comparison to intact FAD012. Higashi et al. reported that hydrogen bonds between molecules with a dimeric structure dissociated as a result of complex formation, producing a higher peak due to a carbonyl group.[31] These findings presumably indicate that the FAD012 dimer in the 3DGMw (FAD012/γCD) dissociated in the solid state and that dissociated FAD012 presumably interacted with the cavity of γCD via hydrogen bonds. In other words, FAD012 presumably enters the cavity of γCD, forming an inclusion complex.

NIR Spectroscopy. FT-IR spectra of the 3DGMw (FAD012/γCD) presumably indicated molecular interaction between the hydroxyl groups of FAD012 and γCD and the alkyl group of FAD012 in the solid state. However, the FT-IR spectra were not able to attribute that interaction to hydroxyl groups or the alkyl group. Thus, NIR spectroscopy was performed with a focus on the hydroxyl groups and the alkyl group. Intact FAD012 produced peaks due to C−H bonds in the aromatic ring (benzene ring) in FAD012 at 4388 and 8684 cm^{-1}, and it produced peaks due to the alkyl group at 4320 and 8356 cm^{-1} (Figure 6bX,Z). In addition, γCD produced a peak due to its hydroxyl groups at 4772 cm^{-1} and a peak due to moisture at 5240 cm^{-1} (Figure 6bY). With the 3DGMw (FAD012/γCD), the peaks due to the C−H bonds in the benzene ring of FAD012 shifted to 4412 and 8652 cm^{-1}, and the peaks due to the alkyl group shifted to 4356 and 8348 cm^{-1} in comparison to intact FAD012. In addition, the hydroxyl groups of γCD produced a higher peak at 4796 cm^{-1}, and the peak due to moisture in γCD broadened. When peaks in the NIR spectra broaden or shift, the functional groups of drugs are interacting.[32] These findings for the 3DGMw (FAD012/γCD) presumably indicate molecular interaction in the solid state due to the cavity of γCD and hydrogen bonds with FAD012.

SEM. DSC and PXRD patterns suggested that a complex is formed in the 3DGMw (FAD012/γCD). SEM and TEM observations are useful as methods for confirming the drug shape. Then, combined with information such as SEM and XRD, it is possible to consider the characteristics of such a complex.[33] In addition, PXRD patterns indicated that the crystalline state of the complex changes. Thus, SEM was performed in order to observe the shape and surface of crystals. FA012 crystals were smooth needles of about 500 μm in size (Figure 7a). γCD crystals were angular with a smooth surface, and particles were about 50 μm in size (Figure 7b). Changes in the surface of particles were not noted in the PM (FAD012/γCD) (Figure 7c). In the 3DGMw (FAD012/γCD), particles were about 300 μm in size, and angular crystals with a smooth surface were evident (Figure 7d). Typically, an inclusion complex with γCD is cubical.[34] The current study similarly found cubical crystals in the 3DGMw (FAD012/γCD), suggesting that FAD012 and γCD may form an inclusion complex in the solid state.

Measurement of ^{1}H-^{1}H ROESY NMR Spectra. ^{1}H-^{1}H ROESY NMR spectroscopy was performed in order to examine the detailed molecular interaction in the 3DGMw (FAD012/γCD) in solution. ^{1}H-^{1}H ROESY NMR spectroscopy can reveal interaction between a guest molecule and the cavity of CD, so it is used to estimate the relative position of the inclusion complex. With the 3DGMw (FAD012/γCD), there was a cross peak between H-3 (3.7 ppm) inside the cavity of γCD and H-b (6.6 ppm), H-c (7.1 ppm), and H-d (2.0 ppm) of FAD012 (Figure 8a). In addition, there was a cross peak between H-5 (3.6 ppm) inside the cavity of γCD and H-a (2.0 ppm), H-b (6.6 ppm), and H-c (7.1 ppm) of FAD012 (Figure 8b). Typically, H-3 is a proton in the wide aperture of the CD ring while H-6 is a proton in the narrow aperture of the CD ring.[35] These findings presumably indicate that the carbonyl group in the FAD012 molecule is included in γCD from the wide to the narrow opening of its torus.

Solubility of FAD012/γCD Systems in Distilled Water. Assessment of physical properties in the solid state indicated that an inclusion complex was formed in the 3DGMw

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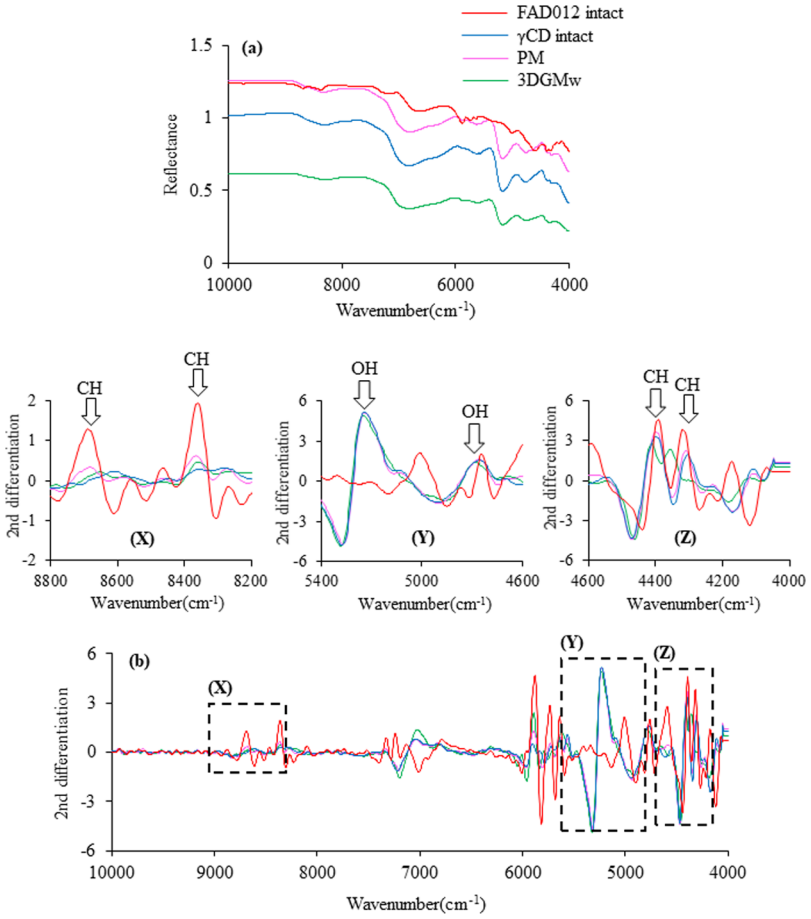

Figure 6. (a) NIR absorption spectra of FAD012/γCD systems observed at 4000−10,000 cm^{-1}. (b) NIR absorption spectra of FAD012/γCD systems: (X) second differential near-infrared absorption spectra of FAD012/γCD observed at 8200−8800 cm^{-1}; (Y) second differential near-infrared absorption spectra of FAD012/γCD observed at 4600−5400 cm^{-1}; (Z) second differential near-infrared absorption spectra of FAD012/γCD observed at 4000−4600 cm^{-1}.

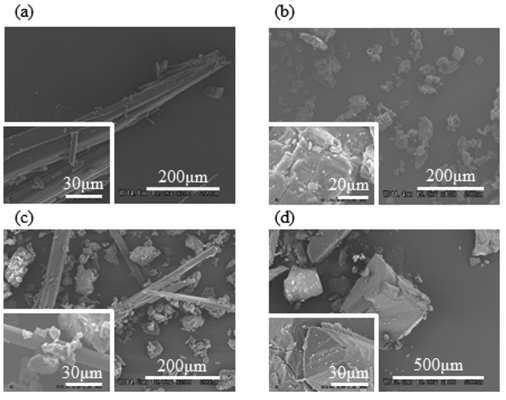

Figure 7. SEM morphology of FAD012/γCD systems: (a) intact FAD012, (b) intact γCD, (c) PM (FAD012/γCD = 1:1), and (d) 3DGMw (FAD012/γCD = 1:1).

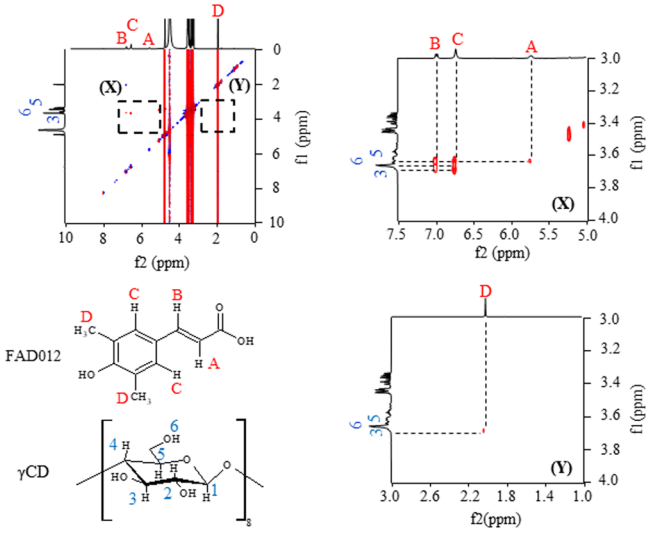

Figure 8. ^{1}H-^{1}H ROESY NMR spectra of FAD012/γCD systems.

(FAD012/γCD) at a molar ratio of 1:1. Thus, a solubility test was performed in order to determine whether the solubility of FAD012 improved as a result of molecular interaction between FAD012 and γCD (Table 1). One hour after the start of the test, the solubility of intact FAD012 was 134.4 μg/mL while solubility in the 3DGMw (FAD012/γCD) was 656.7 μg/mL, indicating that solubility increased about 5-fold. This is presumably due to molecular interaction as a result of the formation of an FAD012 and γCD inclusion complex by cogrinding using a 3D ball mill. In other words, 3DGM has a high-speed 3D rotation that has achieved noncriticality, and it is possible to contribute to a new drug formulation research by enabling a nonaggregated and highly uniform ground mixture. As a formulation design of FAD012, poorly water-soluble

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Table 1. Solubility of FAD012/γCD Systems in Distilled Water (25 ± 0.5 °C)[a]

	solubility (μg/mL)			
sample	1 h	3 h	6 h	24 h
FAD012 intact	134.4 ± 43.9	151.3 ± 65.1	152.8 ± 60.7	149.7 ± 57.0
3DGMw (FAD012/γCD)	656.7 ± 19.7	753.7 ± 6.1	854.7 ± 85.7	840.2 ± 77.3

[a]Results were expressed as mean ± SD (n = 3).

FAD012 formed an inclusion complex with γCD, and the solubility was improved by changing the dosage form from an oral preparation to a parenteral injection or ointment; it can contribute to the expansion of administration routes such as transdermal absorption. In the future, it is necessary to conduct in vivo tests for the purpose of using FAD012.

CONCLUSIONS

The current study prepared an FAD012/γCD inclusion complex by cogrinding using a 3D ball mill unlike the previously reported methods. In the 3DGMw (FAD012/γCD), the carbonyl group of the FAD012 molecule was included in γCD. The 3DGMw (FAD012/γCD) had improved solubility, which was found to have contributed to the formation of an inclusion complex. Based on these results, cogrinding using a 3D ball mill should be useful and the use of FAD012 is expected to expand.

αCD and βCD have different numbers of bonds. In the future, differences in forms of inclusion in αCD and βCD need to be determined.

MATERIALS AND METHODS

Materials. FAD012 (Figure 9a) was purified by the Josai University Pharmaceutical Chemistry Laboratory. γCD (Figure 9b) was provided by Cyclo Chem Co., Ltd. (Tokyo, Japan) and was stored at a temperature of 40 °C and 82% relative humidity for 7 days. 2,2-Diphenyl-1-picrylhydrazyl was obtained from Sigma-Aldrich. Other chemicals were of special grade and were purchased from Fujifilm Wako Pure Chemical Corporation, Tokyo.

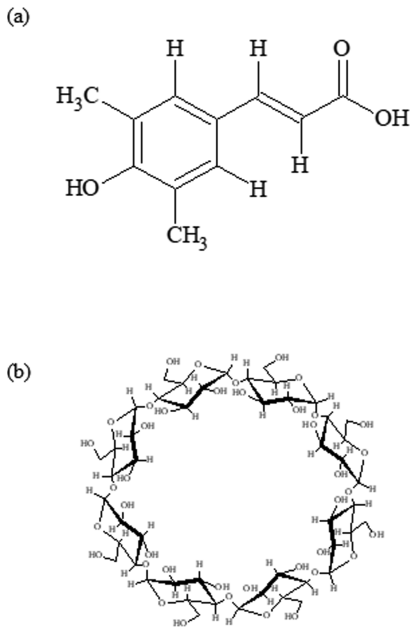

Figure 9. Chemical structures of (a) FAD012 and (b) γCD.

Preparation of a Physical Mixture and a Ground Mixture. The physical mixture (PM) was prepared by mixing FAD012 and γCD at a molar ratio of 1:1 using a vortex mixer for 1 min. The ground mixture (3DGM) of FAD012 and γCD was prepared by grinding the PM (FAD012/γCD) (500 mg total) using a 3D ball mill with a 200 g ball of Φ5 mm for 60 min, and 300 μL of water was added (3DGMw) or not (3DGMnw).

Methods. *Determination of Complexation Stoichiometry.* The molar ratio of inclusion of FAD012 and γCD was determined according to Job's method (the method of continuous variation).[24] The ratio of FAD012/γCD concentrations was changed from 0.1 to 0.9, and the change in absorbance was plotted. After the test solution reached equilibrium, the sample was filtered with a 0.45 μm membrane filter. Absorbance was measured at a wavelength of 308 nm using an ultraviolet visible spectral photometer (UV-2500PC, Shimadzu Corporation).

Phase Solubility Study. Phase solubility studies were performed according to the method of Higuchi and Connors.[36] A supersaturated amount of FAD012 was added to an aqueous solution (10 mL) with a concentration of γCD (0−35 mM). A suspension was obtained via shaking for 24 h (25 ± 0.5 °C) at 100 rpm using a constant temperature shaker (BR42FL, Taitec Co., Ltd.). Once the suspension reached equilibrium, it was filtered through a 0.45 μm membrane filter (hydrophilic poly(tetrafluoroethylene) filter, DISMIC), and the solution was quantified. The apparent stability constant (K_s) of the FAD012/γCD complex was calculated using eq 1 based on the slope of the solubility phase diagram and the solubility (S_0) of FAD012 in the absence of γCD.

$$K_s = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

$$\text{CE} = S_0 \times K_s = \text{slope}/(1 - \text{slope}) \quad (2)$$

Quantitative of FAD012 with HPLC. Solubility was quantified using a high-performance liquid chromatograph (HPLC: LC-20ADvp, Shimadzu Corporation) at a wavelength of 308 nm. The column used was a Cosmosil $5C_{18}$-AR-II packed column (4.6 mm, I.D. × 150 mm), the sample injection volume was 50 μL, and the column temperature was 40 °C. The mobile phase for FAD012 consisted of water/methanol/phosphoric acid (60:39:1), and the retention time of FAD012 was 11 min. In the UV spectrum measurement, the absorbance of FAD012 showed a characteristic absorption maximum at 308 nm (data not shown). Therefore, the UV wavelength was set at 308 nm during the HPLC measurement.

Powder X-Ray Diffraction (PXRD). The diffraction intensity was measured with a NaI scintillation counter using a Miniflex II powder X-ray diffractometer (Rigaku Corporation, Tokyo). Cu rays (30 kV, 15 mA) were used as X-rays, the scanning rate

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for measurement of X-ray diffraction was 4°/min, and the measurement range was 2θ = 5−30°. Measurement was performed by placing a powder sample onto a glass plate so that the surface of the sample was flat.

Differential Scanning Calorimetry (DSC). The Themo plus Evo high-sensitivity differential scanning calorimeter (Rigaku Corporation, Tokyo) was used to perform DSC. Calorimetry was performed by placing about 2 mg of the sample in an aluminum sealed pan and subjecting it to a nitrogen gas stream (60 mL/min) with temperature rising at a rate of 5.0 °C.

Fourier Transform Infrared Spectroscopy (FT-IR). Spectroscopy was performed with the JASCO FT/IR-410 (JASCO Corporation) using the KBr tablet method. The number of integrations was 32, the resolution was 4 cm^{-1}, and the measurement wavenumber range was 400−4000 cm^{-1}. A tablet was prepared by adding potassium bromide (KBr) to the sample at a weight ratio of 1:10 (sample/KBr), mixing, and manual pressing. Background correction was performed using KBr single tableting.

Near-Infrared (NIR) Absorption Spectroscopy. A Fourier transformed near-infrared analyzer (Buchi NIR Flex N-500: Nihon Buchi) was used to perform NIR absorption spectroscopy. Conditions used were a measuring wavenumber of 10,000−4000 cm^{-1}, a measurement time of 8 s, and a measuring temperature of 25 °C. Each sample was filled into a sample cup and measured with an optical path length of 1 nm.

Scanning Electron Microscopy (SEM). SEM was performed using a S3000 N scanning electron microscope (Hitachi High-Technologies Corporation). Each sample was subjected to gold deposition for 60 s and observed under a pressure voltage of 10 kV.

Measurement of ^{1}H-^{1}H Rotating-Frame Overhauser Effect Spectroscopy (ROESY) NMR Spectra. ^{1}H-^{1}H ROESY NMR spectra were obtained using the NMR System 700 MHz (Agilent Technologies). Conditions used were D_2O as a solvent, a resonance frequency of 699.6 MHz, 256 increments, a temperature of 25 °C, a pulse width of 45°, and a relaxation time of 1.5 s.

Solubility of FAD012/γCD Systems in Distilled Water. FAD012 (5 or 50 mg) or the 3DGM (FAD012/γCD) was added to 10 mL of distilled water, and the mixture was shaken for 1, 3, 6, or 24 h at a temperature of 25 °C. After each round of shaking, the mixture was filtered with a 0.45 μm membrane filter. The resulting sample was diluted five times in distilled water/methanol (3:5) and subjected to HPLC.

AUTHOR INFORMATION

Corresponding Author

Yutaka Inoue − *Laboratory of Drug Safety Management, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan;* orcid.org/0000-0003-3419-343X; Phone: +81-49-271-7317; Email: yinoue@josai.ac.jp; Fax: +81-49-271-7317

Authors

Nao Ikeda − *Laboratory of Drug Safety Management, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Yuka Ogata − *Laboratory of Drug Safety Management, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Isamu Murata − *Laboratory of Drug Safety Management, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Xuan Meiyan − *Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Jun Takayama − *Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Takeshi Sakamoto − *Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Mari Okazaki − *Laboratory of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Ikuo Kanamoto − *Laboratory of Drug Safety Management, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Saitama 3500295, Japan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c00277

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