Evaluation of stable and metastable forms of acetaminophen using a combination of X-ray diffraction, thermal analysis, and electron diffraction

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Abstract

It is known that pharmaceutical raw materials can undergo phase transitions to other crystal polymorphs due to temperature and humidity, and that differences in crystal structures affect the bioavailability and safety of pharmaceutical products. Acetaminophen, which has been used worldwide for more than 100 years for a variety of treatments, has been reported to have multiple stable and metastable crystal polymorphs however, the details of its crystallization control, evaluation methods, thermal behavior, and some crystal structure information are not known very well. In this article, a combination of several different evaluation methods using Rigaku technologies was applied to the analysis of crystal structures and physical properties of acetaminophen crystal polymorphs. As a result, the thermal behavior and crystal structure of acetaminophen were clarified by the combination of these analyses, and the details are described herein.

1. Acetaminophen

Acetaminophen is a para-aminophenol dielectric synthesized in Germany in 1873. It was first used medicinally by von Mering in 1893 and was used in earnest as an antipyretic analgesic starting in 1949, when it was shown to be the active metabolite of acetanilide, also known as phenacetin. In the late 19th century, aminovirine was widely used as an antipyretic analgesic along with acetaminophen. However, acetaminophen, which is safer and has fewer side effects, attracted attention because aminovirine had side effects of possible carcinogenicity in the gastrointestinal tract and liver dysfunction, which were considered problematic. Currently, the World Health Organization (WHO) lists acetaminophen as an essential drug, and national guidelines also list it as the first-line drug for analgesic pharmacotherapy[1]. The Japanese Society of Obstetrics and Gynecology recommended acetaminophen as an antipyretic analgesic during pregnancy because it was evaluated to be safer than nonsteroidal anti-inflammatory drugs (NSAIDs), as shown in Table 1. Since 2011, the approved dosage in Japan was raised to a maximum of 4,000 mg per day, the same dosage as in major overseas countries[2]. Furthermore, it is recommended for the treatment of fever caused by coronavirus disease- (COVID-19), and its use as a diverse therapeutic agent is expected to increase in the future. While it is safe as an anti-inflammatory drug, its mechanism of action for antipyretic analgesia is thought to be inhibition of cyclooxygenase in the central system; however, the details are still unknown[3],[4].

2. Crystal Polymorphism of Acetaminophen

Crystal polymorphism is the term used when a compound has different crystal structures but the same chemical formula. It is often present in compounds that are drug substance candidates for pharmaceutical products. The packing arrangement of molecules is different for individual crystal polymorphs of a material, causing them to exhibit different physical properties that may affect the stability, industrial properties and bioavailability of active pharmaceutical ingredients[5].

Table 1. Differences between acetaminophen and NSAIDs

	Acetaminophen	NSAIDs
Mechanisms of action	Inhibition of cyclooxygenase primarily in the central nervous system of the brain.	Suppression of prostaglandins by inhibition of cyclooxygenase.
Antipyretic and analgesic effects	Yes	Yes
Anti-inflammatory effect	Few	Yes
Side effects	Liver damage rarely occurs	Asthma attack, renal dysfunction, digestive symptom, etc.
Recommended age	From under 15 years of age	15 years of age or older

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Table 2. Crystal structure information for the crystal polymorphs of acetaminophen[7]

	Form I	Form II	Form III	
Crystal system	monoclinic	orthorhombic	orthorhombic	monoclinic
T/K	330	298	293	100
Space group	$P2_1/a$	$Pcab$	$Pca2_1$	$Pc11^e$
Z/Z'	4(1)	8(1)	4(2)	2(4)
a/Å	12.872(3)	7.405(3)	11.838(1)	11.755(1)
b/Å	9.370(2)	11.831(4)	8.569(1)	8.572(1)
c/Å	7.085(2)	17.156(6)	14.818(1)	14.516(1)
α/°	90	90	90	84
β/°	116	90	90	90
γ/°	90	90	90	90
V/Å^3	771	1503	1503	1455

In recent years, the molecular structures of pharmaceuticals have tended to become more complex, and the existence of stable but less bioactive crystal polymorphs has become apparent after some products went to market, forcing, for example, the suspension and redevelopment of Ritonavir[6].

In addition, polymorphism has created a number of patent controversies, as it is possible to obtain rights to the invention of a crystal polymorph if it has a novel crystal structure and advantageous properties compared to an already-documented substance, even if the substance is publicly known. Crystal polymorphism is therefore one of the points of attention in preformulation studies, including physical property testing and process studies.

Table 2 lists the crystal polymorphs of acetaminophen[7]. It has three crystal polymorphs, forms I, II and III, and two different crystal systems have been reported for form III. Form I, which has a stable crystal structure and crystallizes easily from solution, is used as a pharmaceutical raw material for a central antipyretic analgesic antiphlogistic drug. Form II has higher solubility than form I and therefore has better compressibility and solubility in tablet formulation[8]. Form III is a metastable form that exhibits even higher solubility than form II. Form III crystallizes only under limited conditions and is known to be highly unstable. It is reported to undergo a phase transition to other crystalline polymorphs within a short time after crystallization[9]. Therefore, controlling the crystallization of form III and evaluation methods for form III are challenging. For example, the unit cell of form III was reported by Céleste A. Reiss et al. in 2018 to be either orthorhombic or monoclinic, and the Cambridge Structural Database (CSD) provided by the Cambridge Crystallographic Data Centre in the UK has only two entries with crystal information. Both were determined using powder X-ray diffraction (XRD) results, and there are still no reported cases of single crystal structure analysis.

3. Evaluation of Crystal Polymorphism of Acetaminophen

In the International Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use guideline 'Q6A', XRD and differential scanning calorimetry (DSC) are described as physicochemical measurements used to confirm the presence of crystal polymorphism. Both techniques are widely used for cases in which the formed crystal phases differ due to differences in the measurement environment, and where it is difficult to distinguish crystal polymorphism or discover a new form using a single measurement only[10]. An instrument for combined X-ray diffractometry and DSC measurement enables simultaneous measurement (hereinafter simultaneous XRD–DSC measurement, product name: X-ray DSC) of the same sample under the same conditions, making it possible to observe changes in crystal polymorphism and thermal behavior at the same time. It is also possible to perform single-crystal structure analysis using electron beams to precisely analyze very small single crystals. In this paper, the crystal polymorphism and thermal behaviors of acetaminophen are clarified by using a combination of XRD, DSC and electron diffraction.

4. Evaluation of Acetaminophen Using Simultaneous XRD–DSC Measurement

4.1 Simultaneous XRD–DSC measurement using two-dimensional semiconductor detector "HyPix-3000"

The X-ray DSC attachment, used to simultaneously perform XRD and DSC on an X-ray diffractometer, was developed by Rigaku. It enables in-situ observation of thermal events such as crystal phase transitions, dehydration, melting, and solidification of samples and the accompanying changes in crystal structure, making it possible to conduct research that relates physical properties and structure on a one-to-one basis. In addition, the use of a two-dimensional detector can

rapidly capture changes in crystal structure.

One-dimensional (1D) detectors are widely used in X-ray diffraction experiments to measure diffraction angles and intensities. In this method, the diffraction lines are measured by moving the goniometer while keeping the angles of the X-ray source and detector equal. When using a 1D detector, this process involves a motion from the end angle back to the start angle, making it possible to miss changes to the sample during that time. In contrast, the use of a two-dimensional (2D) detector allows for exposure measurements with the goniometer axes fixed, thus capturing sample changes over a short period of time. Furthermore, diffraction lines that exist not only in the vertical direction of the detector plane but also in the circumferential direction can be acquired as a two-dimensional image. Thus, the 2D detector can simultaneously acquire information on coarse grains and preferred orientation. Especially for samples containing coarse grains, the coarse grain vibration caused by thermal vibration during heating, variation at melting temperature due to grain size and particle flow in the liquid easily occurred. For data interpretation on samples with coarse grains, measurement with a 2D detector is very effective. Acetaminophen form I often forms coarse grains, and its crystal structure is easily destroyed when the sample is shaped by grinding. Therefore, simultaneous XRD–DSC measurement with a 2D detector is essential for form I.

Figure 1 shows a schematic diagram and a photograph of the apparatus configuration for simultaneous XRD–DSC measurement using a 2D detector. The instrument used was a SmartLab automated multipurpose X-ray diffractometer, and the detector was a HyPix-3000 two-dimensional semiconductor detector, with a rectangular detection surface. This detector was installed vertically so that the detection plane is long relative to the scanning direction.

With this installation method, a two-dimensional image of an angular range of up to approximately 30° can be obtained in a single exposure without moving the goniometer (distance from the sample to the detector: 150 mm). The X-ray DSC attachment keeps a reference sample on one side and a measurement sample on the other side. On the reference sample side, a 4.5 mg aluminum plate was placed on a 7 × 7 mm square aluminum pan. On the measurement sample side, 4.98 mg of acetaminophen (Sigma-Aldrich) was prepared on the same aluminum pan as the reference sample side. The control software used was the XRD Measurement plugin of the SmartLab Studio II integrated X-ray analysis software.

4.2 Results of simultaneous XRD–DSC measurements of acetaminophen form I

The simultaneous XRD–DSC data sets of acetaminophen form I were analyzed with the XRD–DSC plugin of SmartLab Studio II. The results are shown in Fig. 2. The upper graph shows heat flow and temperature on the vertical axis and time on the horizontal axis, while the lower graph shows a 2D diffraction image at each temperature. This is the result of a measurement in which form I was heated from room temperature to 190°C (first heating process, 1st RUN), then cooled to −20°C, and then heated again

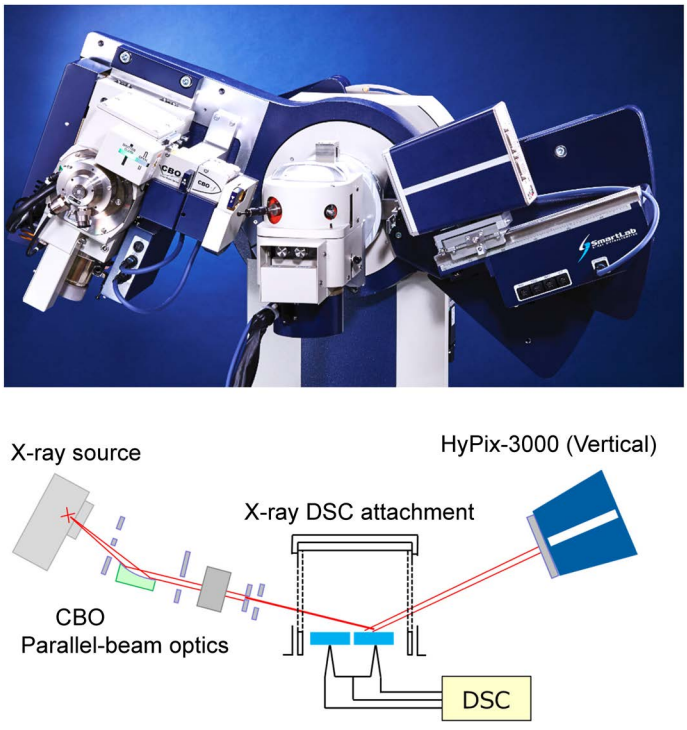

Fig. 1 Photograph (top) and schematic diagram (bottom) of the configuration for simultaneous XRD–DSC measurements using a two-dimensional detector.

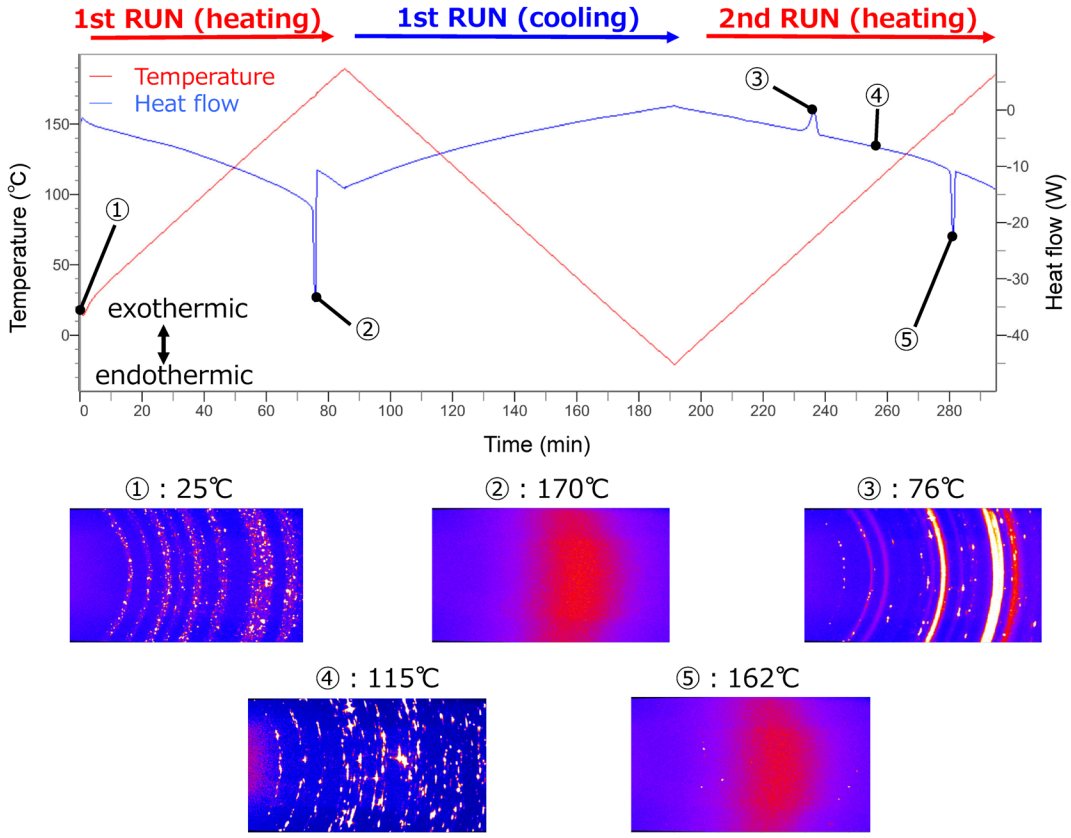

Fig. 2 DSC chart obtained from simultaneous XRD–DSC measurements (top) and two-dimensional diffraction images at various temperatures (bottom).

to 190°C (second heating process, 2nd RUN). The DSC chart obtained from the simultaneous XRD–DSC measurement showed one endothermic peak during the 1st RUN and one endothermic and one exothermic peak during the 2nd RUN.

The 2D images showed spot-like diffraction images at the start of the measurement, indicating that the powder particles formed coarse grains rather than fine grains. Although not shown here, a qualitative analysis of the two-dimensional image was performed. The analysis was performed using the Powder XRD plugin in SmartLab Studio II.

The program has the ability to display Debye rings in a linear fashion for each 2θ angle value based on the information in the two-dimensional image and, furthermore, to automatically convert them into a one-dimensional diffraction pattern. At the beginning, the coarse-grained powder sample was definitely form I from the qualitative analysis.

In the second place, the 2D images were focused on near each thermal reaction peak. At 170°C, where the endothermic peak of the DSC chart was observed during the 1st RUN, the diffraction peaks observed initially were no longer observed, and a halo pattern was confirmed. Therefore, it can be assumed that the endothermic reaction is due to the melting of the crystal phase. The exothermic peak observed at around 76°C during the 2nd RUN, in which the sample was cooled and then heated again, indicates that crystallization has occurred according to the 2D diffraction image obtained at around 76°C. The peak position of the diffraction pattern showing coarse grains is different from that of the initial crystal phase, indicating that a crystal phase different from form I has grown. Upon further heating, the 2D image changed around 115°C, although no change was seen in the thermal profile. Qualitative analysis was performed on the 2D images near 76°C and 115°C during the 2nd RUN. The results of the qualitative analysis are shown in Fig. 3. The upper, middle and lower parts of the figure show the 2D image, the one-dimensional diffraction pattern and the diffraction patterns of form II and III as recorded in the CSD, respectively.

Form II and III were identified at 76°C during the 2nd RUN, and only form II was identified at around 115°C. This indicates that form I crystallizes into forms II and III by reheating, then form III melts or changes to form II, and form II melts at around 162°C. In order to investigate the details of the thermal behavior around 76°C, where form II and III were both observed, DSC measurements were performed with the sample sealed as well.

5. Evaluation by a Single DSC Measurement

DSC was measured using a Thermoplus EVO3 DSCvesta2 equipped with a refrigerated cooling unit (Fig. 4). The temperature conditions were as follows: heating from −30°C to 200°C at 5°C/min (first heating

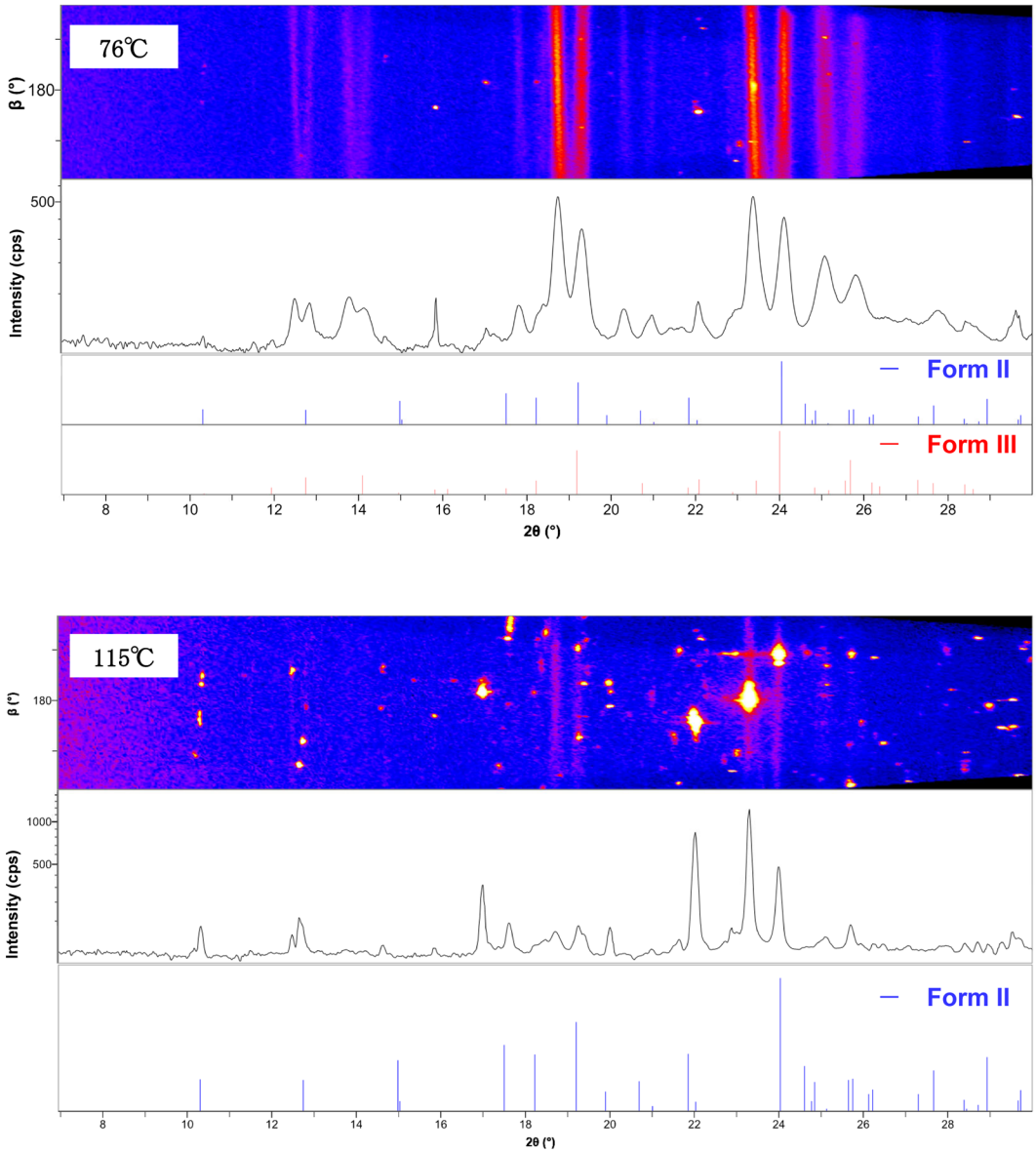

Fig. 3 Quantitative analysis results at 76℃(top) and 115℃(bottom) in 2nd run (heating)

process, 1st RUN), then cooling to −30℃ in 50℃/min, followed by heating to 200℃ at 5℃/min again (second heating process, 2nd RUN). The results of the DSC measurements are shown in Fig. 5. An endothermic peak appeared at 170℃ during the 1st RUN, while exothermic peaks appeared at 75℃ and 121℃, followed by the endothermic peak at 158℃ during the 2nd RUN. The melting temperatures of the sample in the 1st RUN and the 2nd RUN are different, suggesting that the crystalline phases in the two processes are different. These results are also consistent with those observed from simultaneous XRD–DSC measurements, where the endothermic peak at 170°C in the 1st RUN is attributed to the melting point of form I, while the endothermic peak at 158°C in the 2nd RUN is attributed to that of form II. The exothermic peak at 75°C during the 2nd RUN was also confirmed in the simultaneous XRD–DSC measurement and is an exothermic reaction due to crystallization of form II and III. On the other hand, the exothermic peak observed at

Fig. 4 Thermoplus EVO3 DSCvesta2

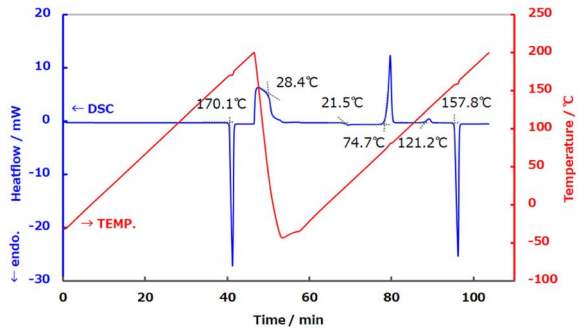

Fig. 5 Result of a single DSC measurement.

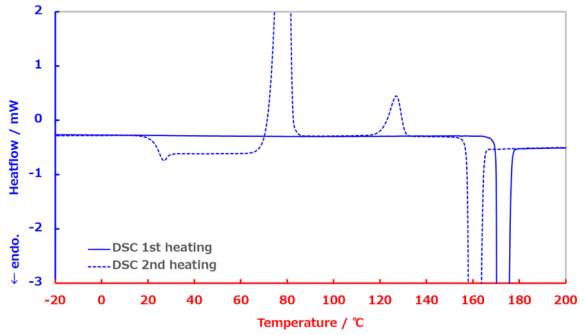

Fig. 6 Multiple magnification chart of 1st and 2nd DSC run (heating).

121℃ was not observed in the simultaneous XRD–DSC measurement. Since only form II crystals were observed in this temperature range in the simultaneous XRD–DSC measurement, this characteristic exothermic peak is due to a phase transition from form III crystals to II crystals, resulting in an exothermic reaction. In general, phase transition from low-temperature stable crystal phase to high-temperature stable crystal phase exhibits an endothermic reaction. However, despite the phase transition between crystals, an exothermic reaction occurs, suggesting that the crystalline phase undergoing this phase transition is a metastable phase.

In other words, results of both the simultaneous XRD–DSC and the single DSC using the sealed crucible clearly indicate that the metastable phase, form III, has undergone a phase transition to the more stable phase, form II. In addition, to examine the DSC baseline in detail, a multiple magnification chart superimposing the results obtained from the 1st RUN and the 2nd RUN is shown in Fig. 6. The DSC baseline includes significant information on the amorphization of crystal and the presence of glass transitions. In Fig. 6, the DSC curve of the 1st RUN is shown as a solid line and that of the 2nd RUN as a dotted line. Around 20℃, no baseline shift could be observed during the 1st RUN, but a distinct shift of the baseline in the endothermic direction was observed during the 2nd RUN.

This suggests that the initial state of the sample was almost 100% crystalline followed by melting and became amorphous during the cooling process. The results of the simultaneous XRD–DSC measurements did not confirm the glass transition, but the results of both techniques suggest that crystals of form II and III are formed at close temperatures after the glass transition. This suggests that nucleation and growth of both crystals may have similar mechanisms. The next challenge is to perform isothermal measurements in addition to non-isothermal measurements at different heating rates to investigate the mechanism more deeply. Through the results of these measurements, it should be possible to gain insight into the detailed control of each crystal.

6. Structure Determination by Single Crystal Structure Analysis

6.1 Outline of structure analysis of single crystals by electron diffraction

Single crystal structure analysis was performed to determine the crystal structure including atomic and molecular positions of the polymorphs identified at each temperature in the simultaneous XRD–DSC and DSC measurements.

The latest single-crystal X-ray structure analyzers, thanks to the development of sources and detectors, have made it possible to visualize the atomic and molecular structures of single crystals that are only several tens of micrometers in size in the case of organic crystals. For many pharmaceuticals, however, it is difficult to grow single crystals that large. Polycrystals that exhibit continuous Debye–Scherrer rings, as shown in Fig. 2, are very difficult to analyze for single-crystal structures using X-rays, because even if one of the grains is

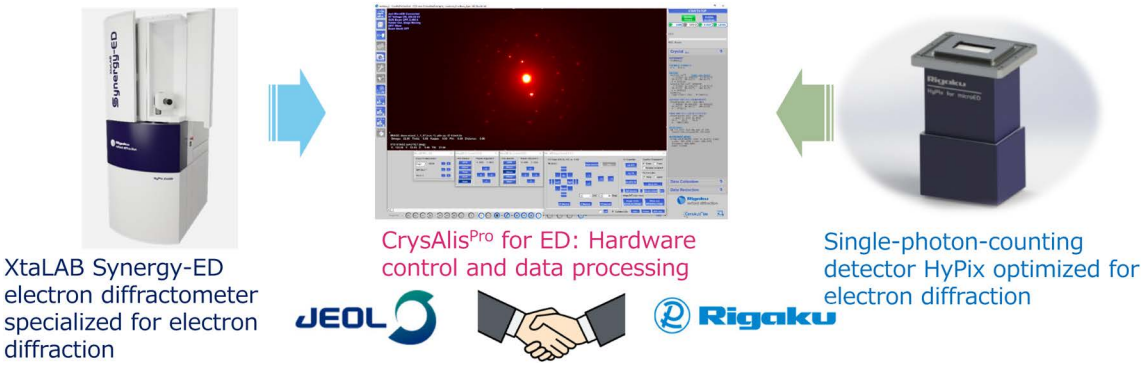

Fig. 7 XtaLAB Synergy-ED and its key components

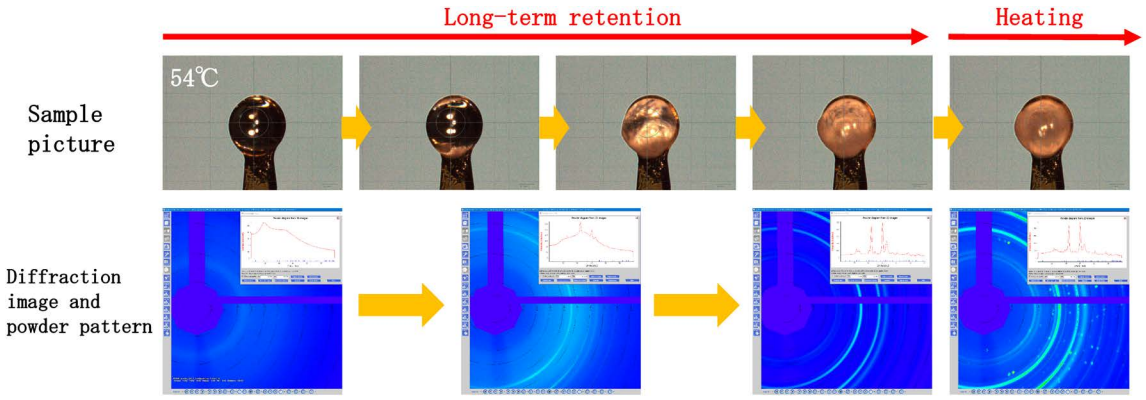

Fig. 8 Sample picture at 54℃ (top) and diffraction image and Powder pattern (bottom).

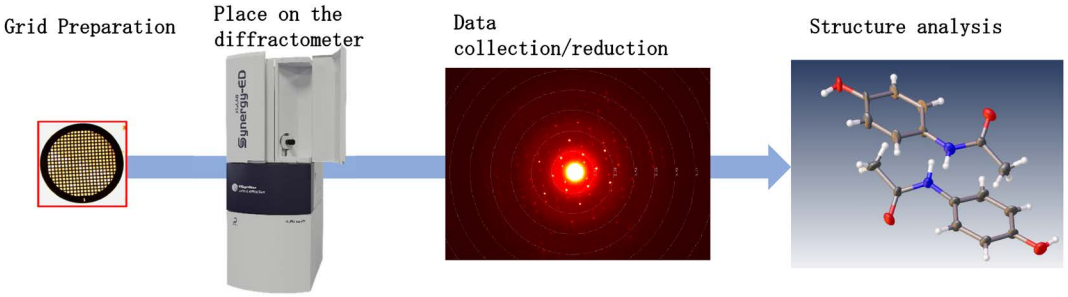

Fig. 9 Flow of single crystal structure analysis

a highly crystalline sample, its size is only a few micrometers.

Recently, Rigaku and JEOL have combined their core technologies to develop the XtaLAB Synergy-ED electron diffractometer, which is capable of structural analysis of single crystals as small as several hundred nanometers. The interaction between electron beams and materials in electron diffraction is thousands to tens of thousands of times stronger than that between X-rays and materials, making it possible to determine the structure of crystals as small as a few thousandths of the size of those used in X-rays. A feature of XtaLAB Synergy-ED is that it has a one-stop workflow that allows for everything from crystal exploration to data measurement and structural analysis(Fig. 7).

6.2 Sample preparation using a single crystal structure X-ray analysis system

Samples were prepared by heating and cooling them in a nitrogen gas-blown temperature controller Cobra and comparing the diffraction patterns observed from simultaneous XRD–DSC measurements with the Debye rings obtained by the single-crystal X-ray structure analyzer XtaLAB Synergy-DW.

Sample preparation was carried out as follows. First, after heating a single grain of a form I single crystal until it melts, the sample was quenched to near room temperature to obtain the amorphous phase. Next, after confirming that the sample was completely amorphous based on the sample observation camera image and diffraction pattern, the sample was gradually reheated from room temperature. Crystals were observed at approximately 54℃. The temperature was maintained for 48 hours, and the formation of crystals was confirmed by the video and diffraction images from the sample observation camera. Further heating resulted in changes in the diffraction images due to the formation of new crystals. The diffraction images obtained were all ring-shaped, indicating that polycrystals were formed during this process.

Table 3. Structural information obtained by XtaLAB Synergy-ED

	Form I	Form II	Form II
Crystal system	monoclinic	orthorhombic	orthorhombic
T/K	293	298	293
Space group	$P2_1/n$	$Pbca$	$Pca2_1$
Z/Z'	4(1)	8(1)	4 (2)
a/Å	7.344(5)	11.77(15)	11.76 (12)
b/Å	9.645(6)	7.5(4)	8.52 (13)
c/Å	12.087 (7)	17.0(2)	14.8 (3)
α/°	90.0	90.0	90.0
β/°	97.37(5)	90.0	90.0
δ/°	90.0	90.0	90.0
V/Å^3	849.1(9)	1488(81)	1481(42)

6.3 Evaluation using XtaLAB Synergy-ED

XtaLAB Synergy-ED was used to analyze the

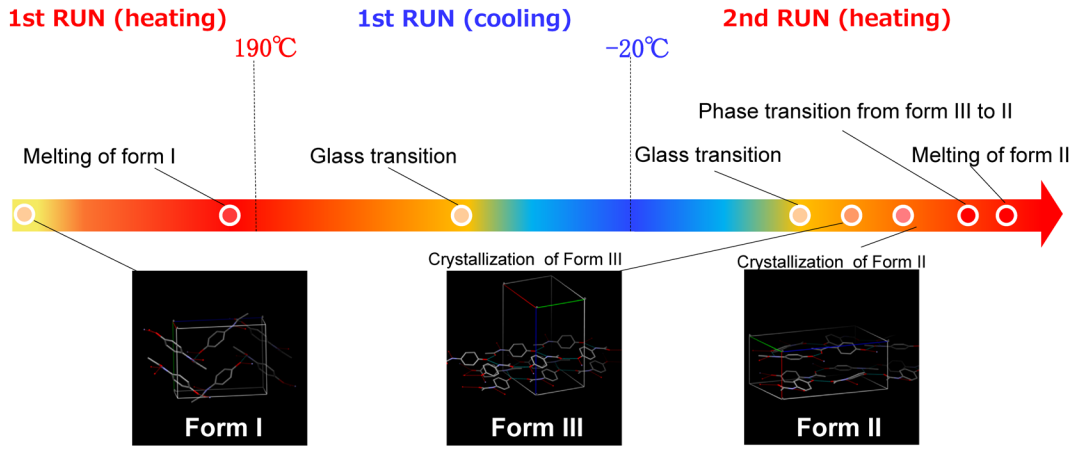

Fig. 10 Crystal structure and change of state of acetaminophen.

structure of one grain each of form I before heating, polycrystals obtained by holding at 54℃, and polycrystals obtained by further heating. The analysis flow is shown in Fig. 9.

In the structural analysis of the two types of heated crystals, the sample was quickly ground so that the crystalline phase would not change due to latent heat in the sample, and the sample was spread on an electron beam diffraction grid for measurement. Further careful sample preparation revealed that the amorphized form I crystals turned into form III crystals at around 54℃ when reheated. The obtained lattice constants for forms I, II, and III are shown in Table 3.

This analysis allowed us to determine a new form III crystal structure from single crystals.

7. Summary

The crystal structures and thermal behavior of acetaminophen were comprehensively evaluated using X-ray diffraction, differential scanning calorimetry, and electron diffraction. As a result, after acetaminophen form I crystals were melted and reheated, the metastable phases, form II and III, crystallized almost simultaneously through a glass transition process. Further heating of acetaminophen form III resulted in a complete crystallographic phase transition to form II (Fig. 10). In addition, by combining electron diffraction and single crystal structure analysis, the molecular structure of form III, which has been rarely reported due to its unstable crystal structure, was successfully visualized for the first time in the world by single crystal structure analysis. This is one insight into understanding the thermal behavior of acetaminophen and controlling its crystalline polymorphism. In pharmaceutical products, there are many polymorphisms that have similar characteristics to acetaminophen, but also present challenges for evaluation techniques. It is hoped that the evaluation techniques described in this article will help to elucidate unknown polymorphisms.

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