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New Preparation Method Using Microwave, Kinetics, In Vitro Dissolution-Diffusion, and Anti-Inflammatory Study of Diclofenac-Proline Co–Crystal

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Abstract

In this study, we developed a novel and simple method to prepare an established diclofenac-proline co-crystal (DPC) using microwave. In addition, we also evaluate the pharmaceutical properties of the co-crystal, including dissolution, diffusion, and anti-inflammatory profile. The kinetics of co-crystal formation was investigated using Fourier-transform infrared spectroscopy. The co-crystal yield was confirmed physically by differential scanning calorimetry and powder x-ray diffraction, while the chemical stability of the drug during and after microwaving was confirmed using thin layer chromatography, ultraviolet spectrophotometry and high-performance liquid chromatography. Experiment results demonstrated that microwave can generate the stable and uniform quality of diclofenac-proline co-crystal efficiently. Furthermore, the process was investigated to follow a second order kinetic, which is different than the neat-grinding process previously reported. Moreover, our findings revealed that co-crystals with L-proline can significantly improve the dissolution, diffusion, and anti-inflammation activity of diclofenac acid.

Conflict of interest

The authors declare no conflict of interest.

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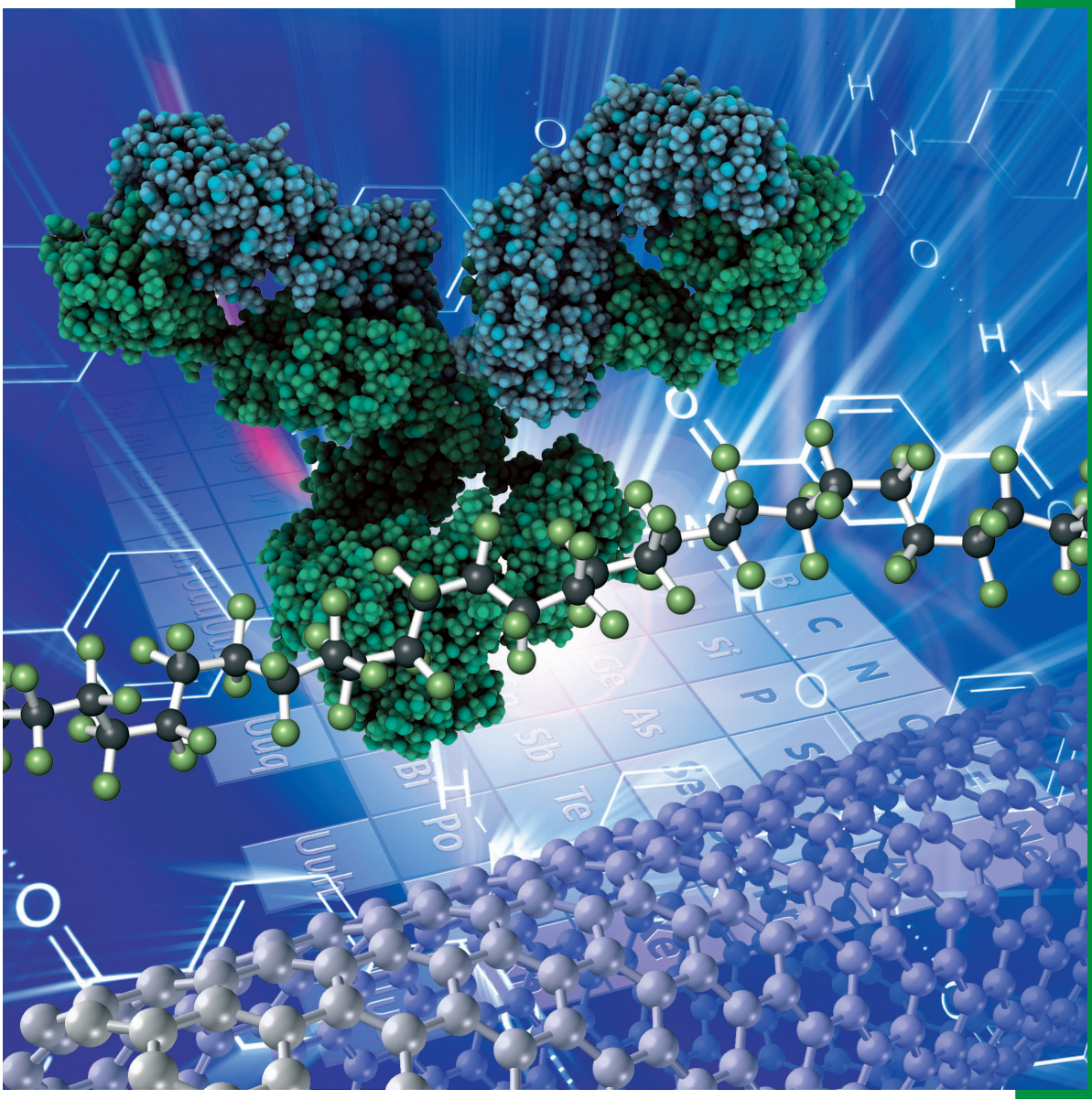

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New Preparation Method Using Microwave, Kinetics, In Vitro Dissolution-Diffusion, and Anti-Inflammatory Study of Diclofenac- Proline Co–Crystal

Ilma Nugrahani,*[a] Dwi Utami,[b] Livia Ayuningtyas,[a] Afrillia Nuryanti Garmana,[a] and Rozana Oktaviary*[a]

In this study, we developed a novel and simple method to prepare an established diclofenac-proline co-crystal (DPC) using microwave. In addition, we also evaluate the pharmaceutical properties of the co-crystal, including dissolution, diffusion, and anti-inflammatory profile. The kinetics of co-crystal formation was investigated using Fourier-transform infrared spectroscopy. The co-crystal yield was confirmed physically by differential scanning calorimetry and powder x-ray diffraction, while the chemical stability of the drug during and after microwaving was confirmed using thin layer chromatography, ultraviolet spectrophotometry and high-performance liquid chromatography. Experiment results demonstrated that microwave can generate the stable and uniform quality of diclofenac-proline co-crystal efficiently. Furthermore, the process was investigated to follow a second order kinetic, which is different than the neat-grinding process previously reported. Moreover, our findings revealed that co-crystals with L-proline can significantly improve the dissolution, diffusion, and anti-inflammation activity of diclofenac acid.

Introduction

Poor homogeneity and low purity are two primary obstacles faced in the development of co-crystal production. Usually, starting materials are still found in the resulting co-crystal product, corresponding to low yield.[1–3] Some methods have been reported to address these issues, including neat grinding, liquid-assisted grinding, hot extrusion, and slow evaporation, all of which have specific advantages and disadvantages.[4–7] Mechanochemical processes such as grinding, with or without solvents, is laborious in terms of maintaining the continuity due to particle size, surface property changes, and the electrostatic effect during the process.[8] Other methods such as slow evaporation have disadvantages, including difficulty maintaining the co-solubility of the parent materials in solution due to their inherent different polarities and dielectric constants,[9–11] while the hot extrusion/melting method has a high risk of chemical instability due to heat exposure.[12–15] Our previous study reported a novel co-crystal from acid form of diclofenac (DA), with L-proline (LP) as the co-former.

We termed the co-crystal a diclofenac-proline co-crystal (DPC) and we screened the 3D structure and studied its co-crystallization using the neat-grinding method.[16–17] To scale up the preparation of the co-crystals in the laboratory, in addition to neat and solvent dropped grinding, we attempted co-melting/extrusion but it failed due to the instability of the components at the high temperature required. Compared with other methods, our findings indicated that the best method for the preparation of DPC was the liquid-assisted grinding technique. However, since this method is performed manually, it is difficult to measure the energy involved as well as to quantify, validate, and standardize the method.[18]

Currently, microwaving is widely employed in chemical research and industry. In general, microwaving works by exciting the rotational energy using the wavelength on a meter–micrometer scale.[19–22] This technique has been tried for various extraction, chemical production, and crystal engineering processes, and has been proven to be more sustainable than conventional methods.[23–27] However, to date, microwaving is not frequently used for co-crystallization, only a few reports about it.[28–32]

The aim of this study was to develop a method for the preparation of DPC using a domestic microwave (MW) with low energy (399 W). This trial was based on previous conventional methods that are solvent-drop grinding and neat grinding.[4–7,16–7] The process was then further investigated to better understand the dynamic co-crystal formation with the MW. In previous research, we used diffractogram profile to determine the kinetic of co-crystal production with neat grinding method. Meanwhile, in present MW method development kinetic study we used Fourier-transform infrared (FTIR) spectra.

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In addition, we evaluated physical and chemical stability of the DPC, along with other crucial factors such as the dissolution and diffusion, which had previously not been studied. Finally, the in-vivo anti-inflammatory activity of the DPC was examined using Wistar rats after ethical and protocol approval. All results demonstrated integrated and consistent data, confirming that DPC improve the pharmaceutical aspects and in-vivo activity of DA. Collectively, this research findings supported data that DPC is a promising candidate for development in dosage form formulations which is able to be generated using MW.

Results and Discussion

Characterization and kinetics study using Fourier-transform infrared (FTIR)

Previous studies of co-crystal dynamics have been performed using infrared instruments such as near infrared, Raman and even rarely FTIR.[16–17,33] In this study, FTIR was used to observe changes in the spectra at wavelength regions of ± 3170–3175 and 3268–3271 cm^{-1}, which presented the intra-molecular hydrogen bond of -NH•••O=C in DPC, as well as the broad spectra at ± 1950–2000 cm^{-1}.[16–17] The result of FTIR analysis are depicted in Figure 2 as the first characterization of the co-crystal formation.

The time when the maximum AUC reached the steady state can be considered as the optimum time for processing. Figure 2a revealed that the DPC produced by MW-assisted-ethanol. was reached optimally after 5–12 minutes. After 12 minutes, the AUC decreased, indicated the breaking of hydrogen bonding. Near the melting point, the specific peaks of DPC were lost, that are the area of 3170–3175 cm^{-1} and 1950–2000 cm^{-1}.

Ethanol system produced co-crystal immediately after the addition of solvent (Figure 2a), While distinctive DPC peaks from methanol system can only be clearly observed after 1 minute of MW treatment (Fig 2b). Furthermore, physically, ethanol and methanol-assisted system melted at different times. Melting occurred after 20 minutes for ethanol and after 25 minutes for methanol. This result revealed that MW-assisted ethanol produced DPC more efficiently than MW-assisted methanol. However, Figure 2a-b indicated that ethanol and methanol systems have similar optimum time for co-crystallization. Figure 2b shows that optimum time for MW-assisted ethanol as well as MW-assisted methanol are 5–12 minutes, indicated by the occurrence of specific DPC peaks after this time. After the optimum time, FTIR spectra is shown to decrease gradually until the melting point. Meanwhile, MW-assisted-water system and neat mixture had optimum times of 15–25 and 30–40 minutes and then melted at 40 and 45 minutes, respectively (Figure 2c and 2d). After melting, the compound produced from these methods was unstable, as shown by the irregular and yellow-brownish color-fused liquid in Figure 3. The conditions of the melted mixtures are shown in Figure 3a-d below.

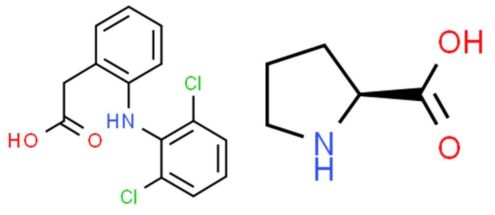

Figure 1. Chemical structure of diclofenac acid (left) and L-proline (right)

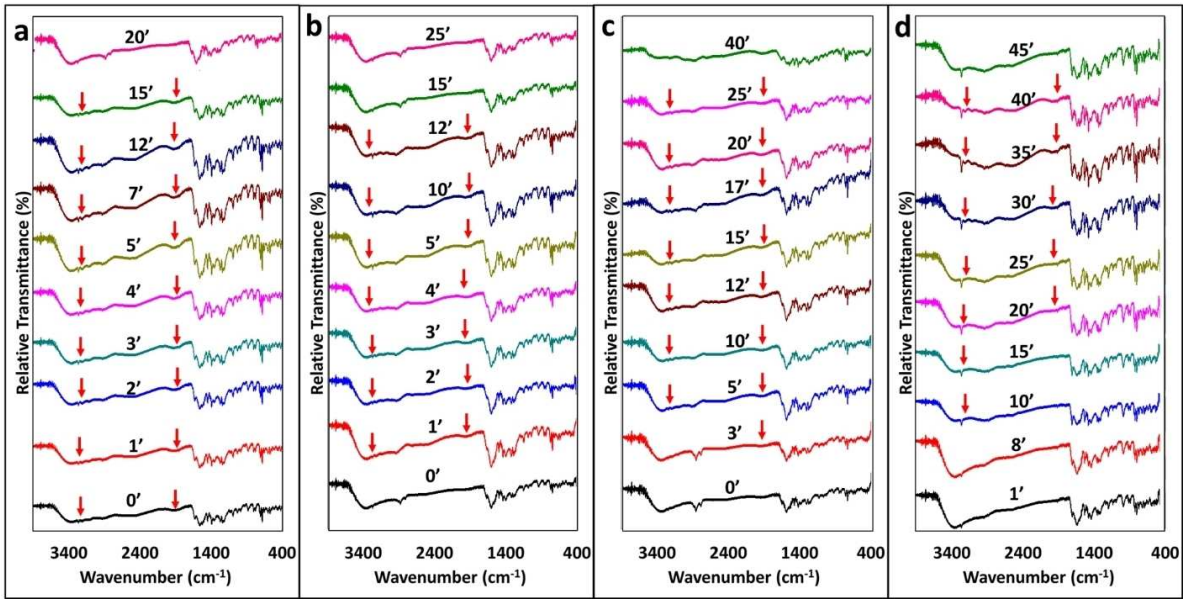

Figure 2. FTIR spectra compilation of DPC from MW treatment: (a) assisted by ethanol, (b) assisted by methanol, (c) assisted by water and (d) neat mixture. Note: red arrows marked the specific peaks of DPC formation.

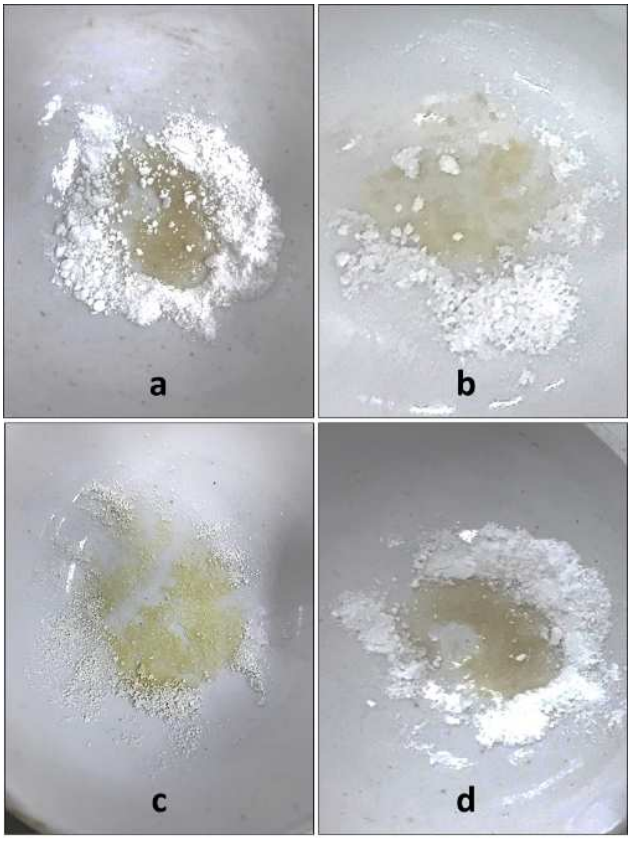

Figure 3. Melted DPC after (a) 20 minutes for MW-assisted-ethanol system, (b) 25 minutes for MW-assisted-methanol system, (c) 40 minutes for MW-assisted-water system, and (d) 45 minutes for neat mixture.

Due to its high specificity and clarity, the spectra at ± 1950–2000 cm^{-1} was selected to measure the kinetics of crystallization. To increase selectivity, the FTIR spectra were first derived and the changes during the MW method are presented in Figure 4. The area under the curve (AUC) of the absorbance was found to be correlated with the amount of DPC. Figure 5 shows the order reaction generated from the increasing curve.

We focused on the specific spectra at 1970–200 cm^{-1} to be monitored as shown in Figure 4. The changes in the DPC vibrational energy due to intermolecular hydrogen bonds formation are shown to increase until the optimum, then decrease.

The kinetics were then analyzed, which resulted in the profile depicted in Figure 5. Our results then revealed the polynomial profile, which resulted in the quadratic equations, indicating that co-crystal formation follows a second order reaction. This data differs from previous reports indicating that the neat-grinding co-crystallization method proceeds with a first order profile.[16–17] It is likely that the differences are due to the different energy types used in the different methods.

The heating characteristics of a particular material (e.g., a solvent) under MW irradiation conditions are dependent on the ability of a specific substance to convert electromagnetic energy into kinetic energy. The MW method require solvent with a high value for this parameter in order to support the rapid nature of this process. Commonly organic solvents are classified according to their heating efficiency in the MW field, with the heating efficiency of ethanol 0.941, followed by methanol at 0.659, and water at 0.123.[34–35] In this case, the hydroxyl group of the ethanol bridges the formation of hydrogen bonds, which are supported by the increasing rotational energy from the molecules of both components and is faster than methanol and water. A longer alkyl chain than methanol causes the -OH to become more active.[36] Without organic solvent support, DPC can be prepared. However, longer time is required to activate the kinetic energy between the two compounds. The longer process can be compared with analog processes such as neat grinding; however, this method is more time intensive than solvent dropped grinding.[17]

This phenomenon can also be explained with other reasons. In addition to the highest rotation energy of methanol and ethanol compared with the other materials used, the volatility/penetration of these solvents is appropriate to reach the deepest layer of the solid molecules.[29] Furthermore, the dielectric constant is congenial to the DPC binary system.[34–35] Finally, other variables are affected by different factors, including the powder's charge, moisture content, and frequency of rotational energy.

Differential scanning calorimetry (DSC) and powder X-ray diffractometry (PXRD) confirmation

The thermograms collected from DSC and diffractogram from PXRD measurement were shown in Figure 6 and 7. DPC products from the optimum time which were 5–12 minutes of MW using methanol and ethanol, 15–40 minutes for water, and 30–45 minutes for the neat system have a distinctive melting point at around 156.7–158.7 °C. The endothermic curves of DPC from methanol, ethanol, and water system were symmetrical and close to the single crystal's reported before, compared to the physical mixture which positioned in the top of Figure 6.[17] However, the thermogram of the neat mixture was not symmetrical. It is indicated that the neat system did not able to yield the cocrystal purely even until 30–45 minutes.

The thermal profile then was confirmed with PXRD data in Figure 7. In this figure, the peak patterns were compared with the previous reports of the standard. All diffractograms show the specific peaks on $2\theta = 4.29°$; 9.73°; 11.54°; 13.18°; 14.31°; 19.42°; 20.48°; and 25.46°; these are equal to the reported DPC as the reference, with the specific peak at $2\theta = 9.84°$. The red star in Figure 7 represents the 1–0-0 plane.[17]

However, confirming DSC result, diffractogram of the neat system still showed some small peaks remained from the starting material's, signed by the yellow stars. Neat system also can produce DPC, however it needs the longer time (30-45 minutes), which caused the breaking of intermolecular bonding before the proper result is yielded. All data lead to conclude that the best systems for co-crystallization are MW-assisted-ethanol and MW-assisted-methanol, followed by water system and the neat system.

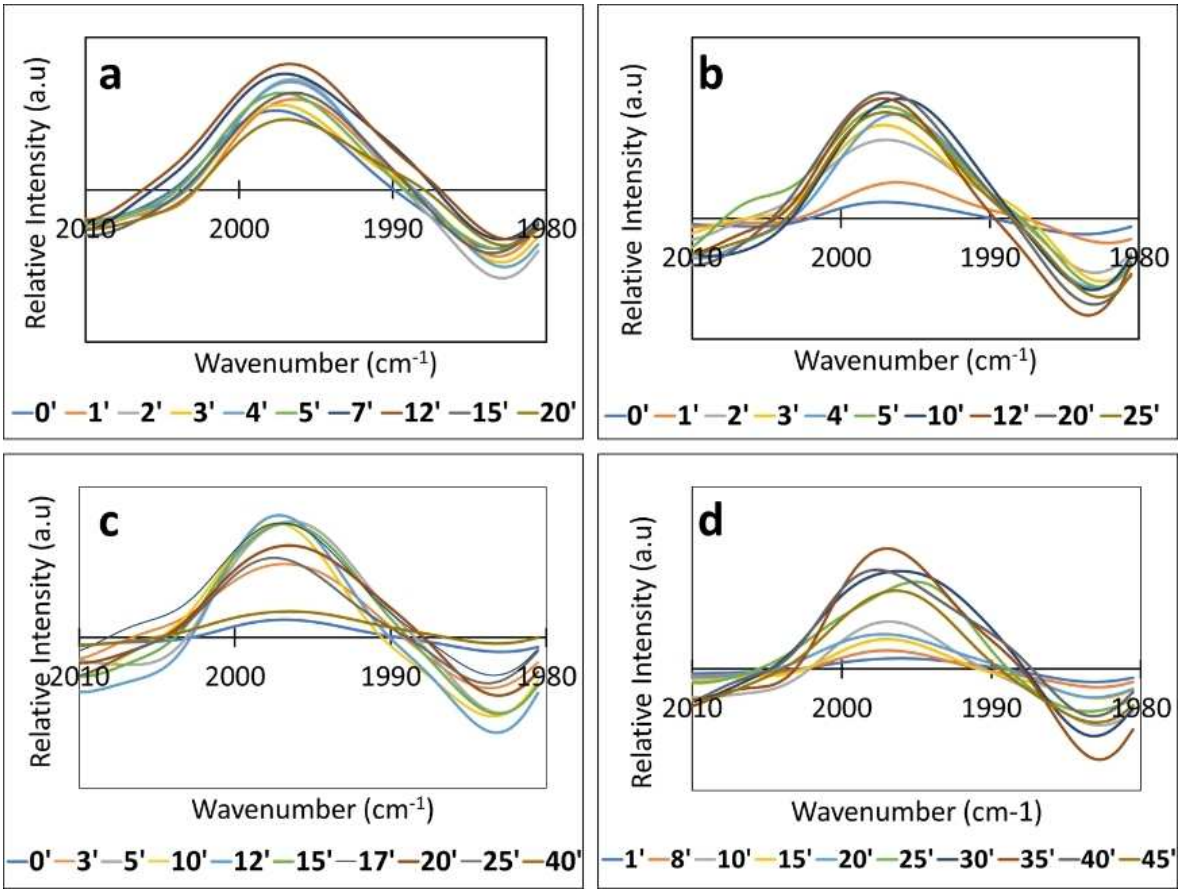

Figure 4. Absorbance intensity changes in FTIR spectra at 1970–2010 cm^{-1} in: (a) MW-assisted-ethanol system, (b) MW-assisted-methanol system, (c) MW-assisted-water system, and (d) neat mixture.

Chemical stability testing using thin layer chromatography (TLC), ultraviolet (UV) spectroscopy, and reverse-phase high performance liquid chromatography (HPLC)

The chemical stability was analyzed by TLC, UV spectrometry, and HPLC. Firstly, TLC result (Supplementary Material, Figure 2) revealed that no other spot except DA exists until the melting point of co-crystals, indicating that the MW did not destroy the active compound. All samples show only the similar diclofenac spot, with no changes shown by L-proline spot. These results confirm that no chemical changes occurred during the MW time of each system. Next, as shown in Figure 8, the UV spectra at 276 nm show no change of the drug in DPC from the MW method.

Lastly, HPLC analysis was conducted to further confirm previous analysis result. The chormatograms are depicted in Figure 9(a) and (b). As shown in Figure 9(a), DPC sampled at optimum MW time period for every production method developed in this research were compared to the physical mixture. All DPC sample generated peaks with similar AUC and retention time to physical mixture after detection at 254 nm. However, upon exceeding optimum time, DPC powder melted, followed by the degradation of co-crystal. After melted, new peaks occurred and the chromatogram was different to that of physical mixture. This observation suggests the possibility of degradation product.

Afterward, all systems were checked by focusing on the time of retention of DA, that is 5.24 minutes as shown in Fig 9(b). Here is shown that all chromatograms from ethanol, methanol, water, and neat system, have the similar retention time and AUC. This confirmed the stability of systems until optimum time period of MW process. By combining the kinetic and stability data, the duration of MW treatment for each solvent system in DPC production can be standardized for future application.

All physical and chemical data support the conclusion that the homogeneous DPC can be obtained quickly using MW treatment due to the efficiency of the energy transferred by the equipment. This is in line with the strategic future goals of environmental conservation, which involves less chemicals and reagent, and includes reduced energy consumption, waste, and pollutants.[37–38] The next experiment results then describe the impact of DPC formation on the drug performance by a series of dissolution, diffusion, and by anti-inflammatory test.

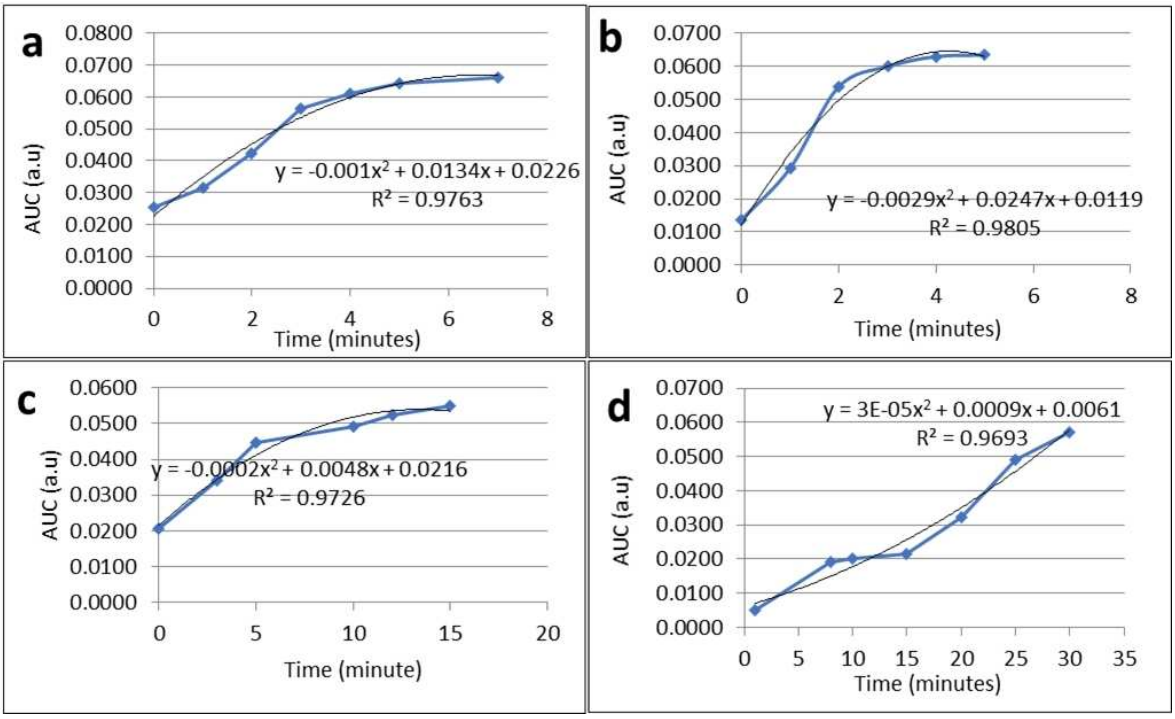

Figure 5. The kinetic equation of: (a) MW-assisted-ethanol system, (b) MW-assisted-methanol system, (c) MW-assisted-water system, and (d) neat mixture.

Dissolution testing

Ethanol and methanol systems were the two most efficient MW co-crystal production method, which generated DPC with almost identical characteristics and stability profile. Therefore, only one representative (MW-assisted-ethanol system) was chosen for in-vitro dissolution and diffusion testing. Along with the solubility improvement, dissolution testing in stomach and intestine medium[39] showed an increasing dissolution rate of DPC (collected from the ethanol system) as depicted in Figure 10. Indeed, DPC released the parent drug faster than did pure DA, as determined by the zwitterionic capacity, which increased the rate and solubility of both media types. At pH 1.2, DA was released at a maximum of 20% from the DPC after 30 minutes, compared with DA was released at a maximum of 12% after 60 minutes and from physical mixture after 45 minutes as much as 15%. This result showed that the dissolution of DA in pH 1.2 was poor, due to the acidity of DA (pKa = 4.8), the total drug released amount was very limited at this pH. As well as the previous data, Figure 10 shows that the DA was completely released at pH 6.8 after 30 minutes from the DPC, 45 minutes from DA, and 60 minutes from physical mixture. However, in pH 6.8, almost all drugs were released. These data confirm that the DPC had an improved dissolution profile from that of DA significantly in the intestinal pH. LP in physical mixture also pushed the dissolution rate due to its properties as a deep eutectic solvent in the solution, which has been established in many reports.[38–41] However, DPC remain superior by increasing the DA dissolution rate compared to DA alone and physical mixture.

Diffusion testing

Diffusion testing was conducted with two similar media samples used in dissolution test and the results are shown in Figure 11. These experiments also revealed a similar phenomenon in that DPC was predominant, with an increasing rate and quantity of DA diffusion up to $\pm$ 4 folds. The DPC diffused maximally after 2 hours, followed by PM and the pure DA. Furthermore, diffusion testing results revealed that the highest penetration occurred from the DPC system in pH 6.8.

Anti-inflammatory activity testing

Inhibition of an inflammatory response is presented as a percentage. Our experimental results from pure DA, DPC from the MW-ethanol and methanol system (chosen as the two most efficient co-crystal production method), and carrageenan as a positive control are shown in Table 1 and Figure 12. Both DPC obtained from MW-water system and neat mixture were omitted from in-vivo study, assuming no potential inflammation inducer (e.g. organic solvent) used during production. Our results had a p value < 0.05, with the data showing a normal distribution. Finally, in-vivo test results are shown in Figure 12. On the histogram, the increase in inflammation in the maximum control group occurred in the third hour. The inflammation was caused by the release of prostaglandin in the third phase so that the resulting inflammation was at a maximum.[42] Carrageenan can induce inflammation in three phases, with the first phase being histamine and 5-HT release.

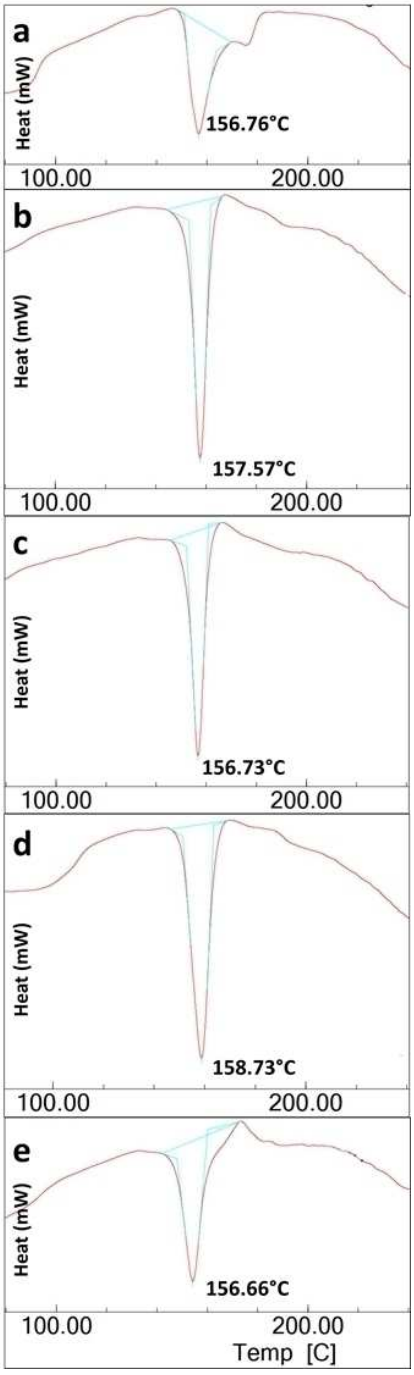

Figure 6. DSC thermogram of: (a) physical mixture, (b) DPC from MW-assisted-ethanol system, (c) DPC from MW-assisted-methanol system, (d) DPC from MW-assisted-water system, and (e) DPC from neat mixture after each optimum time of heating.

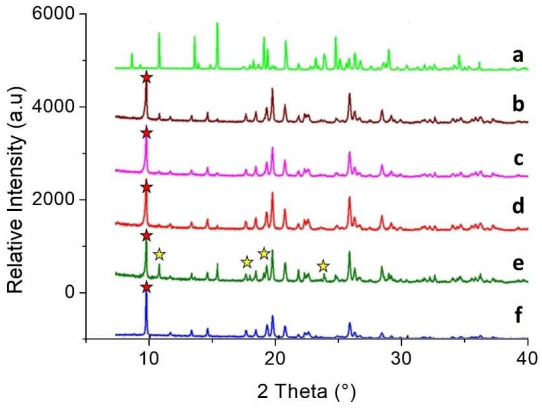

Figure 7. X-ray powder diffractogram of: (a) physical mixture, (b) DPC from MW-assisted-ethanol system, (c) DPC from MW-assisted-methanol system, (d) DPC from MW-assisted-water system, (e) DPC from neat mixture and (f) DPC calculated as the reference. All diffractograms of MW system reveal a different pattern from PM and similar to the calculated standard.

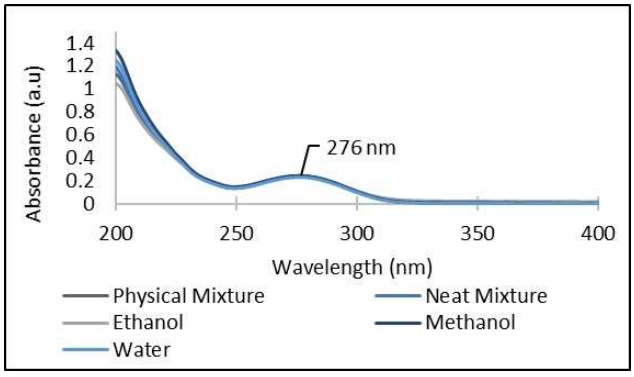

Figure 8. UV absorbance spectra of DPC sampled before melting.

The second phase is mediated by kinin, while the third phase is mediated by prostaglandin.[43]

The administration of DPC can inhibit inflammation from the first hour after carrageenan administration. Inhibition of inflammation by the DA group, which were significantly distinct from the positive control group was achieved at 1 to 3 hours. Meanwhile, the DPC group showed significantly greater inhibition compared with the other groups, from the first to the sixth hour. However, the work-onset factor of the DPC was not signed to be changed. Judging from the data, DPC revealed $\pm$ 1.5 times stronger than DA. This increasing effect is expected to be supported by the increase in solubility, dissolution, and diffusion as explained previously. As the final impact, the rate of absorption should be going up. Therefore, it can be concluded that the solubility of DA is the determinate step for the dissolution, diffusion, and absorption rate of the DPC. As shown in Figure 12, anti-inflammatory activity of DPC prepared with the assistance of either methanol or ethanol are similar statistically.

Conclusions

Present experiment has proven that microwave can produce homogenous diclofenac-proline co-crystal either with the assistance of methanol, ethanol, water, or a neat system and follows a second order kinetics. The ethanol dropped MW was the fastest method, followed by methanol and water. The

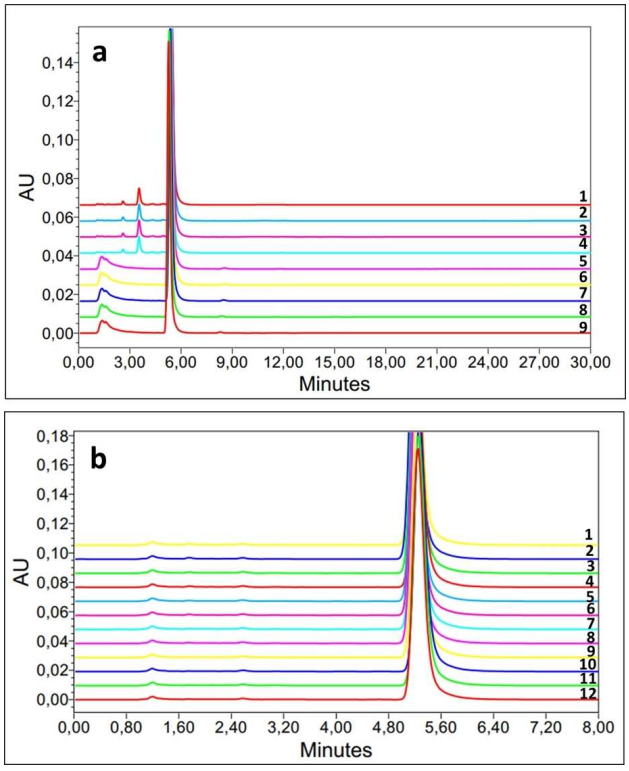

Figure 9. (a) overlaid chromatograms of (1) melted MW-assisted-ethanol system sampled at 20 minutes, (2) melted MW-methanol system sampled at 25 minutes, (3) melted MW-assisted-water system sampled at 40 minutes, (4) MW neat mixture sampled at 45 minutes, (5) physical mixture, (6) MW-assisted-ethanol system at optimum point, (7) MW-assisted-methanol system at optimum point, (8) MW-assisted-water system at optimum point, (9) neat mixture at optimum point and (b) overlaid chromatograms of: (1) physical mixture as the reference; (2-5) MW-assisted-ethanol system after micro-waving for 5, 10, 12 minutes, respectively; (6-8) are the chromatograms of MW-assisted-methanol system after 5, 10, 12 minutes, respectively; (9-10) MW-water-system after 15 and 25 minutes; (11-12) neat system after 30 and 40 minutes (b).

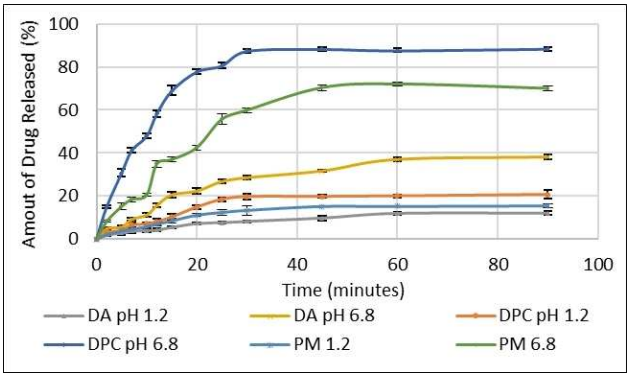

Figure 10. Dissolution profile of MW-assisted-ethanol system (DPC) compared to diclofenac acid (DA) and physical mixture (PM) in pH 1.2 and 6.8 media.

longest process was the neat system without any liquid dropped. The novel findings indicated that diclofenac-proline co-crystal increase the dissolution and diffusion rate of the acid

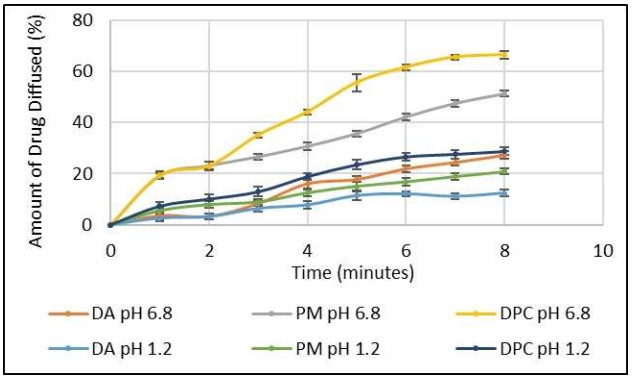

Figure 11. Diffusion profile of MW-assisted-ethanol system (DPC) compared to diclofenac acid (DA) and physical mixture (PM) in pH 1.2 and 6.8 media.

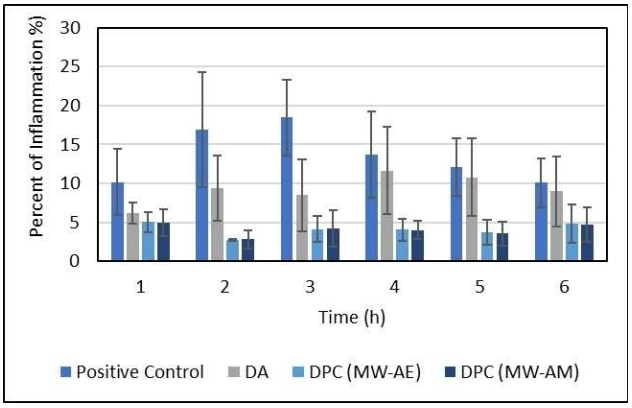

Figure 12. The inflammation volume percentage of carrageenan, diclofenac acid (DA), MW-assisted-ethanol system (DPC MW-AE) and MW-assisted-methanol system (DPC MW-AM) groups in 6 h observation.

form of diclofenac, and produced 1.5 times higher anti-inflammatory activity, with a longer duration of effect. Thus, with a simple and inexpensive process, this technique can be developed as a green and cost-efficient method for scaling up the production of diclofenac-proline co-crystal for further research and drug development.

Supporting Information Summary

Solvent-assisted microwave method for diclofenac-L-proline co-crystal preparation, characterization, performance evaluation and stability study are described. From kinetic model evaluation, ethanol was found to be the best solvent in assisting the formation of intermolecular interaction between diclofenac acid and L-proline which was reached after only 5 minutes of microwaving. The resulting co-crystal will eventually revert to its starting materials after the optimum microwaving period are exceeded. No degradation products were observed after MW treatment until optimum time period was reached.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: crystal engineering · diclofenac · drug delivery · L-proline · microwave chemistry

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