Preparation, characterization and in vivo evaluation of pharmacological activity of different crystal forms of ibuprofen

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Abstract: Different polymorphic forms can affect the performance of the drug product. In addition, isomorphic crystals show different chemical and physical properties due to the changes in the crystal habit. However, it is unclear whether the crystal habit results in different pharmacological activity. The aim of this study was to investigate whether the pharmacological effect of ibuprofen could be affected due to the variety of the crystal habit. Solvent change technique and conventional fusion method were carried out to modify the characteristics of ibuprofen. The physicochemical properties of each were investigated using powder X-ray diffraction (PXRD), Fourier transform infrared (FT-IR) spectroscopy and differential scanning calorimetry (DSC) techniques. Results of scanning electron microscopy (SEM) analysis revealed differences in the surface characteristics of the crystals obtained. Further study revealed that the samples crystallized exhibited the remarkable variation on the dissolution profiles in different dissolution medium. Moreover, in vivo antinociceptive and anti-inflammatory findings demonstrated that the crystal habit modifications resulted in the different therapeutic efficacy. Taken together, these results indicate that the modification of the crystal habit had a great influence on the in vivo pharmacological activity of ibuprofen crystals.

Keywords: Crystal habit, dissolution test, antinociceptive activity, anti-inflammatory activity, ibuprofen.

INTRODUCTION

Polymorphism is a common phenomenon that exists extensively in the pharmaceutical field (Zhang et al., 2013; Hasa et al., 2017). Different crystal forms of a drug substance may affect the solid-state properties, such as melting point, solubility and dissolution rate (Dudognon et al., 2008; Vasileiadis et al., 2015). In addition, emerging evidences have revealed that the polymorphs of a same compound may exhibit different pharmacological activity. It has been reported that three polymorphs of glycine, α-, β-, γ-forms, had different behavioral effects on the genetic catalepsy strain of rats (Markel et al., 2011). Moreover, conversion of crystalline form of repaglinide showed significant improvements in the anti-hyperglycemic activity by microwave method (Zawar and Bari 2012). However, not only changes of the poly morphs can influence the physicochemical properties of pharmaceuticals, isomorphic crystals could also show different properties due to the changes in the crystal habit (Yi et al., 2013; Moriyama et al., 2015). While extensive research is dedicated to the variability in the therapeutic performance of poly morphs, little is known about the effect of crystal habit on the pharmacological activity.

Ibuprofen (Ibup), a widely used non-steroidal anti-inflammatory drug (NSAID), possesses antinociceptive and antipyretic activities (Aranda et al., 2017). There have been published researches on the crystal habit modifications of ibuprofen, which differ considerably in the physical properties, such as solubility (Cano et al., 2001), compressibility (Garekani et al., 2001), tabletability (Rasenack and Muller 2002a) and flowability (Rasenack and Muller 2002b). In addition, the crystal habit modification of ibuprofen by crystallization using water-soluble additives improved the dissolution properties of crystals (Acquah et al., 2009). It has been shown that the dissolution rate of a poorly water-soluble drug plays a significant role in the oral bioavailability of the drug (Lazarevic et al., 2014). Moreover, some reports have also demonstrated that the increase in dissolution rate may provide the rapid onset of action after the drug is taken orally (Mutalik et al., 2008; Elsayed et al., 2014; Yadav et al., 2014; Shazly et al., 2015). However, whether the crystal habit may influence the dissolution rate, and then affect in vivo pharmacological effect has not been proposed up to now.

Herein, ibuprofen was used as a model drug to assess whether the in vivo performance of a poorly water-soluble drug could be affected by crystal habit. The crystal habit modifications of ibuprofen, which were affected by solvent or conventional fusion method, were evaluated using powder X-ray diffraction (PXRD), Fourier transform infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC) and scanning electron microscopy (SEM) analysis. In addition, the influence of the crystal habit on the dissolution profiles and in vivo pharmacological activity will also be explored.
MATERIALS AND METHODS

Materials
High grade ibuprofen (Xi'an Bodyguard Pharmaceutical Co., Ltd., Shaanxi, China) meeting the Pharmacopoeia of the People's Republic of China (PPRC) specification was used as original sample in all experiments. Solvents used in this study, ethanol, methanol, propylene glycol, ethylene glycol, chloroform, diethyl ether and all the other chemicals were of analytical grade with high percentage purity. Water was used in double-distilled quality. Carrageenan was purchased from Aladdin Industrial Inc. (California, USA).

Crystallization procedures
Crystallization from solutions or by conventional fusion method was conducted to obtain the serials of ibuprofen crystal forms according to previously described methodology with minor modifications (Garekani et al., 2001; Liu et al., 2012; Rasenack and Muller 2002b). In the first step, ibuprofen was dissolved in the solvent at 40°C (30g/60mL). Then, precipitation was reached by cooling to 4°C overnight. The obtained crystals were collected by filtration and dried in a desiccator under vacuum conditions. All crystal forms dry powders were sifted through a 100-120 mesh sieve (125-150μm) to ensure the same powder particle size were presented. The solvents used were ethanol, methanol, propylene glycol, ethylene glycol, chloroform and diethyl ether. All experiments were repeated 3 times for repeatability and consistency of crystal morphologies.

Similarly, a suitable amount of ibuprofen was melted completely on a hot stage in a water bath at 80°C. Then, the melt was cooled slowly at ambient conditions for crystallization. The obtained ibuprofen crystals were pulverized in a mortar and dried in a desiccator under vacuum conditions. The final powder samples were sifted as before and kept in a desiccator for further use.

Analytical techniques
(i) Powder X-ray diffraction
Powder X-ray diffraction (PXRD) patterns of the original and crystallized ibuprofen samples were recorded using an X-ray diffractometer (Simadzu XDS 6000 X-ray diffractometer equipped with a Cu Ka X-ray source, with radiations generated at 40mA and 40 kV). The samples were scanned from 2 to 50 of diffraction angle (2θ) at scanning speed of 0.02 2θ/s. These patterns were used to confirm the internal structure of the original and crystallized ibuprofen.

(ii) Fourier transform infrared spectroscopy
Fourier transform infrared (FT-IR) spectra was recorded on a Shimadzu 8400S spectrometer by the conventional KBr pellet method at room temperature in the range 4000-400 cm⁻¹. IR solution 1.10 software (Shimadzu, Tokyo, Japan) was used for recording the data from the FT-IR spectra and further analysis. 2 mg of drug sample and 100 mg of KBr (2 mg sample in 100 mg KBr) were used in preparation of the KBr pellets (Negar, et al., 2013).

(iii) Differential scanning calorimetry
Differential scanning calorimetry (DSC) analysis was performed on a DSC822e Differential scanning calorimetry (Mettler Toledo, California, USA). Samples weighing about 3 mg were loaded in aluminum perforated pans and placed into DSC cell. Thermal analysis was performed in triplicate from 25°C to 150°C at a heating rate: 10.0°C /min.

(iv) Scanning electron micrographs
Scanning electron micrographs (SEM) of crystals were obtained using a SU8010 FE-SEM (Hitachi, Tokyo, Japan). Samples were fixed on an aluminum stub. The specimens were mounted on a metal stub with conductive double-sided adhesive tape and coated under vacuum conditions with gold in an argon atmosphere using a sputtercoater, prior to observation.

In vitro dissolution studies
The release of the original and crystallized ibuprofen samples was studied using a fully automated ZRS-8G dissolution tester system (Tianjin Xinzhou Technology, Tianjin, China). The samples were added to 900 mL distilled water, 0.1mol/L hydrochloric acid solution (pH 1.2), acetate buffer solution (pH 4.5) and phosphate buffer solution (pH 6.8) at 37±0.5°C and the paddle speed was set to 50 rpm. At predetermined sampling times 2mL aliquots were taken and filtered through a 0.45μm filter. The removed fluid was instantly replaced with an equal amount of fresh dissolution medium. The drug release was assayed by a UV-2450 Spectrometer (Shimadzu, Tokyo, Japan). All samples were analyzed in triplicate.

In vivo antinociceptive and anti-inflammatory activity studies
(i) Experimental animals
Adult ICR mice (25-30g) and Wistar rats (200 - 250 g) provided by the Medical Experimental Animal Center of Xi'an Jiaotong University (Shaanxi, China) were used in this research. Animals were housed in a temperature-controlled room at 25±2°C in 12h light -12h dark cycles, with ad libitum access to food and water. All efforts were made to minimize the discomfort of animals used. After a week of acclimatization, animals were randomly divided into five groups, namely, the control group, original ibuprofen group, ibuprofen crystals from methanol, chloroform or fusion groups. The control group (n=10) received placebo and the experimental groups (n=10) were given the powdered original ibuprofen or ibuprofen crystals respectively daily by gavage. All animal experiments were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and performed with the approval of the
Institutional Review Board for animal experiments of Xi’an Jiaotong University.

(ii) Hot plate test
The central antinociceptive properties of the original and crystallized ibuprofen samples were assessed by the hot plate test and the acetic acid-induced writhing response in mice. The hot plate test was conducted as described previously with minor modifications (Martinez et al., 1999; Yin et al., 2003). In brief, female ICR mice were dropped twice on a heated plate (55 ± 0.5°C), separated by a 30 min interval. All animals were familiarized with the test procedure in the first trial. The second trial was served as the control reaction time (licking of a paw or jumping), recorded as the response latency on a hot plate. Mice with baseline latencies of <5 or >30 s were excluded from the study. 0.5 h before this, different groups of animals were treated respectively with placebo (control group), original ibuprofen or ibuprofen crystals from methanol, chloroform or fusion 100 mg/kg. Measurements were performed before (zero time), and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 h after treatment, with a cut-off time of 45 s to prevent development of paw lesion. Pain threshold increase (%) = (Pt - P0)/P0 *100/ P0 and P0 separately represent the basic pain threshold and pain threshold at time interval.

(iii) Acetic acid-induced writhing response test
The antinociceptive effect of the original and crystallized ibuprofen samples was studied using the acetic acid-induced writhing response test in mice. The management and administration were the same as in the hot plate test. The writhes in the male mice were induced by intraperitoneal injection of 0.6% acetic acid (10 mL/kg body weight). The original and crystallized ibuprofen samples were administered 30 min before the injection of acetic acid. The number of abdominal contractions (writhing number) was counted over a period of 15 min. The antinociceptive activity of the samples was expressed as the reduction in the number of abdominal contractions.

(iv) Carrageenan-induced paw edema test
The in vivo anti-inflammatory effect of ibuprofen crystals was determined by the carrageenan-induced paw edema test in rats and xylene-induced ear edema response in mice. Fifty Male Wistar rats were randomly divided into five groups. Dose (50 mg/kg) of ibuprofen crystals from methanol, chloroform or fusion and dose (50 mg/kg) of original ibuprofen were administered to the experimental groups, respectively. 30 min after the administration, acute paw edema was induced in the right paw by sub plantar injection of 0.05 mL of 1% w/v freshly prepared carrageen an suspension in normal saline. Paw volume was measured immediately before (V0) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 h after carrageen an treatment (Vt) using a plethysmometer, as previously described (Elhabazi et al., 2006). Mean increase in the paw volume and % edema was calculated for all time intervals by using following formula: % edema= (Vt - V0)*100/ V0.

(v) Xylene-induced ear edema test
The xylene-induced ear edema test used in this study was investigated in mice. The management and administration were the same as in the acetic acid-induced writhing response test. After 30 min of the drug administration, an edema was induced on the right ear of each mouse by topical application of 20 µL xylene (on the inner and outer surface). After 1h, the animals were sacrificed by cervical dislocation. 9 mm diameter disks were removed from each ear and weighed immediately. The swelling was estimated as the difference in weight between the punches from right and left ears, and expressed as an increase in ear thickness.

STATISTICAL ANALYSIS
The results were presented as the mean ± standard error of the mean (SEM). All statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test, when appropriate. P <0.05 was considered statistically significant.

RESULTS
Crystal habit modifications of ibuprofen
The crystalline structure of the ibuprofen crystal was analyzed by PXRD, FT-IR spectroscopy, DSC and SEM. The results indicated that the properties of ibuprofen crystals that were obtained by the solvent change technique and conventional fusion method differed significantly from the common samples. To verify that only the crystal habit has been changed and the crystal internal structure remains intact, PXRD experiments were conducted. PXRD is a very useful tool to identify whether a series of crystals is polymorphs or not (Garekani et al., 2001). As shown in fig. 1, PXRD patterns of original ibuprofen sample and crystallized ibuprofen were collected and analyzed in transmission by an X-ray diffractometer with radiation generated at 40 mA and 40 kV. The PXRD patterns displayed a prominent peak at 2 theta values (2θ) =6°, which was the characteristic peaks for ibuprofen (Dhumal et al., 2010). Moreover, the series of samples exhibited spectra with similar peak positions (2θ). Superimposition of all the X-ray patterns showed that peak positions on the PXRD patterns were identical, indicating that the internal crystal structure remains intact after recrystallization. However, there were some variations in the relative intensities of the peaks of all samples. This may be attributed to the variety of crystal habit of the samples (Inoue and Hirasawa 2013).

Similar result was obtained in the FT-IR spectroscopy study. The IR spectra of original and crystallized ibuprofen are shown in fig. 2. In the region 4000-2000
cm\(^{-1}\), the samples exhibited a broad band at about 3200-2600 cm\(^{-1}\) due to the stretching of the carboxyl O-H group which was subjected to intermolecular hydrogen bonding, especially in the ibuprofen crystals from ethanol. In the low-frequency region (1500-600 cm\(^{-1}\)), the bands observed present slightly distinct IR spectra in the fingerprint region as well in all the samples. From the experiments, a change in all the hydrogen bonds of the drugs may be interpreted from the variations in some bands (broadenings and intensity reductions).

In order to investigate the morphology microscopically by using higher resolution pictures, SEM analysis of crystals was taken. The SEM micrographs of crystals obtained are shown in fig. 3 and the differences in morphology of the crystals are discernible. The morphology of the crystals may be described as follows: from methanol or ethanol, plate-like crystals were obtained; from chloroform or propylene glycol were formed irregularly; from diethyl ether, needle-like crystals were the result and from fusion or ethylene glycol, crystals were showed irregular in shape having rough surface with pores. It has been revealed that plate shaped crystals were taken from ethanol, while needle-like crystals were formed from non-polar solvents like diethyl ether (Rasenack and Muller 2002b). Garekani et al. (2001) and Cano et al. (2001) also obtained plate-shaped or grain-like crystals from methanol and ethanol. In correlation to the above cases, the influence of solvent or crystallization method on the modification of crystal habit of ibuprofen was clearly shown.

**In vitro dissolution test studies**

All these differences described above concern the properties of ibuprofen crystals. Beside these there exist differences concerning the drug release. *In vitro* dissolution test was conducted to perform a comparison in dissolution rate between the original and crystallized ibuprofen. fig. 4a showed that the ibuprofen crystallized samples dissolved only a little bit slower than the original ibuprofen in the distilled water. The further properties variety in dissolution behavior was observed in 0.1 mol/L hydrochloric acid solution, acetate buffer solution (pH 4.5) and phosphate buffer solution (pH 6.8) (fig. 4 b, c, d). In brief, ibuprofen crystals from fusion presented the fastest dissolution rate than any other crystal forms in all dissolution medium. On the other hand, crystals from methanol demonstrated the apparent slowest dissolution profile, and ibuprofen crystals from chloroform performed with the middle level. We assumed that different *in vitro* dissolution rate may affect the *in vivo* pharmacological effect. Therefore, *in vitro* dissolution test was conducted to perform a comparison in dissolution rate between the original and crystallized ibuprofen. In order to confirm the assumption, the three crystal forms, ibuprofen crystals from fusion, chloroform or methanol, were chosen to be investigated in the animal models employing the hot plate test, acetic acid-induced writhing response, carrageenan-induced paw edema test and xylene-induced ear edema assay procedures.

**In vivo antinociceptive activity evaluation studies**

The pharmacological effect of various crystals was evaluated using several *in vivo* tests with differences regarding stimulus quality in four different animal models. To obtain a complete picture of the antinociceptive properties of the ibuprofen crystals, the thermal stimuli (hot plate) and chemical stimuli (acetic acid) were used respectively. In the hot plate test, the drug substances...
which had good antinociceptive effect may be considered as potent analgesics. The time course of the effect of the original and crystallized ibuprofen in the hot plate test is shown in fig. 5. The antinociceptive effect of the samples reached its maximum at 2h after the administration of drugs and slowly decreased without reaching control values until 4h. Compared to the control group, ibuprofen samples significantly enhanced the increase (%) of pain threshold, with the exception of ibuprofen crystals from methanol (0.5, 1.0 and 4.0 groups, $P>0.5$). The maximum increase of pain threshold from ibuprofen crystals from fusion, chloroform to methanol was 150.70, 118.30 and 72.29% separately, but that of original ibuprofen was 133.06%.

The acetic acid-induced writhing test is a very sensitive method for evaluation of potential antinociceptive agents. In this test, acetic acid, the irritating agent, is injected to induce abdominal contractions evidencing visceral pain (Bars et al., 2001). As shown in fig. 6, the pre-treatment
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of the animals with original ibuprofen or ibuprofen crystals from methanol, chloroform or fusion significantly reduced the number of writhing episodes induced by acetic acid, as compared with the control group (P<0.05). However, relative to the original ibuprofen, crystals from methanol and chloroform showed significant differences on the number of writhes (P<0.01 and P<0.05, respectively).

**Fig. 5**: Effect of the original and crystallized ibuprofen samples on the hot plate test in mice. The hot plate test was performed 0.5 h after the administration of ibuprofen samples. Results are expressed as Mean ± SEM of the time course of the effect of the original and crystallized ibuprofen. *P<0.05, **P<0.01 significantly different from Control; *P<0.05, **P<0.01 significantly different from original ibuprofen. ANOVA followed by the Newman-Keuls post hoc test (n=10).

**Fig. 6**: Effect of the original and crystallized ibuprofen samples on the acetic acid-induced writhing in mice. The writhing test was performed 30 min after the administration of ibuprofen samples. *P<0.05, **P<0.01 significantly different from Control; *P<0.05, **P<0.01 significantly different from original ibuprofen. ANOVA followed by the Newman-Keuls post hoc test. Results are expressed as mean ± SEM of the number of writhes (n=10).

**In vivo anti-inflammatory activity evaluation studies**

Ibuprofen crystal samples made obvious anti-inflammatory effects in the carrageenan-induced paw edema in rats and xylene-induced ear edema in mice. Carrageenan-induced paw edema model has been commonly used to assess anti-inflammatory activity and efficacy evaluation of a variety of anti-inflammatory
agents (Kumar and Kuttan 2009; Williams et al., 2012). Here, we used the carrageenan-induced paw edema assay to evaluate the anti-inflammatory effect of the produced samples of ibuprofen. As shown in fig. 7, the time course of the anti-inflammatory effect of ibuprofen samples was presented. The original and crystallized ibuprofen samples decreased the paw swelling volume after carrageenan treatment at the intervals of 0.5h to 4h. The maximum paw edema were sequenced as follows: Original ibuprofen (Ibup_original, 41.36%) > Ibuprofen crystals from fusion (Ibup_fusion, 52.72%) > Ibuprofen crystals from chloroform (Ibup_chloroform, 59.49%) > Ibuprofen crystals from methanol (Ibup_methanol, 63.04%). It was suggested that the original and crystallized ibuprofen inhibited the paw edema induced by carrageenan in different stage, but there was no significantly statistical difference between the original samples and crystals from fusion (P > 0.5). Here, we clearly demonstrated that the various ibuprofen crystals showed significant differences on the carrageenan-induced paw edema.

The similar result was obtained from xylene-induced ear edema assay in mice. Ibuprofen samples were administrated 30 min prior the administration of xylene on the right ear of each mouse. Results are expressed as mean ± SEM of ear thickness induced by xylene in mice. *P<0.05, **P<0.01 significantly different from Control; †P<0.05, ††P<0.01 significantly different from original ibuprofen. Newman–Keuls post hoc test after ANOVA (n=10).

DISCUSSION

It has been reported that the ibuprofen crystals carried out using the cooling hot solution method and precipitation from solution were determined as polymorphic forms (Labhasetwar et al., 1993; Khan and Jiabi 1998). However, in the present study, we proved that the internal crystal structure remained intact after recrystallization. These results seem to be consistent with the fact that crystals carried out using the solvent evaporation method were determined as isomorphic by DSC and X-ray analysis (Rasenack and Muller 2002b). In combination of the findings support the notion that the crystal habit of ibuprofen was affected by different solvents and conventional fusion method.

Accumulating evidences suggest that the modification of crystal habit in drug substance would influence the wettability and subsequent dissolution (Blagden et al., 2007; Varshosaz et al., 2008; Modi et al., 2013; Jagtap et al., 2014). Some reports have also revealed that the crystal habit modification of celecoxib influenced its powder dissolution profile (Lakshmi et al., 2013; Modi et al., 2014). In our present work, we showed that the crystal habit modification of ibuprofen resulted in the significant changes of dissolution rate in the four different dissolution medium. In light of these findings, the crystal habit modification may affect the dissolution rate of a particular drug. However, there is still much to learn about the relationship between the pharmacological activity of different crystal forms and crystal habit.

Recently, the potential of dissolution rate of poorly water-soluble drugs on the therapeutic effect has begun to
emerge. It has been reported that spray drying technique enhanced the dissolution rate and improved the anti-inflammatory activity of meloxicam (Shazly et al., 2015). In addition, self-nanoemulsifying drug delivery systems increased the dissolution rate, and then enhanced the pharmacodynamic effect of hydrochlorothiazide (Yadav et al., 2014). In the present study, we found that ibuprofen crystals from fusion exerted considerable increase in dissolution rate and pharmacological activity as compared with crystals from methanol. These accumulating data reveal that the increase in dissolution rate by the crystal habit modification may provide rapid onset of action or better performance of poorly water-soluble drugs. Yahya et al. (2016) differentiated alternative therapies of various pharmaceutical alternatives of clopidogrel bisulfate based on the cost minimization analysis. Thus, further economic evaluation of various crystal forms may be potential to facilitate more rational crystallization procedures. Moreover, various morphologically different crystal forms of arteether possessed various antimalarial activity (Chadha et al., 2011). Interestingly, our present study also showed that modifications of crystal habit of ibuprofen affected pharmacological effect in different animal models. Meanwhile, ibuprofen crystals from fusion, with faster dissolution rate, have better in vivo antinociceptive and anti-inflammatory activity. Taken together, all results in the present study suggest that the crystal habit may influence the dissolution rate, and then affect in vivo pharmacological effect.

CONCLUSIONS

In summary, we have demonstrated that the various crystal habits of ibuprofen were obtained by varying crystallization conditions. Although these crystal habits have identical crystal structure, significant differences were observed in their antinociceptive and anti-inflammatory activity. Furthermore, differences in pharmacological effect of ibuprofen crystals could be ascribed to the different abundance of dissolution rate. This study establishes the potentially significant contribution of crystal habit on the therapeutic performance of poorly water-soluble drugs, such as ibuprofen.

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