Research article

Cocrystal construction between the ethyl ester with parent drug of diclofenac: structural, stability, and anti-inflammatory study

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ABSTRACT

This study aimed to collect the crystallographic data of ethyl diclofenac and discover a cocrystal from this ester with its parent, diclofenac acid, and to investigate their physicochemical properties and anti-inflammation activity. Firstly, ethyl diclofenac single crystal was isolated and continued by the cocrystal screening and isolation. Solid characterization was conducted by thermal analysis, infrared spectroscopy, powder x-ray diffractometry, followed by structural determination using a single crystal x-ray diffractometer. The stability of the cocrystal toward heating and high humidity, followed by the anti-inflammatory activity, was also studied. Ethyl diclofenac and the cocrystal were successfully isolated and subsequently subjected to lattice system determination. Interestingly, the new cocrystal can be generated directly by Fischer equilibrium reaction during esterification of diclofenac acid. Structurally, ethyl diclofenac reveals a P2$1$/c monoclinic and the cocrystal between this ester with its parent drug is a P-1 triclinic system. A hydrophobic interaction -C-Cl-, which is rarely found in a cocrystal, involved in the molecular interaction between ethyl diclofenac and the parent drug, besides the hydrogen bonds. The newly isolated cocrystal has a melting point ±103–104 °C, which is higher than that of ethyl diclofenac (±67.5 °C) but lower than that of diclofenac acid (±173 °C). Hence, this cocrystal is stable towards accelerated stability testing by heating in a microwave, as well as storing in high relative humidity. Moreover, the anti-inflammation test also showed promising activity improvement.

1. Introduction

Currently, diclofenac acid is a commonly used anti-inflammation drug. This active compound is effective to relieve pain [1] and is widely used in topical, regular systemic, extended-release, and transdermal dosage forms. Recently, some studies have explained the production and advantages of its pro-drug, ethyl diclofenac, which possesses higher activity than the acid [2, 3, 4]. The higher effectiveness of ethyl diclofenac was reported to be caused by its lower plasma bond. Another advantage is that the acidic parent drug will be released from the ester in the intestine, protecting the stomach from irritation [2, 3, 4].

Ethyl diclofenac can be produced using the esterification process under acidic conditions by refluxing in the absence of water. The two-dimensional structure of ethyl diclofenac has been characterized by infrared spectroscopy and nuclear magnetic resonance [2]. The ester formation is indicated by infrared spectrum at carboxylic –OH region of

the acidic parent drug (around 1600-1700 cm^{-1}) which disappears and be replaced by ester on around 1721 cm^{-1}. In addition, new peaks which indicate N–H strain and C–O–H strain of ethyl should appear. Meanwhile, nuclear magnetic resonance analysis should reveal a triplet at around 1.31 (CH_3) and a singlet around 3.83 (OCH_2) [2].

However, to date, the crystallographic data has not been published [2, 3, 4]. Crystallographic data are necessary to be studied because they relate to physicochemical properties that will influence pharmaceutical performance. During esterification, a cocrystal between the ester with the residue of the parent substance can result as a side-product [5]. Salts may also be produced with this cocrystal besides esters [6, 7]. During esterification, the presence of water can disturb the process, causing a reversible reaction between the product and educt, namely, the Fischer equilibrium reaction [8]. When the mixture is recrystallized, intermolecular bonds are constructed and co-arranged into a new solid phase.

Cocrystal formation is one of the approaches in crystal engineering to improve the physicochemical properties of pharmaceutical substances [9, 10]. The properties that can be changed by the new phase arrangement are the melting point, stability, solubility, dissolution rate, and bioavailability [11, 12, 13, 14, 15, 16, 17]. Hence, cocrystal formation between the ester products and starting material could be reviewed from two sides: negatively as the side product and positively as a prospective way to generate new material in drug design.

The interaction between ethyl diclofenac and diclofenac acid (termed EDDA) was identified, and its complete structure was determined by the crystallographic study. The stability of the product toward heating and high humidity during storage was also investigated. Finally, the impact of cocrystallization on the anti-inflammation activity was evaluated.

The EDDA crystal was constructed using two methods. The first method involved solvent-drop grinding, followed by recrystallization (SDGR) [16, 18, 19], while the second was a combination of Fischer reaction esterification and cocrystallization (FR) [5, 6, 7, 8].

2. Aim of the study

This study was aimed to isolate a single crystal of ethyl diclofenac and its cocrystal to characterize and determine its structure, evaluate the stability both phases towards heating and high humidity, and compare their in-vivo anti-inflammation activity.

3. Materials and methods

3.1. Materials

Diclofenac acid, sodium sulfate, ethanol, ethyl acetate, hexane, toluene, acetone, potassium bromide, and silica gel 60 GF-254 were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Aquadest was prepared by the School of Pharmacy, Bandung Institute of Technology. Male Wistar rat for the anti-inflammatory test was supplied by the Animal Laboratory, School of Pharmacy, Bandung Institute of Technology.

3.2. ED synthesis

In this experiment, ethyl diclofenac crystals were synthesized from diclofenac acid by esterification using 99% ethanol. This process involved reflux and recrystallization under cold conditions (6–12 °C) to push crystal formation because the supersaturated condition will be reached more quickly under low temperature. One μg (0.003 mol) of diclofenac acid was dissolved in 99% ethanol in an excess volume (100 mL), and 3 mL of 6N H_2SO_4 was gradually added. The mixture was then refluxed under two conditions, at 80 °C [2] for 45 min and 90 °C for 60 min. The ester was extracted by hexane and was washed with water three times in a separatory funnel. The hexane phase was added to sodium sulfate anhydride to adsorb the water residue, and then the solution was evaporated at 6–12°C/1 Atm/RH 72% to collect the ethyl diclofenac crystal. As noted, due to its low melting point, ethyl diclofenac was stored in a refrigerator at 6–12 °C.

3.3. EDDA cocrystal isolation

3.3.1. Cocrystal screening using electrothermal AI9000 (Keison, UK)
Ethyl diclofenac and diclofenac acid were weighed in a series of molar ratios and then were crushed to homogeneity to compose physical mixtures. A capillary tube was filled with a small quantity of each mixture, which was subjected to melting point determination. A phase diagram was constructed based on the result to predict the profile and molar ratio of the interaction [20].

3.3.2. Cocrystal isolation
After the phase diagram was confirmed to indicate the solid molecular interaction, the corresponding molar ratio of ethyl diclofenac and

diclofenac acid mixture was grounded, assisted by ethanol dropping. This method then is named solvent dropped grinding assisted crystallization (SDGR). Next, the mixture was crystallized using a slow evaporation method at 6–12°C/1 Atm/RH 72%, followed by direct cocrystallization using the Fischer reaction [8], named as FR method, which was performed using a similar procedure for ethyl diclofenac with 95% ethanol as the solvent. The existence of water maintains the equilibrium state between ethyl diclofenac with diclofenac acid, which was then expected to form cocrystals after the solvent has evaporated. The EDDA cocrystal was then characterized using a differential scanning calorimeter, infrared spectroscopy, and powder X-ray diffraction. Finally, the single crystal was analyzed using single crystal X-ray diffractometry to determine the 3D structure [21].

3.4. Purity test, powder characterization, and structure determination of ethyl diclofenac and EDDA cocrystal

The purity of diclofenac acid and the cocrystal were then analyzed using thin-layer chromatography and melting point measurement using Electrothermal (IA9000; Keison, UK). The pure substance will show one only spot, without any other strange spot on the chromatogram plate. By electrothermal, the purity will be represented by the short range of melting point (± 2 °C) for the pure phase, while the impurity existence will show the wider range. The 2D structure of ethyl diclofenac was confirmed using Fourier transform infrared spectroscopy (FTIR) Jasco 4200 type A, Japan) at around 1600-1700 cm^{-1}, which is a region of carboxylic –OH. Besides it, a nuclear magnetic resonance analysis using NMR-Agilent 500 MHz, USA also was performed to strengthen the structure elucidation. In nuclear magnetic resonance analysis, a singlet peak indicating –OH moiety will disappear due to the formation of ester [2]. Thermal profiling was performed using differential scanning calorimetry (DSC 2830; Thermoplus Evo, Rigaku, Japan) to know the thermal profile of ethyl diclofenac and also EDDA, which should be different from their starting materials. Finally, crystallography was completed by diffractometry using SMART-LAB X-ray diffractometer (Rigaku, Japan) to characterize the crystal pattern and single-crystal X-ray diffractometer (R-AXIS RAPID II, Rigaku, Japan) to determine the 3D structure thoroughly.

3.4.1. Thin layer chromatography analysis
The purity of the esterification product ethyl diclofenac was tested using the system of toluene-acetone (1:2), n-hexane-ethyl acetate (8:1), and n-hexane-ethyl acetate (8:2). A small quantity of the physical mixture and cocrystal were dissolved and spotted onto a thin layer chromatography plate (silica gel 60 F-254), followed by elution with the mobile system. The result was then visualized under an ultraviolet (UV) 254 nm lamp (Bandung Institute of Technology).

3.4.2. Thermal analysis using electrothermal IA9000
This apparatus was used to test the purity and physicochemical characteristic by measuring the melting point of the powder sample. It was also used to screen the formation of interaction. Electrothermal IA9000 (Keison, UK) was completed using an 8× viewing magnifier and LED lamp to assist the observation and has three holes to analyze three samples simultaneously. A small amount of the sample was used to fill a capillary tube, which was then placed into the hole in the instrument for observation. The starting temperature was set from 50 °C, and the heating rate was 10°/min.

3.4.3. Differential scanning calorimetry analysis (Thermoplus Evo 2830, Rigaku, Japan)
Approximately 5–10 mg of samples were accurately weighed and were used to fill an aluminum pan. The pans were heated at an increasing temperature from 25 °C to 250 °C with a heating rate of 5 °C/min under a nitrogen purge of 50 mL/min. All pans were closed during the measurement.

3.4.4. Infrared spectroscopy analysis

Infrared spectrophotometer using a Fourier transform infrared (FTIR Jasco-4200 type A, Japan) recorded the spectra of diclofenac acid, ethyl diclofenac, the physical mixture, and the cocrystal using a potassium bromide beam splitter. The potassium bromide pellet method was employed, and the background spectrum was collected. The range was set from 450 to 4000 cm^{-1} with a 16-cm^{-1} resolution.

3.4.5. ^1H nuclear magnetic resonance analysis

The ^1H nuclear magnetic resonance (NMR) spectrum of 20 mg of ethyl diclofenac was recorded in an NMR-Agilent 500 MHz system (USA) using tetramethylsilane as the internal standard and acetone-d6 as a solvent operating at a frequency of 500 MHz (^1H). Ethanol pro analysis (99%) was carried out on the results of esterification (ethyl diclofenac) from diclofenac acid.

3.4.6. Powder X-ray diffractometry

Powder X-ray diffraction measurement was performed using a SMART-LAB X-ray diffractometer (Rigaku, Japan). The samples were placed in between the Myler® films. The powder diffraction pattern was collected from $2\theta = 3°$–$40°$ at ambient temperature with a step and a scan speed of $0.01°$ and $3°$ min^{-1}, respectively, using the Cu–Kα source at 45 kV and 200 mA.

3.4.7. Single-crystal X-ray diffraction analysis and refinement

The single crystal X-ray diffraction (SCXRD) collected data at 173 K for ethyl diclofenac crystal and EDDA cocrystal. The measurements were carried out in the ω-scan mode using the SCXRD-R-AXIS RAPID II system (Rigaku, Japan) as the diffractometer and Cu–Kα radiation obtained from the rotating anode source using a graphite monochromator. The integrated and scaled data were empirically corrected for absorption effects using ABSCOR. The original structure was solved using a dual space algorithm implemented in SHELXT and was refined on F2 using SHELXL-2017/1. All the nonhydrogen atoms were refined anisotropically. The hydrogen atoms attached to oxygen or nitrogen atoms were located using the differential Fourier map and were refined isotropically. Other hydrogen atoms were determined geometrically and were included in the calculation using the riding model. The molecular graphics were produced using Mercury 3.7.

3.5. Stability study

Accelerated stability was performed to evaluate the stability of the EDDA cocrystal compared with the physical mixture. Samples were exposed to heat and high humidity. A microwave (SHARP, R-230R(S), Japan) at the power of 399.5 W was used as a model for heating treatment [18, 19, 22, 23]. The high humidity condition was prepared in the chamber of saturated potassium nitrate solution, which was measured as the relative humidity of $\pm 84\%RH/25$ °C [24]. The infrared spectra were studied to evaluate the particular cocrystal binding change during the stability test. Thin layer chromatography was used to detect the chemical stability after the stability test, using the silica gel (60 F-254) plate and the mobile phase of n-hexane-ethyl acetate (8:1) and toluene-ethyl acetate (1:2).

3.6. Anti-inflammation test

3.6.1. Materials

Before observation was conducted, ethics commission approval (No.13/KEPHP-ITB/4–2018, approved in Bandung, April 20th, 2019) was received for the study of five groups of male Wistar rats, each comprising six animals, which were treated with carrageenan (Sigma), Na-CMC (Sigma), ethyl diclofenac, diclofenac acid, EDDA cocrystal (EDDA-CC), and the physical mixture (EDDA-PM).
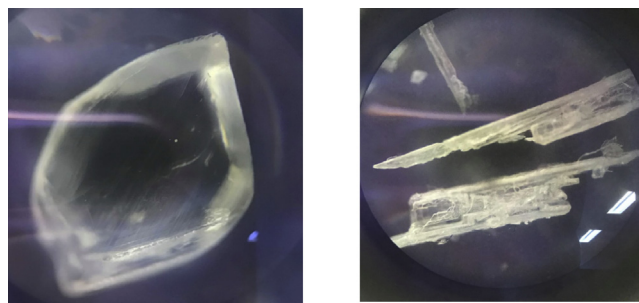

Figure 1. The rectangular ethyl diclofenac crystal (left) and needle-like diclofenac acid (right) crystal photograph (40x).

3.6.2. Procedure

Male Wistar rats were used following approval by the Animal Care and Ethics Committee, School of Pharmacy, Bandung Institute of Technology, Indonesia, 2018. The five groups of rats with a body weight 150–200 g were acclimatized for one week before treatment. The carrageenan and drug suspension was prepared the day before the test. The carrageenan suspension was prepared by weighing as much as 10 mg and dissolving it in 1 mL of aqua pro injection. The solution was mixed thoroughly and incubated at 37 °C for 24 h. In making the drug suspension, a 1% Na-CMC suspension was prepared first. Na-CMC was weighed to 1.5 g and was dissolved in 45 mL of hot water. After expanding, the mixture was grounded to homogeneity. The Na-CMC suspension was added to a final volume of 150 mL using distilled water. For the drug suspension preparation, the drug sample was mixed and ground with 1% Na-CMC until it reached a final volume of 50 mL. Before the test began, male Wistar rats were weighed, and each dose was calculated based on the body weight. The rat's left foot initial volume was measured using a plethysmometer and was recorded as Vo before the drug was given. The test group was divided into five treatments: positive control group (injected with carrageenan for inflammation induction without the drug), diclofenac acid -treated group, ethyl diclofenac -treated group, ethyl diclofenac - diclofenac acid physical mixture-treated group, and EDDA cocrystal-treated group. Each group was orally administered the drug suspension according to the dose equal to diclofenac acid at 1 mg/kg of the body weight. Thirty minutes later, the two groups were injected with carrageenan 0.05 mL of 1% (w/v) by the intraplantar route. The positive control group was only given 1% (w/v) carrageenan. One hour after the administration of 1% carrageenan (w/v), the volume of the left foot of each rat was measured again using a plethysmometer. Every hour, the volume of the foot was measured until the sixth hour. The data were then converted to the inflammation inhibition value, calculated by reducing the measured inflammation volume of each rat with the inflammation volume of the control group at the same time. The data were then analyzed statistically using 2-way ANOVA, followed by Dunnett's and Tukey's test.

4. Results

4.1. Isolation, characterization, and crystallographic study of ethyl diclofenac

The ethyl diclofenac crystal was obtained from the esterification of diclofenac acid through refluxing in 99% ethanol, with a concentrated of H_2SO_4 as a catalyst. In the first condition, reflux was conducted at 80°C/ 45 min [2]. Next, the process was optimized by increasing the temperature and time to 90°C/60 min. The recrystallization was performed under 6–12 °C because cooling can induce the supersaturation of ester solution and maintain the stability of the crystal.

From the two processes, the resulted yields were 75.31 ± 2.78 and $90.64 \pm 4.78\%$ w/w (n = 3). Refluxing in higher temperature and longer
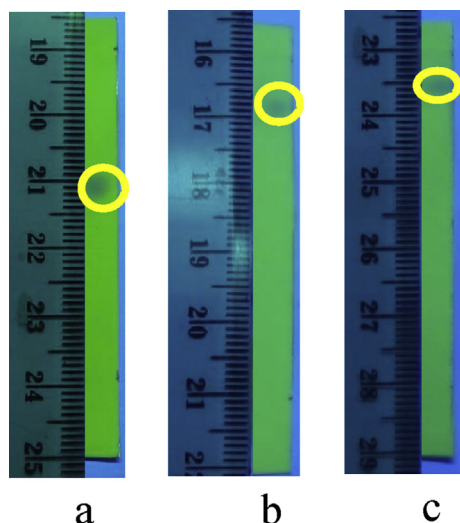
Figure 2. Chromatogram of ethyl diclofenac on silica gel 60 F-254 using three types of mobile phases: a) n-hexane: ethyl acetate (8:1), b) ethanol: ethyl acetate (2:3), and c) toluene: acetone (1:2)—under UV 254 nm lamp.
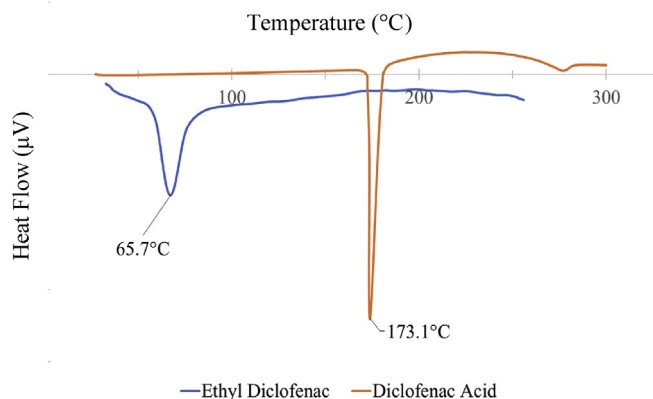

Figure 3. Thermogram of ethyl diclofenac as the product and diclofenac acid as the parent drug and the melting points.

period (90°C/60 min) increased the amount of yield and produced the more transparent crystal. Therefore, the characterization was conducted on ethyl diclofenac crystals obtained from the optimized method.

The ethyl diclofenac crystal, depicted in Figure 1 as an asymmetrical rectangular shape, was compared with the needle-like crystal of diclofenac acid, in 40x magnification.
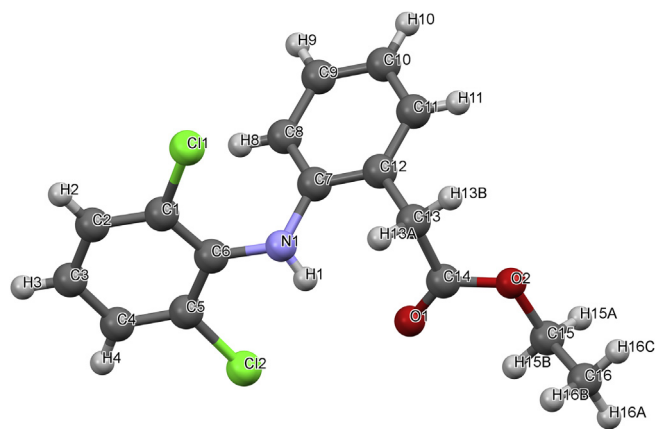

Figure 5. The 3D structure of ethyl diclofenac.

4.2. Purity test

The purity test on ethyl diclofenac was carried out by thin layer chromatography (Figure 2) as well as thermal analysis, including melting point range determination and differential scanning calorimetry.

Determination of melting point range using an electrothermal system revealed a result of ±67–68 °C (1 Atm/25 °C). Meanwhile, the thermogram of ethyl diclofenac compared with that of the parent drug, as shown in Figure 3.

4.3. Structural confirmation

In chemical analysis, infrared spectroscopy is the most common qualitative method to elucidate structure quickly [25]. The spectrum of ethyl diclofenac showed that no O–H strain carboxylic peak at 1693 cm^{-1} was evident because it was replaced by the ester spectra on 1708.62 cm^{-1}. Two specific peaks also appeared on 3293.82 cm^{-1} (N–H strain) and 1238.08 cm^{-1} (C–O–H strain of ethyl), and no spectrum of the starting material, diclofenac acid nor the ethanol.

The 2D structural data was supported by the result of ^{1}H nuclear magnetic resonance (500 MHz) measurement, which revealed specific spectra as follows: a triplet in 1.24–1.27 ppm (CH$_3$), a singlet in 3.01 ppm (NH), a singlet in 3.8 ppm (CH$_2$), a quartet in 4.16–4.2 ppm (OCH$_2$), and a multiplet in 6.46–7.48 ppm (Ar–7H). Thereofore, the substance yielded has been fixed chemically as the pure ethyl ester of diclofenac, and then its 3D structure can be determined using a single crystal diffractometer.

4.4. Ethyl diclofenac crystal characterization and 3D structure determination

The ethyl diclofenac crystal was analyzed using powder X-ray diffractometer. Figure 4 depicts the diffractogram of ethyl diclofenac that
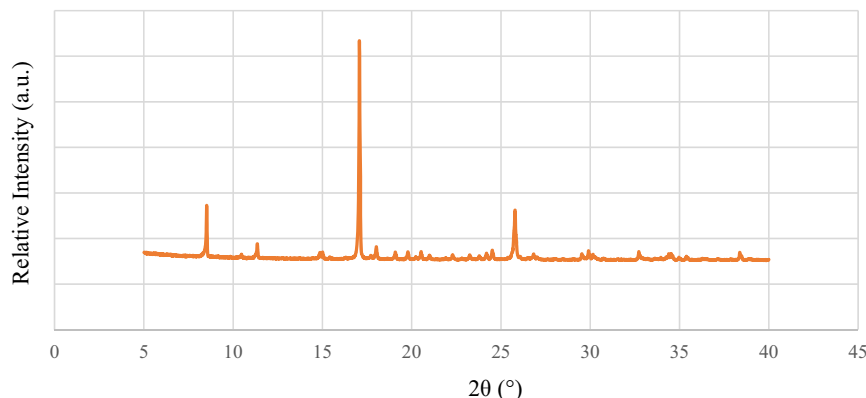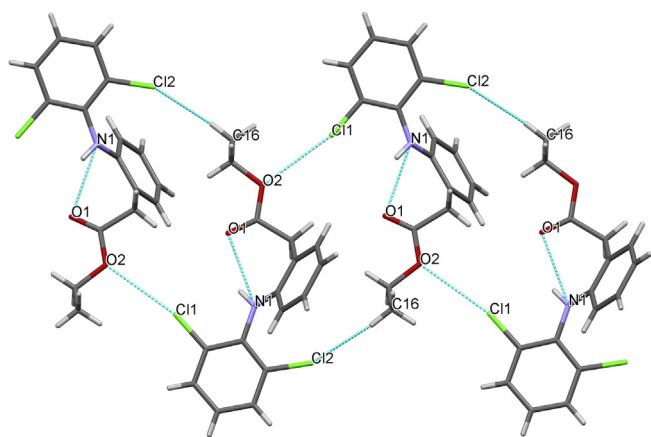

Figure 4. Difractogram of ethyl diclofenac.

Figure 6. Hydrogen bonds in the ethyl diclofenac crystal system.

Table 1. Hydrogen bonds in the ethyl diclofenac crystal lattice.

D–H···A	D–H (Å)	H···A (Å)	D···A (Å)
C16–H16A···Cl1	0.980	2.864	3.844
C15–H15B···Cl2	0.990	2.886	3.876
C13–H13B···O1	0.990	2.716	3.706
N1–H1···O1[a]	0.81(2)	2.214	3.024

[a] Intermolecular hydrogen bond.

has not yet been listed in the *Cambridge Crystallographic Data Centre* before.

The diffractogram in Figure 4 showed the distinctive peaks at $2\theta =$ 8.52°, 17.07°, and 25.8°. Thereafter, the experiment can be continued by using a single crystal X-ray diffractometer to solve the detailed 3D structure of ethyl diclofenac. The resulting 3D-conformational structure is shown in Figure 5, and the interaction binding is revealed in Figure 6.

The image in Figure 6 below, constructed with the review of hydrogen bonds in Table 1, depicts the intermolecular bonds that construct the ethyl diclofenac lattice. These are N (1)-H (1)•••Cl (2), N (1)-H (1)•••O (1), and C(15) –H(15B)•••O(1).

The hydrogen bonds that support the new coarrangement are explained as follows (Table 1):

Four ethyl diclofenac molecules are involved in one lattice, as illustrated in Figure 7, composing a P21/c configuration, the layer of ethyl diclofenac crystal has a different appearance when viewed from the a-axis, b-axis, and c-axis, as shown in Figure 7.

Table 2 listed the complete crystallographic data of ethyl diclofenac.

The diffractogram of the ethyl diclofenac crystal was compared with the calculated result. Both revealed a similar pattern, as shown in Figure 8.
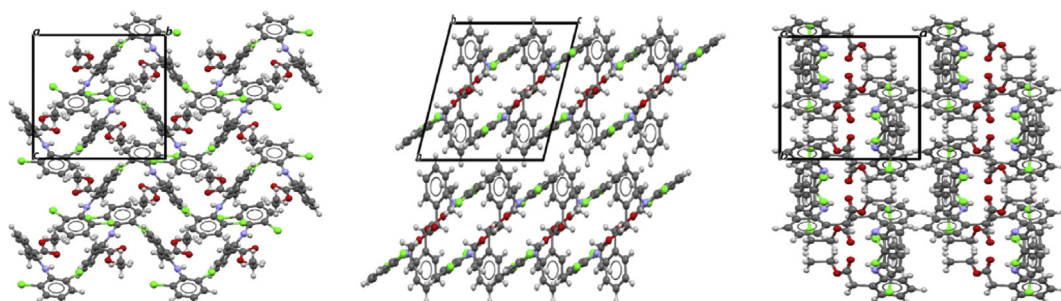
4.5. EDDA cocrystal screening, isolation, characterization, and structure determination

Initially, screening was conducted to fix the molar ratio of the cocrystal, followed by single-crystal isolation, characterization and 3D structure determination.

4.5.1. Screening of the EDDA cocrystal

Screening was conducted to make cocrystals of ethyl diclofenac and its parent acid using the Electrothermal IA9000 system. From the melting temperature data of the binary system in Table 3.

Melting point data then was plotted to a phase diagram, as shown in Figure 9, as follows. The pattern indicated the physical interaction of ethyl diclofenac with its parent acid and formation of a cocrystal at a 1:1 M ratio because this point is in the middle of the curve between the two melting points [20, 26].

4.5.2. Cocrystal isolation

This part of the experiment was aimed to collect cocrystals from ethyl diclofenac with the parent acid using two different methods. In the first method, diclofenac acid and ethyl diclofenac powders at an equimolar ratio were grounded, assisted by solvent dropping, followed by slow evaporation recrystallization to obtain a single crystalline. To brief, as has been mentioned in Introduction, it was named SDGR method. Solvent-drop grinding using 99% ethanol was performed to initiate the interaction, which uses the principle of grinding to increase the energy [27, 28]. The solvent-drop grinding mixture was then recrystallized using ethanol under cold conditions, 6–12°C/1 Atm/RH 72%.

Table 2. Crystallographic data of the ethyl diclofenac crystal from the single crystal X-ray diffraction-assisted Mercury® program.

Parameter	ED system	
Moiety formula	$C_{16}H_{15}Cl_2NO_2$	
Molecule weight	324.19 g/mol	
Temperature (K)	93(2) K	
λ	0.71075 Å	
Crystal system	Monoclinic	
Space group	P2$_1$/c	
Cell unit dimension	a = 10.8554(5) Å	$\alpha = 90.0°$
	b = 11.6850(5) Å	$\beta = 108.2990(10)°$
	c = 12.5092(5) Å	$\gamma = 90.0°$
Volume	1506.49(11) Å³	
Z	4	
Density (calculated)	1.429 g/cm³	
Absorption coefficient	0.434 mm^{-1}	
F (000)	672	
Final R indices [I > 2sigma(I)]	R$_1$ = 0.0350	
CCDC Deposit number	1904253	

Figure 7. Ethyl diclofenac crystal layers on the (left) a-axis, (middle) b-axis, and (right) c-axis.
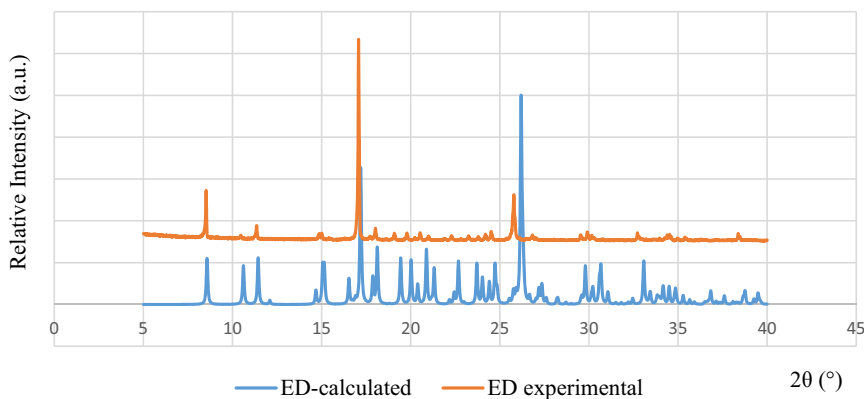
Figure 8. Diffractogram of crystalline ethyl diclofenac (ED) obtained from the experiment compared with the calculated pattern.

Table 3. Melting point data of the ethyl diclofenac - diclofenac acid binary system.

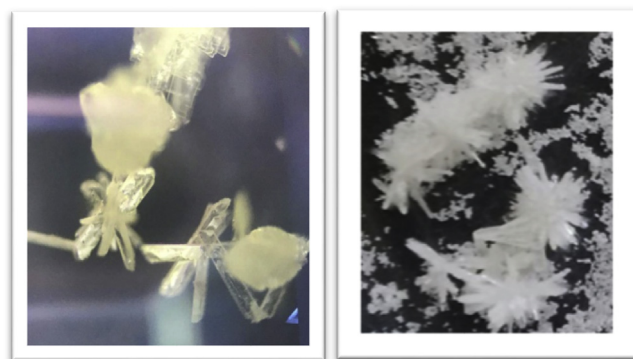| Molar ratio of ethyl diclofenac:diclofenac acid	Melting point (°C) (n = 3)
0:10	69.0 ± 0.3
1:9	67.3 ± 0.5
2:8	62.1 ± 0.9
3:7	70.1 ± 0.8
4:6	82.1 ± 1.2
5:5	102.3 ± 0.2
6:4	110.2 ± 0.4
7:3	115.1 ± 0.4
8:2	112.3 ± 0.7
9:1	155.2 ± 0.2
10:0	173.2 ± 0.2

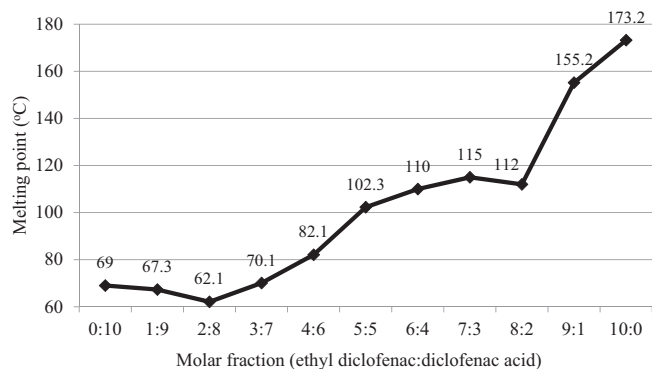**Figure 10.** EDDA crystalline from the SDGR method (left) and Fischer reaction (right).

Figure 9. Phase diagram of the ethyl diclofenac - diclofenac acid binary system.

Figure 11. Thermogram of ethyl diclofenac, diclofenac acid, EDDA-SDGR and EDDA-FR. The melting point of EDDA was revealed to be between that of ethyl diclofenac (67.7 °C) and diclofenac acid (173.1 °C)—that is, 103 °C for SDGR and 104.3 °C for FR yield.

The second method was performed by arranging simultaneous reflux as performed in ethyl diclofenac production, replacing pure ethanol with 95% ethanol. In addition to the replacement of 99% ethanol, all parts of the process were performed similarly to the previous method explained for ethyl diclofenac crystal construction. This method was based on the Fischer reaction, which maintained a small amount of water to facilitate an equilibrium state between ester formation and hydrolysis. It then was briefly named as the FR method.

Yellowish and white rod-shaped single crystals were obtained from the two methods of cocrystal arrangements. The crystal's shape was similar to each other and different from ethyl diclofenac or diclofenac acid, as shown in Figure 10. Thereafter, the purity, powder character,
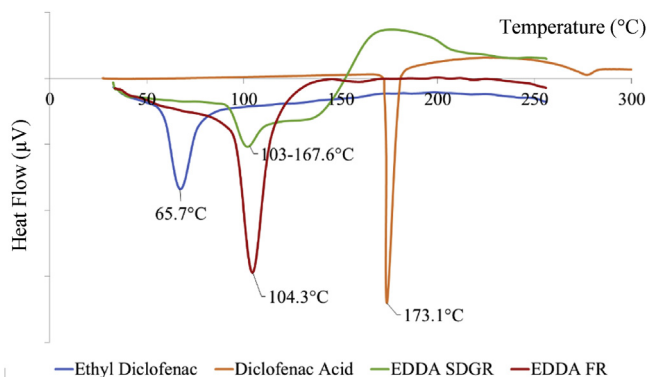
crystal structure, and stability of the cocrystals were analyzed and characterized thoroughly.

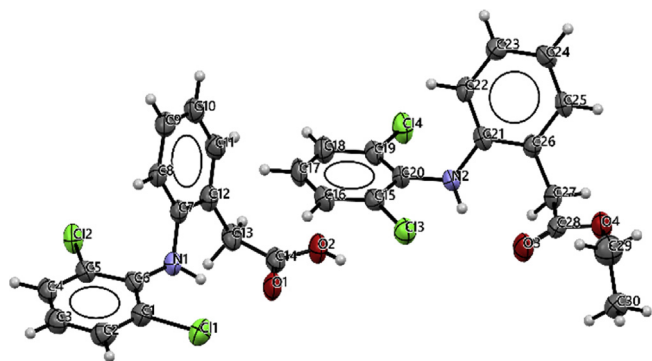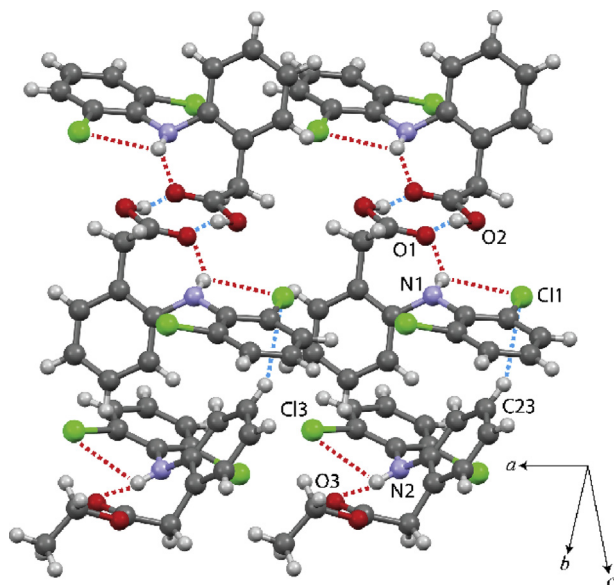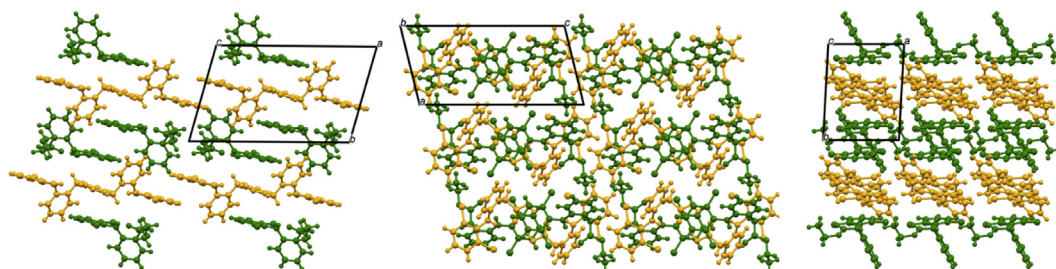Figure 10 shows that the cocrystal yielded from FR method looks more white than SDGR result.

4.5.3. Thermal analysis of EDDA

The thermal profile of cocrystals was examined using differential scanning calorimetry, and thermograms are depicted in Figure 11.

The thermogram in Figure 11 shows the range of melting temperatures of cocrystals (103 °C for SDGR and 104.3 °C for FR yield) compared to those of ethyl diclofenac (67.7 °C) and the acid parent drug (173.1 °C).

Table 4. FTIR data of the cocrystal compared with the physical mixture of ethyl diclofenac and diclofenac acid.

Moiety	λ (cm^{-1})	
	Physical mixture	Cocrystal
C–Cl	771.387	767.53
C–Cl	748.245	744.388
C=O	1704.76	1700.91
N–H•••O/O–H•••N	1897.61	1909.18
N–H•••O/O–H•••N	2595.72	2526.29
N–H	3332.39	3324.68

Table 5. Hydrogen bond data on EDDA.

D–H•••A	D–H (Å)	H•••A (Å)	D•••A (Å)	D–H•••A (°)
N(1)–H(1) •••Cl(1)[a]	0.88(5)	2.57(5)	3.002(4)	112(4)
N(1)–H(1) •••O(1)[a]	0.88(5)	2.10(6)	2.871(4)	146(5)
O(2)-(1A) •••O(1)[#1]	0.96(5)	1.77(6)	2.710(5)	165(5)
N(2)–H(2) •••Cl(3)[a]	0.856(19)	2.65(5)	2.987(4)	105(4)
N(2)–H(2) •••O(3)[a]	0.856(19)	2.27(3)	3.008(5)	145(4)
C(23)-(23) •••Cl(1)[#2]	0.95	2.82	3.668(5)	149.0

[#1] -x+1, y+1, z+1; [#2] -x+1, y, z+1.
[a] Intramolecular hydrogen bond.

Figure 12. The 3D structure of the EDDA cocrystal.

Figure 13. Hydrogen bonding of EDDA.

Table 6. Crystal data of EDDA.

Parameter	EDDA system	
Molecular formula	C$_{14}$H$_{11}$Cl$_2$NO$_2$, C$_{16}$H$_{15}$Cl$_2$NO$_2$	
Molecular weight	620.33 g/mol	
Temperature (K)	173(2) K	
Crystal system	Triclinic	
Space group	P-1	
Cell unit dimension	a = 8.2320(2) Å	α = 74.721(2)°
	b = 10.4496(4) Å	β = 76.443(2)°
	c = 17.4339(5) Å	γ = 89.403(2)°
Volume	1404.26(8) Å^3	
Z	2	
Measured ref	16493	
Independent ref	4941 [R$_{(int)}$ = 0.0750]	
Refined parameter	371	
Goodness-of-fit on F^2	1.066	
Final R indices [I > 2σ(I)]	R$_1$ = 0.0753	
CCDC deposit number	1904270	

4.5.4. EDDA structural study using infrared spectroscopy

The binding interaction was investigated firstly using FTIR. New peaks appeared at 2518.58 cm^{-1} and 1897.61 cm^{-1} and some others shifted to 3332.39 cm^{-1}, 1704.76 cm^{-1}, 771.387 cm^{-1}, and 748.245 cm^{-1} (Table 4). Those shifts represented the strains of N–H, C=O, C–Cl, and the interaction of N–H•••O or O–H•••N, respectively.

4.5.5. Crystallographic study of EDDA

Diffractograms were explored to confirm cocrystal formation. The diffractogram of EDDA has different patterns from that of the physical mixture of EDDA with distinctive peaks at 2θ: 5.74, 10.94, 14.27, 16.65, 20.36, 21.66, and 25.16°. The 3D crystal structure of EDDA was determined using single crystal X-ray diffraction to determine the accuracy of all hypotheses. A clear single crystalline was selected to be analyzed. Due to the high stability of EDDA, the analysis does not need any special condition and can be treated in the ambient temperature and pressure (measured as 20 °C, 1 Atm). The result is illustrated in Figures 12, 13, and
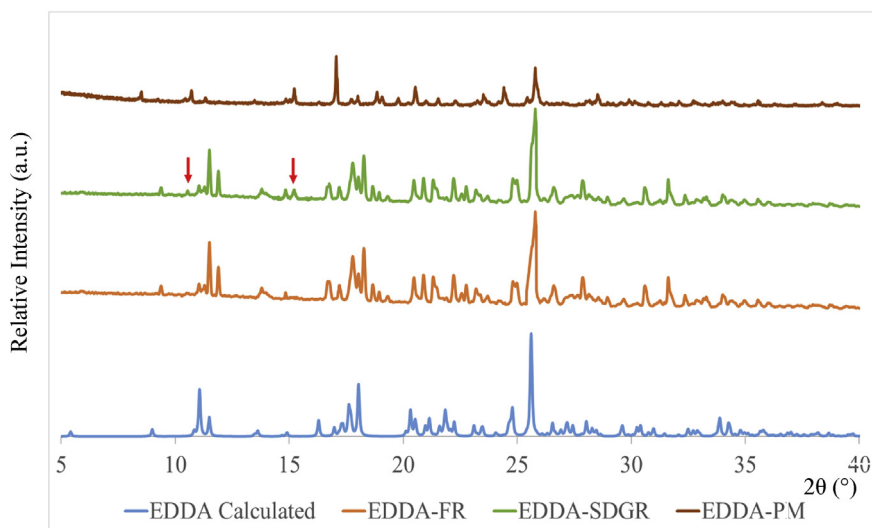

Figure 14. The 3D structure layer of ethyl diclofenac (green) with diclofenac acid (orange) on the (left) a-axis, (center) b-axis, and (right) c-axis.

Figure 15. Diffractogram comparison of EDDA-calculated and EDDA-FR with EDDA-SDGR and EDDA-PM.

14. Meanwhile, the intermolecular bonds and EDDA lattice system data are listed in Tables 5 and 6, respectively. This new cocrystal data has been submitted to CCDC with the deposit number 1904270.

The structural analysis result in Figure 12 depicts the 3D configuration of EDDA completed with element labeling. Meanwhile, Figure 13 reveals the hydrogen bonds in the structure. The red cycle points to the bonding location.

The cocrystal in Figure 13 was revealed to have several hydrogen bonds between the moieties. The type and strength of each hydrogen bond are shown in Table 5. Based on the data, another type of bond also existed, the T-shaped C–H•••π interaction, which binds the dichlorophenyl moiety (from ethyl diclofenac) with H-phenyl (of diclofenac acid). Halogen binding to the H-phenyl is classified as a weak hydrogen bond and collaborates with the presence of strong hydrogen bonds such as OH•••O, NH•••O, NH •••Cl and other van der Waals bonds [11].

The crystallographic data of EDDA are listed in Table 6.

Figure 14 depicts the structure layer of the EDDA crystal, which shows a different appearance when viewed from the a-axis and c-axis, represented as a P-1 triclinic system.

Moreover, to confirm the solid experimental crystalline structure, Figure 15 reveals that the experimental data matched the calculated diffraction pattern. Compared with the calculated diffractogram, the pattern of EDDA-SDGR and EDDA-FR were similar.

4.5.6. Stability study against heating and high relative humidity

The stability test monitored the change of samples using infrared spectra data revealed in Figure 16. The first specific peak of the EDDA cocrystal is depicted at 3324 cm^{-1} (noted by "I" in Figure 16A), which is a result of the interacted shifting of N–H at 3322.75 cm^{-1} and 3293.82 cm^{-1} from the physical mixture spectrum, which is placed at the top of the overlaid spectra. Meanwhile, the second spectrum (II) at 1700.91 cm^{-1} was a new spectrum generated from the interaction of the two spectra of C=O from the starting material, originally laid at 1710.55 and 1698.02 cm^{-1}. The more clear position was revealed in Figure 16 B as the zooming on the determinant peak.

Storage under high humidity for 7 days (RH 84%/25 °C) showed no changes in the physical appearance of EDDA. Infrared data revealed that the spectra of samples showed persistence under the high humidity test, as shown in Figure 17.

To ensure the chemical stability of the drug, thin layer chromatography was performed to check the changes during the test, especially after microwaving. All the results showed a similar pattern on the plate chromatograms that consisted of 2 spots, ethyl diclofenac and diclofenac

acid, after the stability test. These data proved that the EDDA cocrystal is chemically stable, as shown in Figure 18 below.

4.5.7. Anti-inflammatory test

A test was also conducted to evaluate the effect of cocrystallization on the antiinflammation effect. The result is very interesting, revealing synergic activity. Figure 19 describes the different pattern of anti-inflammatory inhibition.

5. Discussion

We managed to obtain ethyl diclofenac from an optimized method, as explained in the *Method and Material (*subsequence *4.1)*. Figure 1 depicts the yield of ethyl diclofenac crystal appearance, which totally different from its acid parent drug. The thin layer chromatography used three mobile phase systems (Figure 2) obtained ethyl diclofenac crystal was chemically pure. All chromatograms only showed one spot after elution, confirming the complete reaction between diclofenac acid with ethanol to produce the pure ethyl diclofenac. In the thermal analysis using differential scanning calorimeter, a similar melting temperature was observed between the obtained ethyl diclofenac and published data, that is ±67.5 °C [2], as revealed in Figure 3. With the small range of melting point, it also can be concluded that the ester yielded was pure. The data then was confirmed by 2D structure by infrared spectra, which indicated the disappearance of carboxylic moiety and the occurrence of the ester group. This result was also supported by ^{1}H nuclear magnetic resonance, which showed triplet peaks at 1.24–1.27 ppm (CH$_3$) and quartet peaks at 4.16–4.2 ppm (OCH$_2$), indicating ethyl ester moiety, as well as the absence of –OH singlet peak which supposedly appear in carboxylic acids [2]. As a conclusion from this step, the 2D structure of ethyl diclofenac was confirmed.

The crystallographic data are needed to be collected due to its importance of solid character on the physicochemical properties that may influence drug's performance, explained previously as being related to stability, hygroscopicity, compacting property, flow, solubility, and absorptivity [11, 12, 13, 14, 15, 16]. Figure 4 showed a new diffractogram profile of ethyl diclofenac, which never been listed before in the *Cambridge Crystallographic Data Center,* thereof it can be stated that a new phase was found.

The next study was performed using single crystal X-ray diffractometry, which is revealed the data illustrated in Figure 5 and Figure 6. The 3D molecule position was depicted in Figure 5 and the interaction which was presented in Figure 6, involves weak hydrogen bindings, and also are
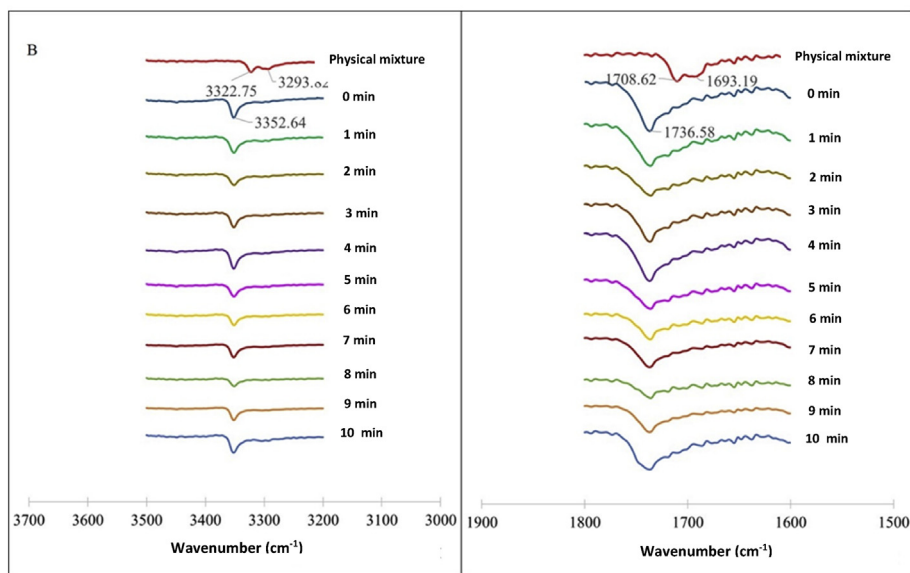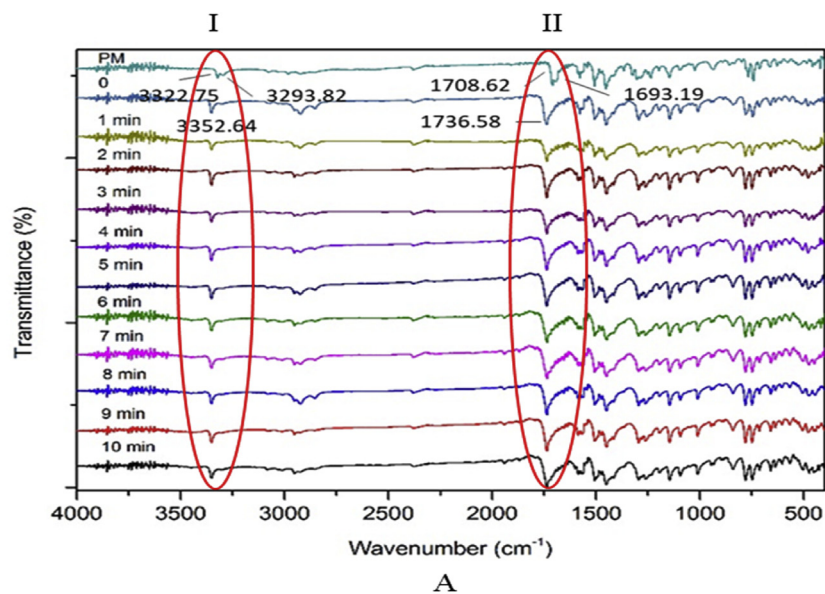
Figure 16. A. Infrared spectrum of EDDA from microwave-heating (399.5 W) stability, at the top position is the physical mixture. B. The distinctive spectra of the EDDA cocrystal (I and II). Noted: PM = physical mixture; 0 min = EDDA before heating.

listed in Table 1, causing the melting point of ethyl diclofenac to become relatively low. Thus, it is suggested to store ethyl diclofenac in the refrigerator due to its instability towards heating. Although the stability study of ethyl diclofenac in high humidity was not performed yet, according to Waterman [29], ester is a potentially hydrolyzable functional groups, therefore, ethyl diclofenac is predicted sensitive to moisture and tend to hydrolize and produce its carboxylic acid. However, the further stability study of EDDA cocrystal, we found that ethyl diclofenac in the EDDA cocrystal was stable in high humidity in 7 days presenting as one spot of TLC analysis in Figure 18. Therefore the EDDA cocrystal formation is indicate to enhance the stability of ethyl diclofenac toward moisture. Investigation of stability ethyl diclofenac in comparison with its cocrystal is suggested in advance.

The shape of space configuration depicted in Figure 7 is different only on the b-axis appearance (in the middle of the figure). When it is correlated to the crystal data in Table 2, edge β = 108.299, while the other edges are as follows: α = γ = 90.0°. Thus, the data confirmed that the ethyl diclofenac crystal is a monoclinic system with a different β angle. Furthermore, diffractogram of the ester crystal obtained from the

experiment matched calculated data (Figure 8). Therefore, we can conclude that ethyl diclofenac was successfully isolated. The new crystal structure then was submitted into CCDC with deposit number 1904253.

The discussion is then continued with cocrystal screening and composing results. Based on the data in Table 3, which plotted into a phase diagram in Figure 9, two eutectic points, at molar ratios of 2:8 and 8:2, were observed, forming a "W" profile and indicating the formation of a molecular interaction [20, 30]. As shown in Figure 9 the center of the "W" curve is a 1:1 ratio. Several investigation in cocrystal formation, the stoichiometry ratio of cocrystal component correspond to the "W" center point [31, 32, 33]. Therefore, the stoichiometry ratio of ethyl diclofenac and diclofenac acid is predicted as 1:1 M ratio. This prediction is confirmed by single crystal analysis that showed the stoichiometry cocrystal component of EDDA is 1:1 ratio.

Afterward, SDGR and FR were used to isolate EDDA cocrystal. Solvent dropped grinding was conducted to facilitate the intermolecular bonding before recrystallization [34, 35]. Meanwhile, the equilibrium condition in FR method was created by maintaining the existence of only a small amount of water to produce EDDA spontaneously. As a result, the
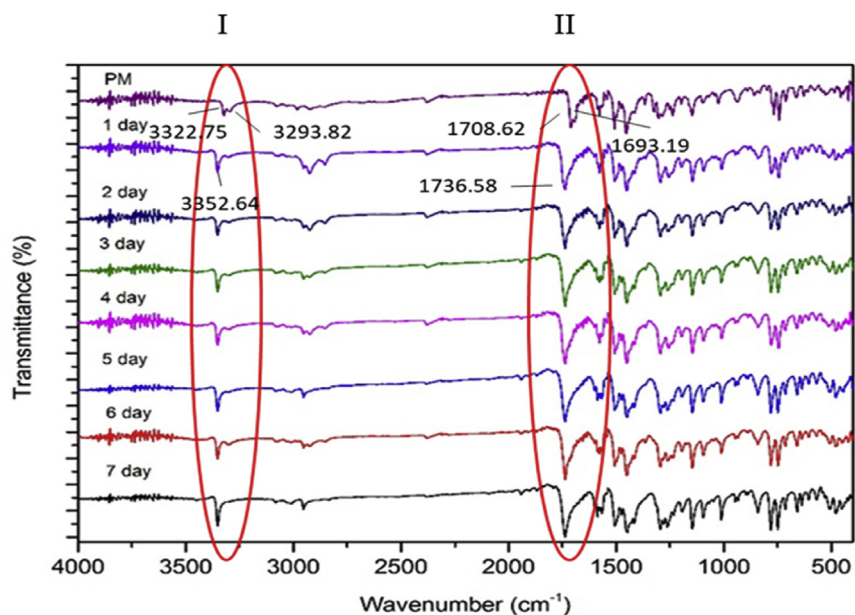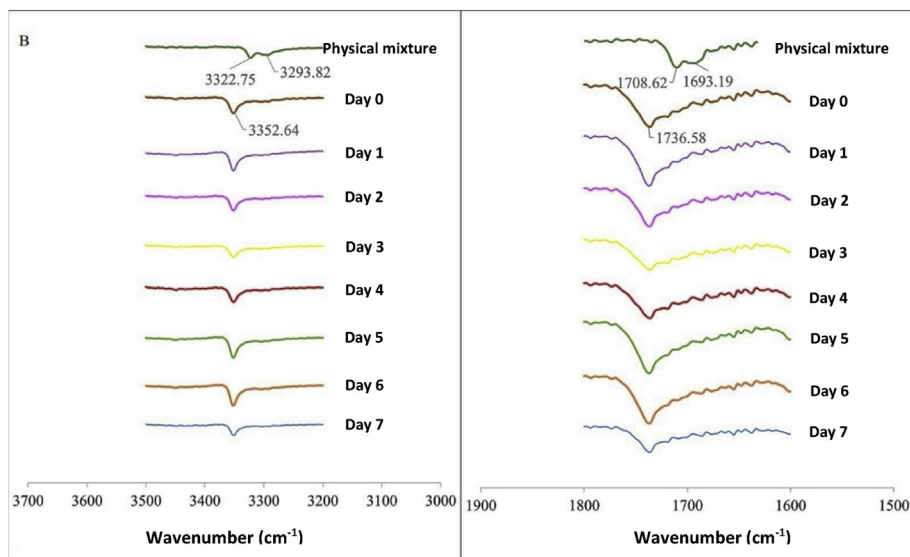
Figure 17. A. Infrared spectra of EDDA during storage at 84% RH/25 °C, day to day. At the top is the physical mixture spectrum; B. The distinctive spectra of EDDA cocrystal (I and II). Noted: EDDA-PM = physical mixture of ethyl diclofenac with diclofenac acid; Day 0 = EDDA before heating.

yellowish - rod-shaped crystals were obtained from SDGR, and the white one from the FR method, as revealed in Figure 10. However, the appearance of both results was similar. The brighter appearance of FR's crystal was predicted due to the higher purity.

Cocrystals from both methods also have a similar thermal pattern, with their endothermic peaks at 103–104 °C (Figure 11). However, the SDGR cocrystal still has the starting material's peak, indicating that the SDGR cocrystal was not pure, as shown by the very broad endothermic curve and existence of a diclofenac acid peak residue at 167.6 °C. Some retrials were attempted; however, the results were almost the same and still need further optimization. Nevertheless, pure EDDA could be collected successfully from the FR method, as indicated by a single endothermic peak at 102–104 °C in the thermogram. The higher purity of the EDDA from FR was predicted due to the heating during esterification supporting the homogeneity and kinetics of the molecules and solvent. Thus, the interaction between two substances can be constructed by FR more intensively than the SDGR method. Due to its purity, the cocrystal

from the FR method was chosen for solid characterization and structural study using the single crystal x-ray diffractometer.

The interaction site inside EDDA was studied two-dimensionally using infrared spectroscopy and three-dimensionally using single crystal diffractometry. The spectrum in Table 4 shows a new peak at the wavenumbers 1909.18 cm^{-1} and 2526.29 cm^{-1}, indicating hydrogen bonds of N–H•••O or O–H•••N. The wavenumber shifts occur at 3324.68 cm^{-1} (strain of N–H), 1700.91 cm^{-1} (strain of C = O), and at 767.53 cm^{-1} and 744.388 cm^{-1} (C–Cl bonding). The shift of the N–H strain is shown in diclofenac acid. The C–Cl shift occurs at two peaks, the first is caused by ethyl diclofenac, and the other is caused by its parent acid. Van der Waals and hydrogen bonds are the most common interactions between compounds to construct a cocrystal [13, 16]. However, these data indicate that the molecular interaction between ethyl diclofenac and diclofenac acid involves the hydrophobic interaction of C–Cl.

The 3D data resulted in Figures 12, 13, and 14 reveal the final cocrystal structure, the hydrogen, and the 3 axes appearance of the cocrystal,
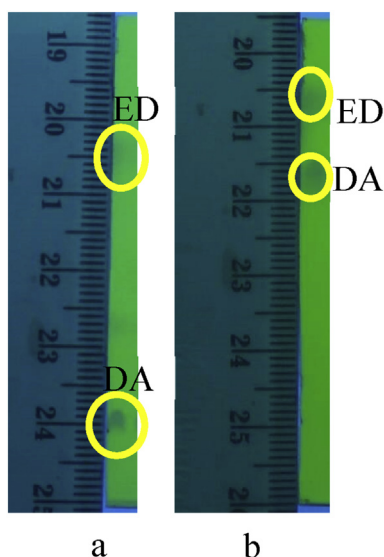
data, it can be concluded that a novel cocrystal from ethyl diclofenac and its parent acid can be arranged intentionally by SDGR or spontaneously during FR esterification. However, the second method, FR, is superior.

Some references have reported that the interaction will be degraded and separated into its components under high temperature and a humid environment [17, 36, 37, 38, 39]. Thus, we performed a simple stability study to evaluate the significance of EDDA towards heating and high humidity storage. The sample was then analyzed by infrared spectroscopy which revealing that EDDA was stable under 10 min of microwaving. If 1W = 1 J/s, it means that the energy involved was (399.5 × 10 × 60) J = 239.7 kJ. As noted, in that time ethyl diclofenac has been melted. Nevertheless, no changes were observed in the two cocrystal distinctive peaks at 3351.68 cm^{-1} and 1737.55 cm^{-1}, which are marked with red circles (Figure 16). A higher melting point of EDDA indicates better stability towards external energy compared with the ester alone.

No principal changes were observed between the EDDA cocrystal sample before and after 7 days of storage in high humidity. As identified by FTIR, the interaction was not reverted to its physical mixture, which is presented on the top of Figure 17. Also, there are not the other new peaks, indicated that no degradation product was yielded. By the addition interaction with an ester, diclofenac acid will more resistance toward humid. Moreover, the thin layer chromatogram in Figure 18 revealed that EDDA was stable by showing that the spots of both compounds, the ethyl and the acid, appear clear with no other spots. Briefly, EDDA showed the appropriate stability, which higher than ethyl diclofenac toward heating and better than diclofenac acid on its humid absorption property. Totally, this cocrystal is expected to combine the advantage of the starting compounds.

This new cocrystal data hopefully will be useful for subsequent studies about the production process of other synthetic ester drugs. On the one hand, this study provides information to understand how to handle cocrystallization as a side product because it may occur in some cases [5, 6, 7]. From another perspective, a cocrystal arrangement is one of the leading terms in the new approach to drug development. As known widely, the physicochemical improvements will follow the crystal change [9, 10, 11, 12, 13, 14, 15, 16, 17, 36, 37, 38, 39].

In addition, as stated in a reference [3], ethyl diclofenac has a lower bond with plasma so that the free drug in blood will be higher, causing better activity. All combinations of properties between the ester as a pro-drug with the parent are prospective to be used for drug development. The anti-inflammatory test is shown in Figure 19, which depicts that EDDA has the highest activity, followed by the physical mixture, ethyl diclofenac, and diclofenac acid. All the data were checked by 2-way ANOVA, followed by Dunnett's and Tukey's test, which revealed a significant difference in the response between the groups [40]. The reason for this phenomenon is assumed to be the following. The activity of ethyl diclofenac was reported to be more potent than the parent drug due to its
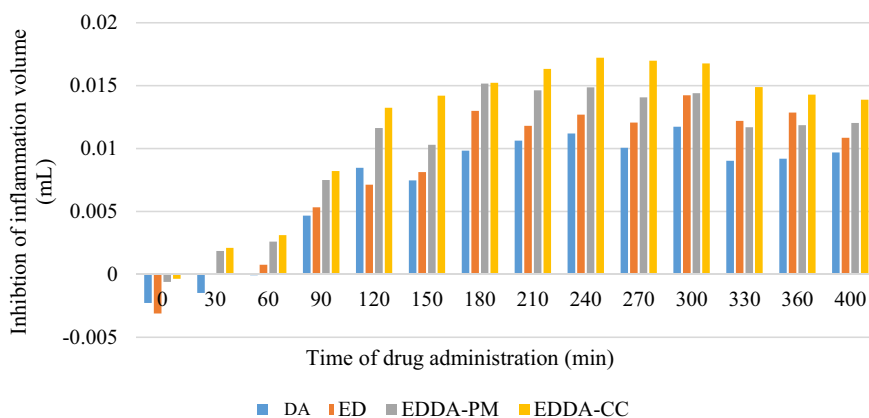

Figure 18. Chromatogram of EDDA after the stability test under heating (equals with the result of stability test toward humidity), from the mobile phase: a) n-hexane-ethyl acetate (8:1) and b) toluene-ethyl acetate (1:2). Viewed under a UV lamp at 243 nm. Noted: ED: ethyl diclofenac, DA: diclofenac acid.

respectively. Figure 12 revealed the 1:1 interaction between ethyl diclofenac with its acid parent drug, co-arranged a new phase. The hydrogen bonds which are in the form of the intramolecular interactions N–H•••Cl and N–H•••O in both ethyl diclofenac and its parent acid form are explained in Figure 13 and supported by the data in Table 5. Additionally, there are hydrogen bonds in O–H•••O (strongest hydrogen bond) and C–H•••Cl (hydrophobic bond), confirmed the infrared data. Figure 14 illustrates the different appearances of EDDA lattice when viewed from 3 axes, reflecting the three different degrees of the crystal edges. The cocrystal consists of ethyl diclofenac and its parent drug in the intermittently ordered layer. In addition, Table 6 revealed EDDA as a triclinic crystal system with a larger volume than ethyl diclofenac —that is, 1404.26(8) Å^3 versus 1506.49(11) Å^3.

In powder X-ray diffractometry, a small difference was revealed between the diffractogram from the experimental data with the calculated pattern. A peak at 2θ = 20.41° of the calculated diffractogram appeared at 20.16° in the experimental crystalline data of EDDA-FR result (Figure 15). Nevertheless, the diffractogram of EDDA from SDGR still remained some parent peaks, as marked in Figure 15, due to incomplete cocrystal formation. This result is in line with thermograms that showed endothermic peaks of the starting material. From all the crystallographic

Figure 19. Inflammation volume-inhibition of the cocrystal (EDDA-CC) compared with each component ED (ethyl diclofenac), DA (diclofenac acid), and the physical mixture (EDDA-PM). Note: n = 6.

lower plasma binding in the blood [1, 2]. The physical mixture also shows the increasing effect than both single starting materials, but it is lower than the cocrystal. The cause may be the increasing acidity in the gastrointestinal tract due to the presence of diclofenac acid affects the solubility and absorptivity of the drug. However, compared with the more ordered and regularly lattice structure of the EDDA, the physical mixture has no intermolecular bonds which can increase the effect more.

Based on the cocrystal structure in Figure 14, the crystal layer comprising the non-polar ethyl diclofenac and its polar-parent acid that causes EDDA to act as a semipolar compound, which commonly has higher absorption capacity than the polar system. However, by having the diclofenac layer as the polar site involved in the interaction, the solubility of ethyl diclofenac will increase. Furthermore, EDDA will separate into its constituents in the intestinal liquid and will enter and be absorbed there. Based on its pKa = 4.18 [41], the acidic form will be absorbed more promptly.

Additionally, EDDA can be absorbed in the two forms, the unchanged ethyl diclofenac, and its parent drug after the hydrolysis reaction. The combination of the immediate release of diclofenac acid with the prolonged action and lower plasma-binding of ethyl diclofenac, enhance the activity. Nevertheless, these explanations are still assumptions. The synergic performance of ethyl diclofenac and its parent drug is probably also based on an unknown mechanism that needs to be investigated further, thoroughly. Other than the oral dosage form, the EDDA cocrystal can be suggested to be formulated for topical or transdermal preparation, which may control the release of diclofenac into the blood system.

6. Conclusion

The 3D structure of ethyl diclofenac has been solved, as well as its new cocrystal with its parent drug, diclofenac acid, namely EDDA. The Fischer reaction is the best method to produce the cocrystal. Crystallography data revealed that the ethyl diclofenac crystal system reveals a P*21/c* monoclinic lattice, while the cocrystal is a P-*1* triclinic. Ethyl diclofenac and EDDA structures were recorded as CCDC deposit no. 1904253 and 1904270, respectively. This cocrystal constructed a typical hydrophobic intermolecular interaction C–H●●●π between dichlorophenyl and phenyl besides the hydrogen bonds. Physically, EDDA has a higher melting point than ethyl diclofenac and, consequently, is more stable towards heating. On the other hand, by its lipophilic interaction, it was proven to be more resistant from water adsorption in the highly humid environment than diclofenac acid. Briefly, the cocrystal can combine the advantage of each component and then improved the anti-inflammatory activity of diclofenac acid and ethyl diclofenac. This report hopefully provides valuable scientific information to control the esterification process or to be employed for drug development from another perspective.

Declarations

Author contribution statement

Ilma Nugrahani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Rahel Hasianna, Aisyah Amalia: Performed the experiments; Analyzed and interpreted the data.

Yuda Prasetya Nugraha: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hidehiro Uekusa: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Dwi Utami: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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