Crystal modifications of 1H-[1]Benzoxepino[5,4-c]pyrazole-3-carboxamide, 8-chloro-1-(2,4-dichlorophenyl)-4,5-dihydro-N-1-piperidinyl and (+) (S)-methyl α-5(4,5,6,7-tetrahydro(3,2-c) thieno pyridyl) (2-chlorophenyl)-acetate benzenesulfonate using certain crystal modifiers.

1 Introduction

1.1 The Organic Solid State and its modifications

Within an organic solid, molecules are held together by intermolecular forces (e.g. hydrogen bonds) that limit or restrict mobility (1-4). The aggregation of molecules in solids creates a single entity termed super molecule. The structural arrangement or packing within the crystal is influenced by the sizes (5, 6), shapes (7, 8), and functionalities of organic molecules (1, 9). Indeed, strategies based on principles of crystal engineering, which involves the understanding and investigation of fundamental properties that dictate molecular arrangement, is essential for the rational design, control, and useful applications of organic solids (10-12).

One of the means to design a target super molecule is with the use of a supramolecular synthon (1, 13). Supramolecular synthons provide adhesive forces in the form of non-covalent bonds that establish specific connectivity between molecules in organic solids. These relatively robust interactions commonly impose directionality on molecular assemblies (14). There are two

types of synthons: the homosynthon and the heterosynthon (1). The former is comprised of self-complementary functional groups [Figure 1(a)] and the latter is composed of complementary groups that differ in functionality [Figure (1b)] (15, 16).

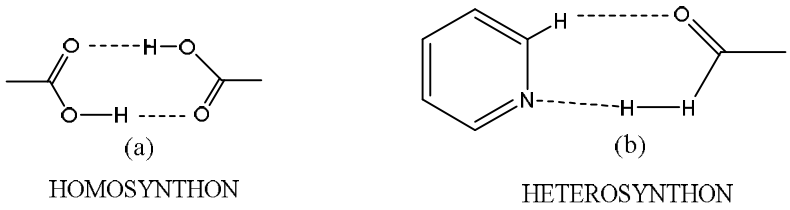


Figure 1: Schematic representation of complementary hydrogen-bonded Supramolecular homo- and heterosynthons: a) carboxyl-carboxyl synthon; b) carboxyl-pyridyl synthon.

For years, controlling the physical properties of solid APIs through modification of the solid phase without changing the molecular structure of the API itself has remained a prime research interest of academic and industrial research groups (17). One of the most important and most used methods of non-covalent modification of APIs is salt formation, in which the API is neutralized by an acid or a base to make a salt (18). More recently, co-crystallization (broadly defined as the crystallization of two non-covalently interacting neutral compounds in the same crystal lattice) has also been used for this same purpose (19, 20). Co-crystallization has even more scope than salt formation because there appears to be no theoretical limit on the types of APIs that can be incorporated into co-crystals. Researchers have been able to make new co-crystals year after year (21) by combining design strategies such as the concepts of supramolecular synthons with high throughput screening methods such as solvent-assisted grinding.

The synthesis of inorganic and organic materials with a specific size and morphology has recently received much attention in the material science research area. Morphology control or morphogenesis is more important for the chemical industry than size control. Many routes have been reported to control the crystal growth and eventually modify the morphology of the crystals. For crystal-habit modification, crystals are grown in the presence of

naturally occurring soluble additives, which usually adsorb or bind to the crystal faces and influence the crystal growth or morphology.

Crystal growth modifiers can be used to achieve a range of outcomes. They can dramatically affect particle shape and size and therefore can be used in a particle engineering sense, i.e. to obtain the desired physical properties of the particles in question. Additives can also inhibit nucleation and growth which has applications such as scale inhibition, for example, as mentioned above. They may even increase the rate of crystallization and be used as promoters. For the most part, most studies of crystal growth modifiers interpret results using a thermodynamic approach only. There is good reason for this: measuring the kinetics of crystallization is much harder, especially when thermodynamic events (such as adsorption at growth features) can lead to kinetic impacts (slowing down of step growth).

1.2 Co-crystals: Definitions, applications, and preparation

A co-crystal can be defined as a crystalline solid composed of two or more molecules that are solids at ambient temperatures that interact via charge neutral non-covalent bonds (22, 23). Pharmaceutical co-crystallization is defined as the formation of a "co-crystal", a combination of an API and a co-crystallizing agent or coformer, very often an organic molecule safe for pharmaceutical utilization [e.g. GRAS (generally accepted to be safe) compounds from Food and Drug Administration (FDA)) (24). Attempts have been made by several authors in the research field (25), especially to better distinguish the concept of co-crystal from more classical salt formation. The FDA also recently provided in its guidance for industry a regulatory classification of pharmaceutical co-crystals taking into account the notion of pKa difference between the species involved in the structure (Salt-Co-crystal Continuum Model) (26).

A comprehensive definition of co-crystal has been provided by Tilborg (27) as follows: a co-crystal is a multi-component crystal in which at least two components are solid under ambient conditions (to distinguish them from

pure solvates). These components co-exist as a stoichiometric ratio of a target molecule or ion and a neutral molecular co-crystal former(s) (to introduce the idea of zwitterionic compounds in co-crystals), bound together through non-covalent interactions, often including hydrogen bonding (hydrogen bonds are the most important intermolecular interactions playing a role in the structure formation of a co-crystal, even if they are not the only ones). For example, metallic coordination bonding could be considered as the principal interactions for metallic salt or metallic coordination complexes linked to a drug molecule, also called sometimes ionic co-crystals (28).

A common approach of modifying or altering the physiochemical properties of an active pharmaceutical ingredient (API) is to create a salt. Salt formation, however is limited to an API with an acid or base ionizable site (29). Co-crystals represent a viable alternative to salt formation in pharmaceutics. For instance, Itraconazole is a highly water-insoluble antifungal drug (Figure 2) (30).

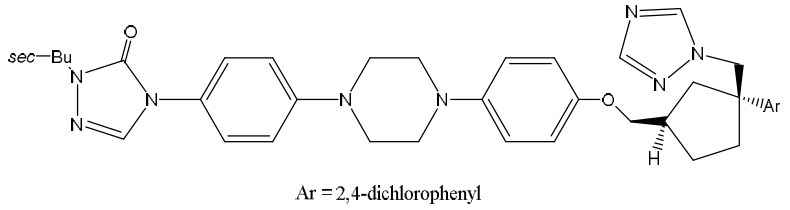


Figure 2: cis-Itraconazole

Currently, the drug is formulated in an amorphous form (Sporanox capsule) that has to be ingested with acidic beverages in order to obtain the maximum absorption through dissolution. The patent literature reveals that attempts to modify the properties of the drug via salt formation proved unsuccessful. Almarsson et al. (30) were able to alter the solid-state properties of the drug with the use of co-crystals. Figure 3 shows Itraconazole co-crystallized with succinic acid. Two molecules of Itraconazole are located anti and bridged by a succinic acid via triazole-carboxyl hydrogen bonds. Dissolution profiles of the Itraconazole co-crystals revealed higher concentrations than that of the commercial Sporanox capsule. The finding demonstrates an excellent application of the use of co-crystals.

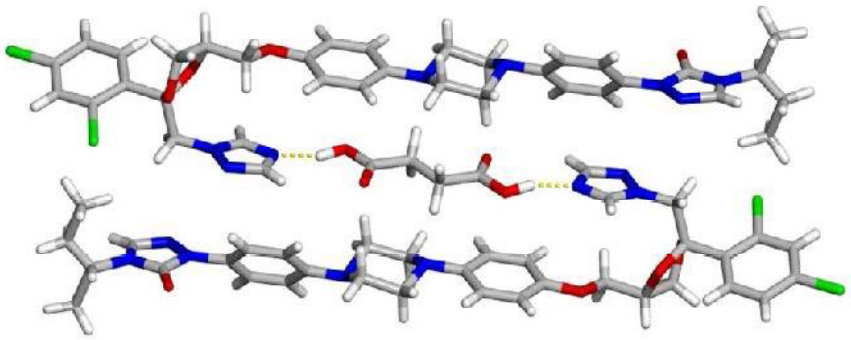


Figure 3: Wireframe representation of 3-component assembly of succinic acid and Itraconazole co-crystal from the single crystal X-ray structure

The emergence of applications of co-crystal solids has motivated solid state chemists to develop alternative or efficient means of their preparation (31, 32). Traditionally, a co-crystal has been prepared by dissolving the solid components in a solution and allowing co-crystallization via slow evaporation or sublimation (33). It is well established that solution-based crystallization provides well-defined and highly-ordered crystals with excellent opportunity for purification; however, there are disadvantages associated with the conventional routes. Solution crystallizations may sometimes require toxic solvents, which can have a harmful impact on the environment as well as can be costly due to challenges in waste disposal. Moreover, these solvents can also form solvates with the individual components rather than form the pure co-crystal or may lead to undesired metastable polymorphs (34).

To circumvent the disadvantages of solution-based crystallizations, mechano-chemical methods have been employed to generate co-crystals with little or no use of solvent (31, 32). Mechanochemistry is the act of grinding or milling solids to induce the formation or breaking of a chemical bond (35). Typically, the grinding is carried out using a mortar and pestle or an automated ball and mill. Substantial increases in heat and pressure are exerted on the solids. For co-crystal formation, non-covalent interactions are, thus formed and broken. Moreover, given the significance of co-crystals,

obtaining these highly useful materials with little or no use of solvent makes the materials appealing from numerous perspectives (32).

The crystal-habit modifiers may be of a very diverse nature, such as multivalent cations, complexes, surface active agents, soluble polymers, biologically active macromolecules, fine particles of sparingly soluble salts, and so on. These crystal modifiers often adsorb selectively on to different crystal faces and retard their growth rates, thereby influencing the final morphology of the crystals. The strategy uses organic additives and/or templates with complex functionalization patterns to control the nucleation, growth, and alignment of inorganic and organic crystals.

The aim of the present study was to check whether it is possible to alter the crystal habits of compounds (**1A**) & (**1B**) (below) by using certain hydrophilic polymers and gel matrices which are routinely used in pharmaceutical formulations.

2 Preparation of co-crystals of compounds (1A) & (1B)

The co-crystals of 1H-[1]Benzoxepino[5,4-c]pyrazole-3-carboxamide 8-chloro-1-(2,4-dichlorophenyl)-4,5-dihydro-N-1-piperidinyl and (+) (S)-methyl α- 5(4,5,6,7-tetrahydro(3,2-c) thieno pyridyl) (2-chlorophenyl)-acetate benzene sulfonate [compounds (1A) & (1B) respectively, Figure 4]

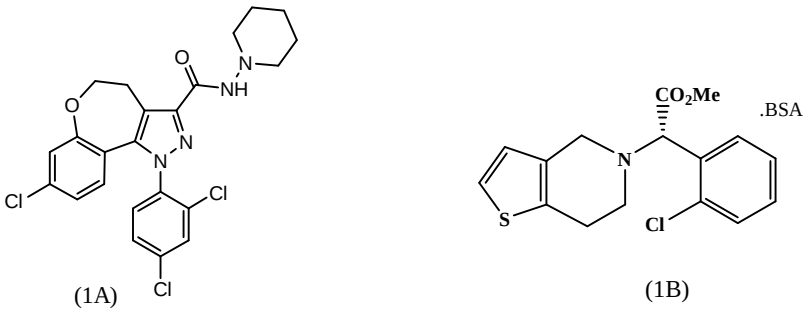


Figure 4: Compounds (1A) & (1B)

were prepared through crystallization experiments wherein each of these compounds was dissolved in suitable solvents to which was added crystal co-formers selected from those that are generally regarded as safe (GRAS)

chemicals. Specifically, the following hydrophilic polymers and gel matrices were used:

- PEG 200;
- PEG 300;
- PEG 4000;
- Polyvinyl alcohol (PVA);
- Polyvinylpyrrolidone K-30 (PVP K-30)

These compounds have been used to control the morphology of a number of inorganic salts (36-38); however, they have not been used for modifying the crystal habits of organic compounds, especially pharmaceutical substances.

The co-crystals of the compounds (1A & 1B) were crystallized using standard techniques such as solvent-assistant grinding, melting, and rotary evaporation, neat grinding, etc. as appropriate. All the novel solid phases were characterized by thermal, spectroscopic, and X-ray diffraction, photographic techniques. Solubility and dissolution experiments were conducted in 10% EtOH-water medium to gauge the effect of different counterions on the solubility and dissolution rate of the compounds (1A) and (1B) were tested. Biological studies to check for the efficacy of the modified compounds were also carried out.

3 Results and discussions

As discussed in Chapter 2 earlier, the compound (1A) was obtained as a crystalline solid and it was very difficult to alter its crystal structure or morphology. Attempts to prepare alternate polymorphic forms were unsuccessful. The amorphous form was obtained with some level of difficulty using dichloromethane as a solvent. Dichloromethane is a low boiling liquid (boiling point ~39.6-40 °C), making the industrial scale production of compound (1A) in amorphous form challenging.

The crystalline form of the compound (1B), the benzenesulfonate salt, has a propensity to form solvates with certain solvents (Chapter 4). However, in

the non-solvated form, it exists only in one polymorphic form. Attempts to prepare alternate polymorphic forms of the compound (1B) were not successful. Use of different solvents, reaction conditions, solvent-antisolvent mixtures etc. resulted in either formation of the amorphous form, one or more solvates most of which were unstable or the same crystalline form as reported in Chapter 4.

Therefore, present study was aimed at trying to alter the crystal habit of the compounds (1A) and (1B) by using certain hydrophilic polymers and gel matrices selected from:

- PEG 200;
- PEG 300;
- PEG 4000;
- Polyvinyl alcohol (PVA);
- Polyvinyl pyrrolidone K-30 (PVP K-30)

The resultant substances were checked for their

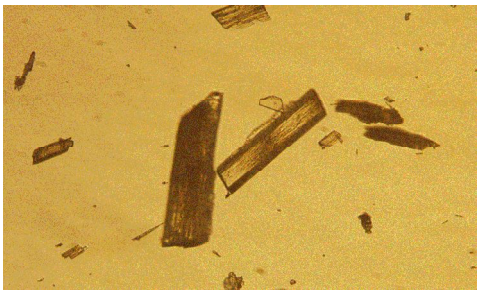
- ✓ Crystallinity;
- ✓ Differences in their melting points as measured through DSC;
- ✓ Changes in their crystal morphology by checking their crystal structure under microscope in presence of a monochromatic light;
- ✓ Checking the external morphology of the crystals using scanning electron microscopy (SEM);
- ✓ Compare the solubilities and dissolution profile of the modified crystals with the compounds (1A) and (1B) respectively;
- ✓ Compare the efficacy of the modified crystals with the compounds (1A) and (1B) respectively.

3.1 Compound (1A):

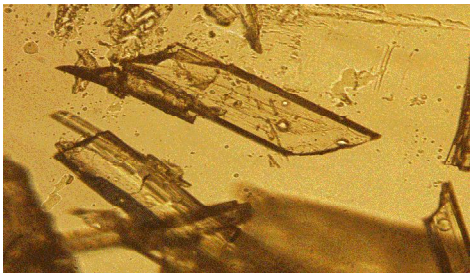
3.1.1 Morphology

The crystals of (1A) appeared to be cubic under microscope (Figure 5(a)). No crystalline substance was obtained using PEG 200. When compound (1A)

was crystallized with PEG 300, there was no change in morphology when seen under microscope using monochromatic light (Figure 5(b)). However upon crystallization with PEG 4000, PVA and PVP K-30, the crystal morphologies were changed [Figures 5(c) to (e)]. Similar phenomenon was also observed when the crystals were studied using SEM. Thus, crystals obtained using PEG 300 exhibited similar morphology under SEM [Figure 6(b)] to the morphology of the compound (1A) [Figures 6(a)], while, use of PEG 4000, PVA and PVP K-30, significantly changed the morphology of the crystals [Figures 6(c) – 6(e)].



(a)



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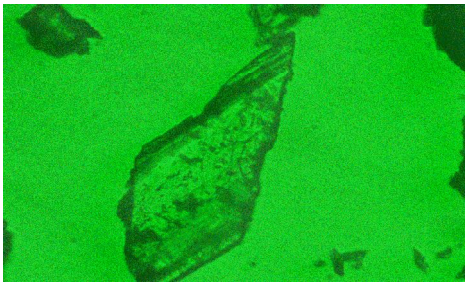
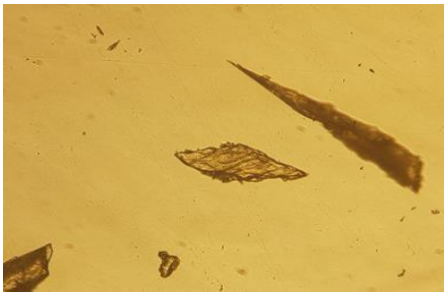


(c)



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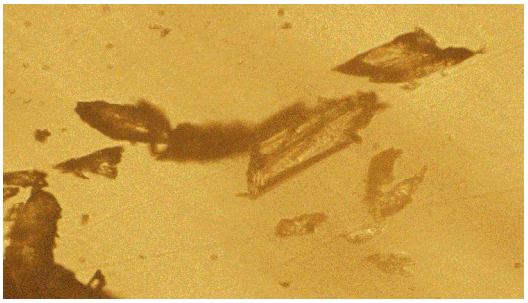
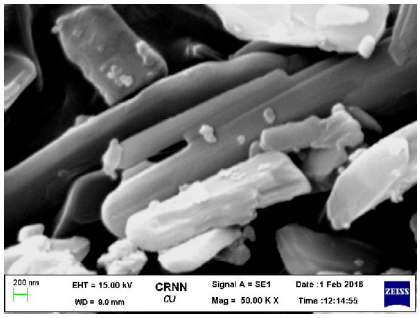
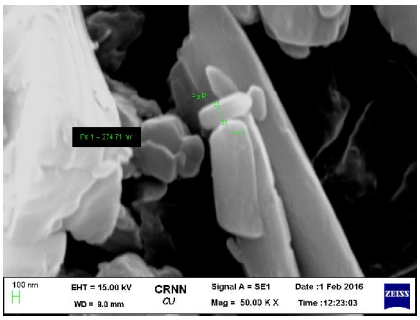


(e)

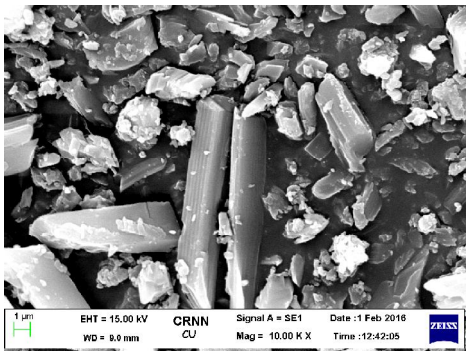
Figure 5: Crystal morphology of (a) compound (1A), (b) with PEG 300, (c) PEG 4000, (d) PVA & (e) PVPK-30



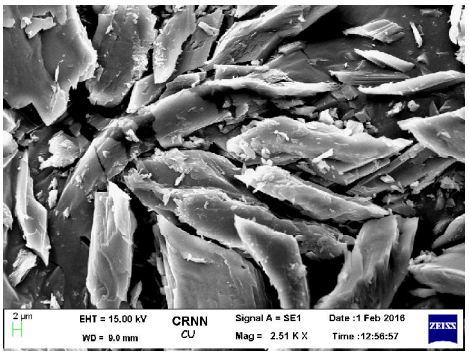
(a)



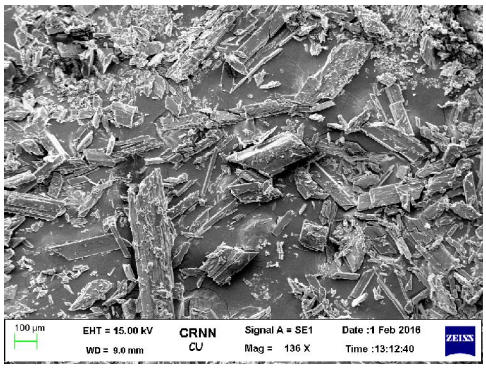
(b)



(c)



(d)



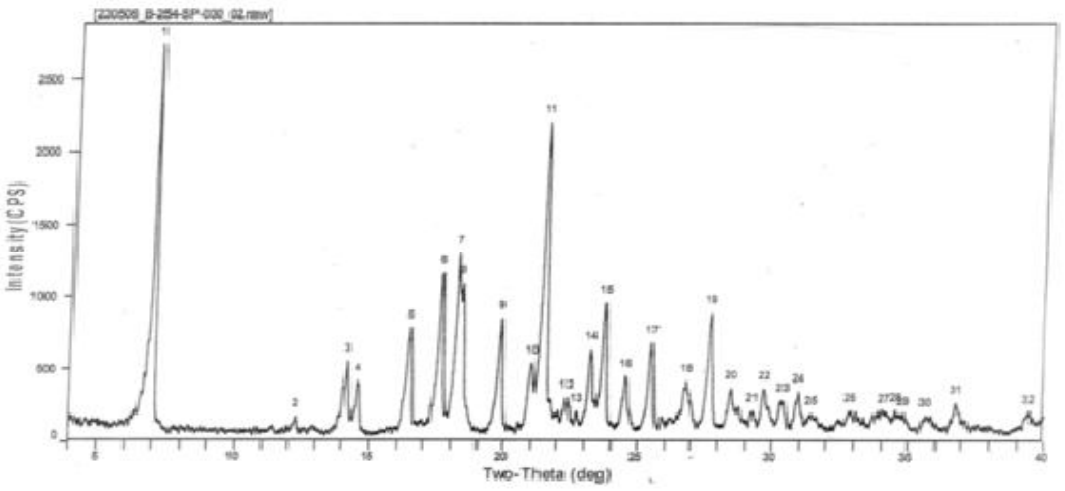
(e)

Figure 6: Crystal morphology of (a) compound (1A), (b) with PEG 300, (c) PEG 4000, (d) PVA & (e) PVPK-30 using SEM.

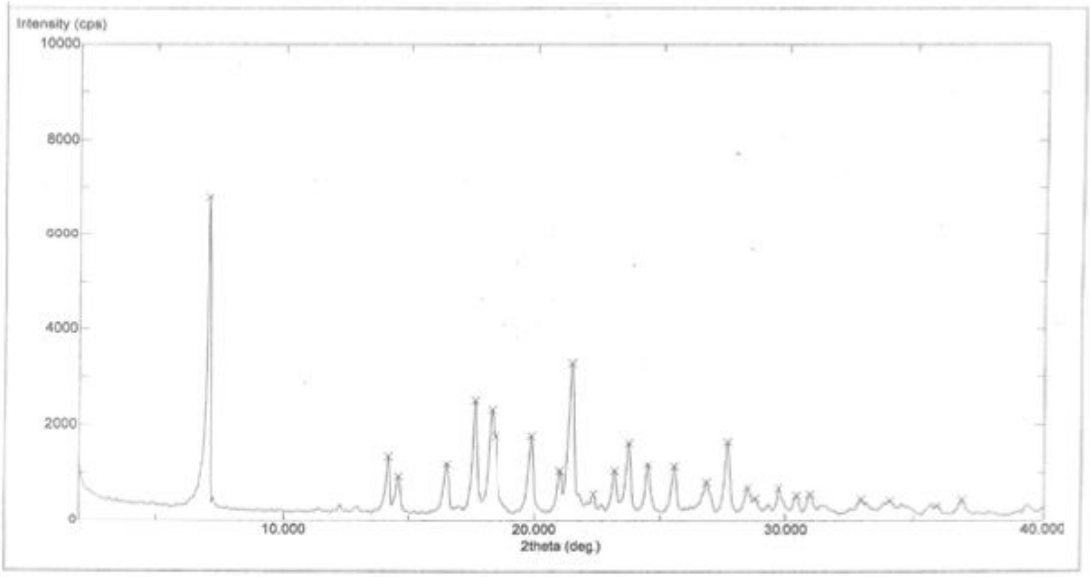
3.1.2 X-ray diffractogram

The XRD pattern of crystalline (1A) is provided in Figure 7(a). There was no change in the XRD pattern when PEG 300 was used. This confirms that PEG 300 did not change the morphology or the crystalline structure

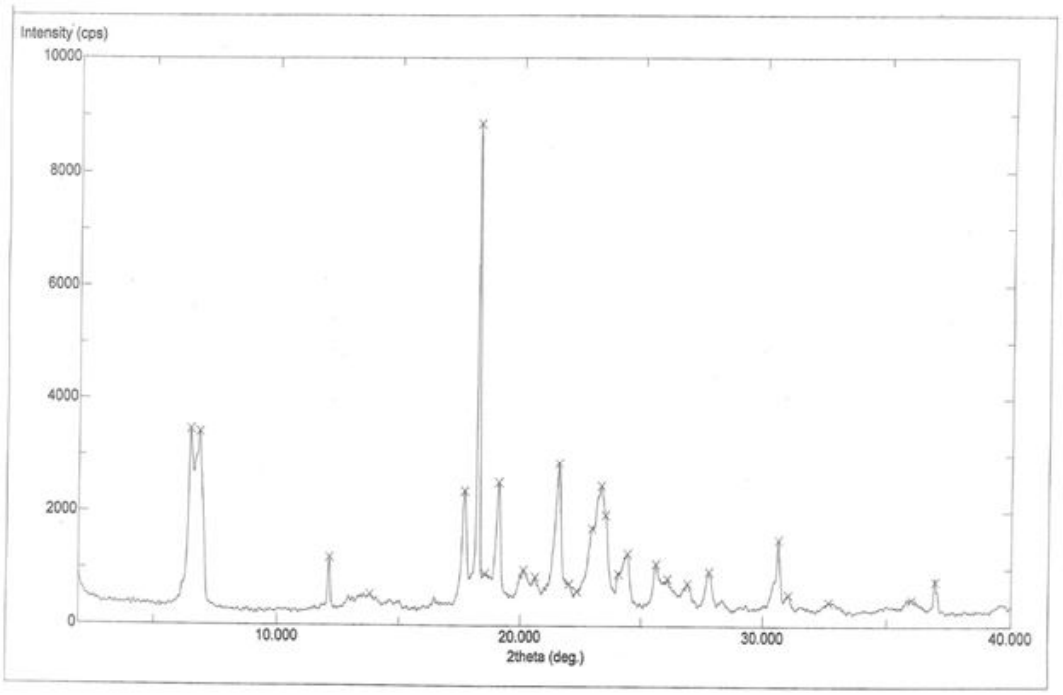
of compound (1A). However, with each of PEG-4000, PVA & PVPK-30, the XRD pattern changed substantially from the pattern of (1A) (Figure 7(c) to (e)), clearly indicating that these polymers were able to modify both the morphologies as well as the lattice structure of compound (1A).



(a)



(b)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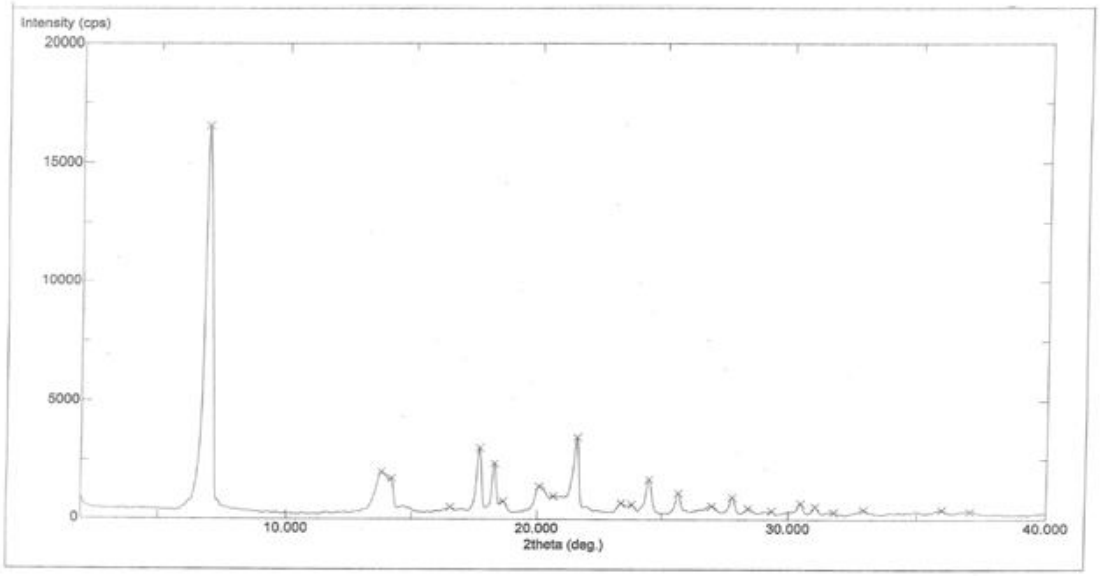
(c)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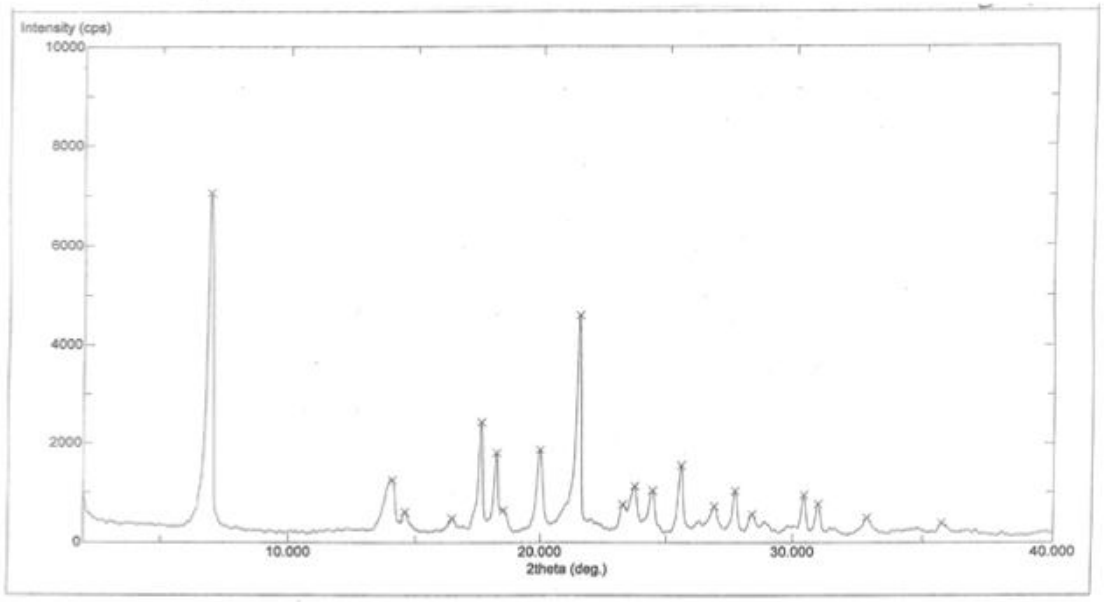
2theta (deg.)

(d)



(e)

Figure 7: XRD pattern of (1A) (a), and with 9b) PEG-300, (c) PEG-4000, (d) PVA & (e) PVPK-30

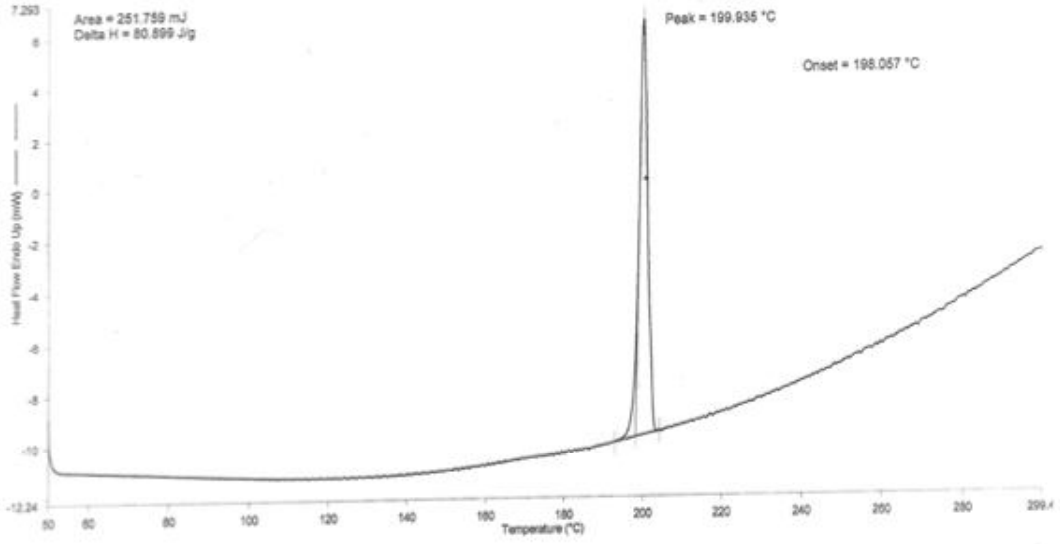
3.1.3 DSC thermogram:

The DSC thermogram of compound (1A) showed a sharp peak at 199.93 °C, with an onset temperature of 198 °C (Figure 8, (a)).The DSC thermogram using PEG-300 did not show any difference from that of (1A).

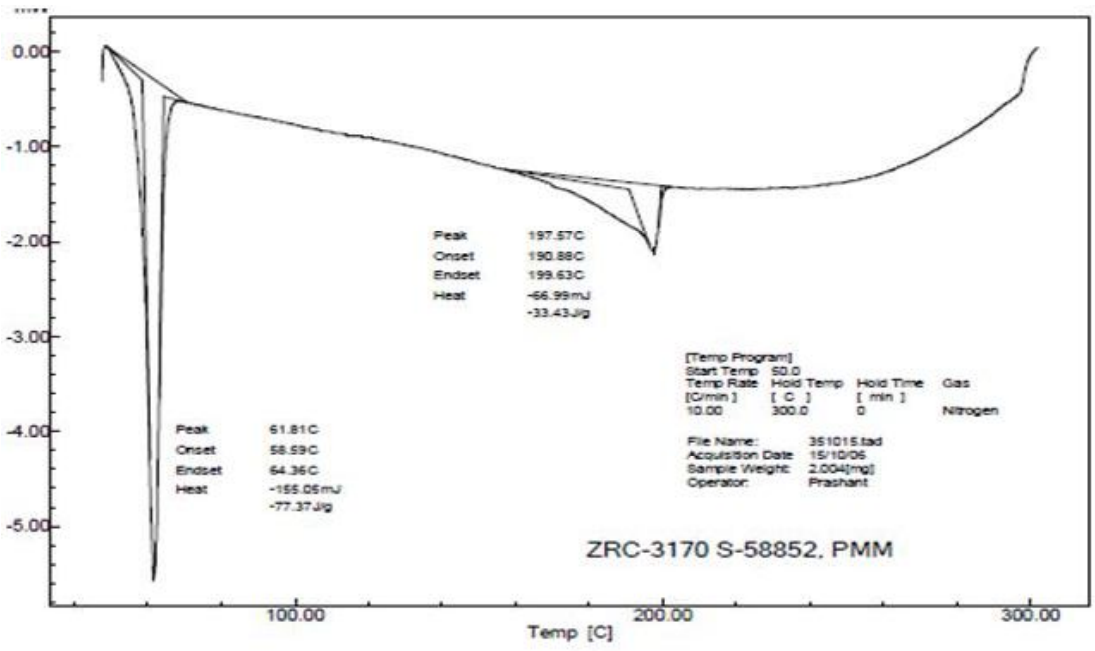
The DSC thermogram of the crystal obtained with PEG-4000 showed one sharp endotherm at 61.81 °C and a second small peak at 197.6 °C (Figure 8(b)). The former peak clearly indicates that the PEG-4000 was loosely bound to the compound (1A), providing the endothermic peak close to its melting point (54-58 °C). Coupled with the fact that the XRD pattern has changed, one can conclude that the PEG-4000 forms a co-crystal with the compound (1A). The second endotherm at 197.6 °C also supports the formation of a co-crystal.

Interestingly, the DSC thermogram of the crystals obtained using PVA & PVPK-30, showed a single endotherm peak at 199.32 and 199.19 °C respectively [Figures 8(c) & 8(d) respectively]. These clearly rules out the

formation of any co-crystal. The possibility of formation of alternate polymorphic forms can be concluded based on the XRD pattern.



(a)



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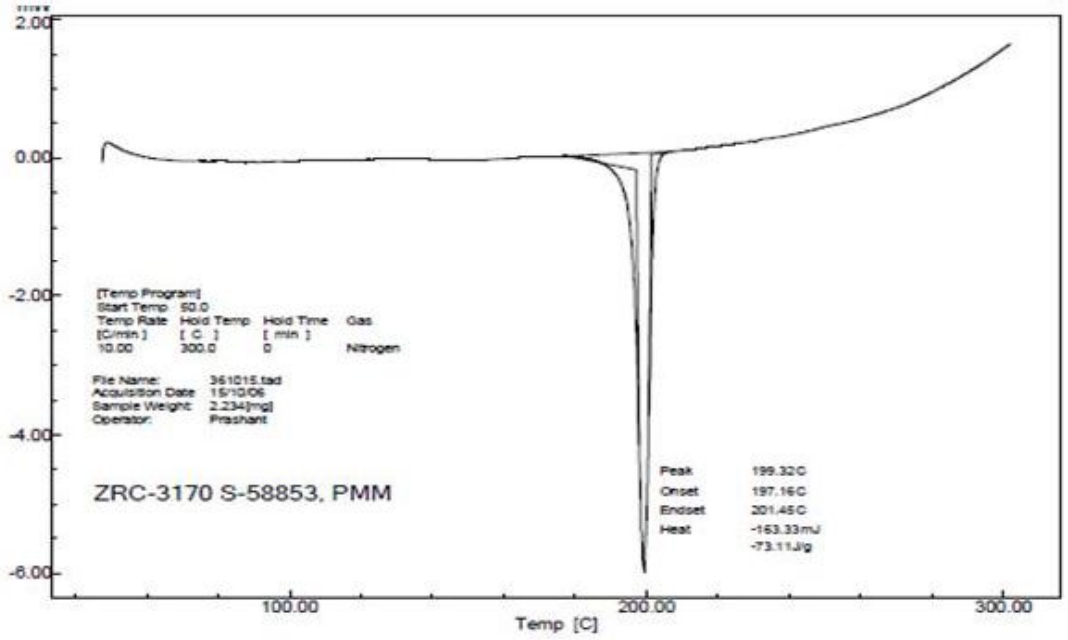
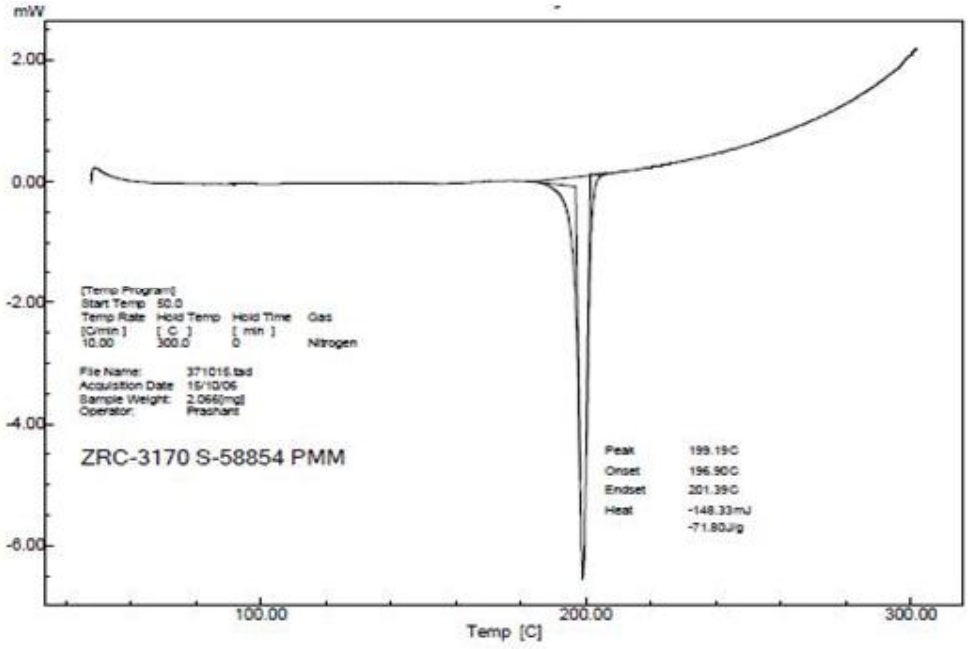


(c)



(d)

Figure 8: DSC endotherm of (a) compound (1A), (b) with PEG-4000, (c) with PVA & (d) PVPK-30

3.1.4 Solubility studies:

Saturated solubility studies of the samples were performed in water at room temperature for 24 hours.

The results are provided in Table 1 below:

Table 1:

Sample ID	Solubility (24 hour at Room Temperature)
Compound 1(A)	Not Detectable (ND)
Compound 1 (A) with PEG 4000	ND
Compound 1 (A) with PVA	ND
Compound 1 (A) with PVPK	ND

The data indicates that the solubility of compound (1A) did not change when crystallized from different organic polymers. Therefore, for the compound (1A), making of the amorphous form is the only way to improve its solubility.

3.1.5 *In Vivo* Efficacy study

Study objective:

Effect of test compounds on inhibition of fasting induced food intake in C57 mice.

The effect of the modified compounds of (1A) was studied on inhibition of fasting induced food intake in C57 mice after single intraperitoneal dose at 30mg/kg. The results are provided in Table 2.

Table 2:

S.N.	Treatment (30 mg/kg)	Food intake (g) 4 h						Food intake (g) 20 h					
		Feed consumption in 4 h (g)			% Change in feed consumption Vs Control			Feed consumption in 20 h (g)			% Change in feed consumption Vs Control		
1	Vehicle Control (IP)	4.5	±	1.4				8.6	±	1.4			
2	Comp 1(A)	3.4	±	0.2	-23.5	±	3.5	7.5	±	0.5	-18.7	±	18.7
3	Comp. 1(A)-PEG-4000	3.3	±	0.8	-26.5	±	17.0	7.5	±	0.5	-12.7	±	6.1
4	Comp. 1(A)-PVA	3.2	±	0.4	-27.5	±	8.2	7.9	±	0.4	-7.5	±	4.9
5	Comp. 1(A)-PVPK	3.3	±	0.4	-26.0	±	9.6	7.9	±	0.7	-7.4	±	8.2
6	Comp. 1(A)-PEG-300	3.3	±	0.8	-26.5	±	17.0	8.0	±	0.8	-6.0	±	8.8

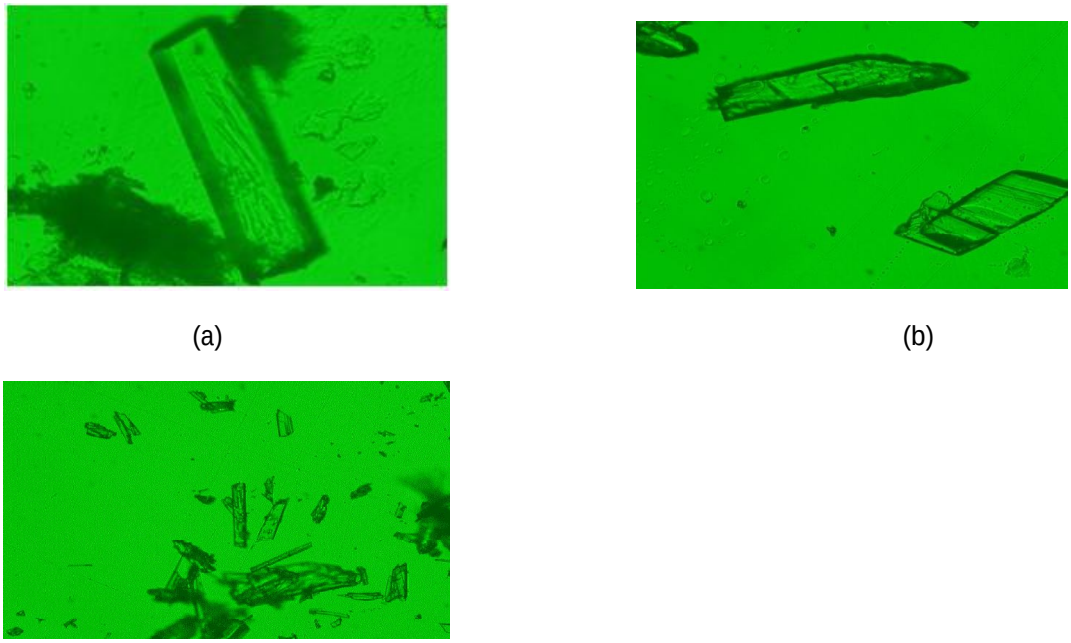
Results (mean ± SD, n=5) indicate that there was reduction in the fasting induced food intake after the test compound administration. The reduction was prominent for the first four hours. However, there was no significant difference amongst the different treatment groups.

Thus, the crystal modifiers modified the crystal lattice of the compound (1A) but such changes did not have any effect on the *in vivo* efficacy of the modified compounds. Therefore, it was concluded that the use of these crystal modifiers can affect the polymorphic nature, morphology, solubilities but may not have an impact on the biological activity of the compound (1A).

3.2 Compound (1B)

3.2.1 Morphology

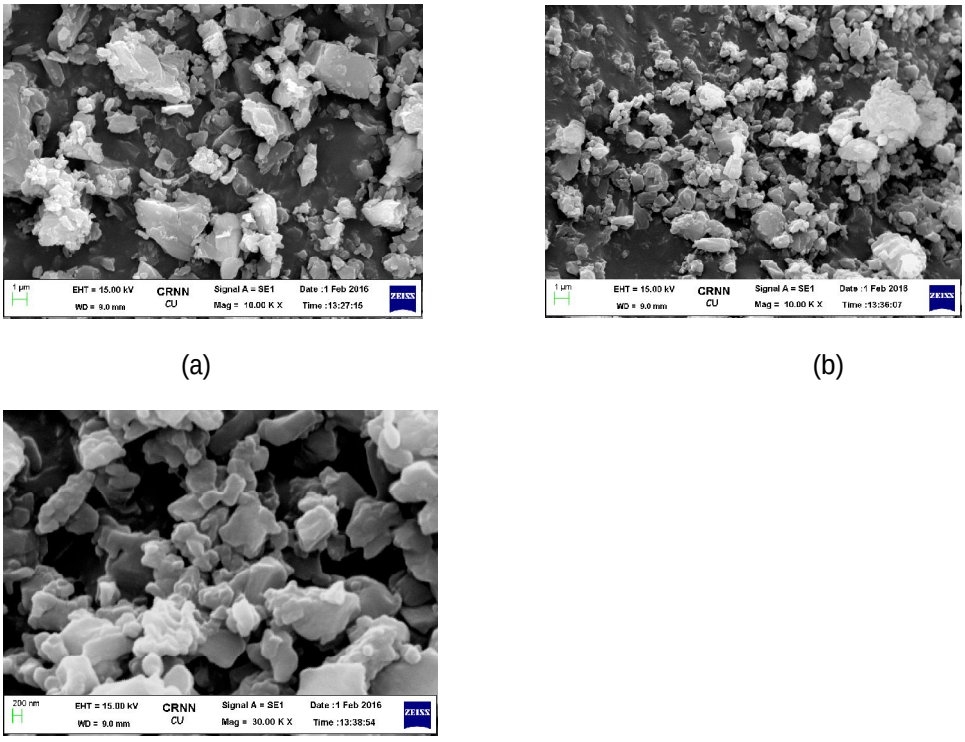
The crystals of compound (1B) appeared cylindrical under the microscope [Figure 9(a)]. Interestingly, when the compound (1B) was crystallized from PEG-200, PEG-300, PEG 4000, PVA & PVPK-30, the morphology of the compounds obtained changed. Thus, when PEG-300 was used, the compound obtained was planar [Figure 9(b)]; with PEG-4000, the compounds with irregular morphology was obtained (Figure 9(c)]; However, with PEG-200, PVA & PVPK-30, no specific structures could be identified, probably indicative of their amorphous nature. However, under SEM, no difference between the compound (1B) [Figure 10(a)], and those obtained using PEG-300 [Figure 10(b)] or PEG 4000 [Figure 10 (c)] could be seen, probably due to the breaking down of the structures.



(a) (b)

(c)

Figure 9: Crystal morphology of (a) compound (1B), (b) with PEG 300 & (c) PEG 4000.



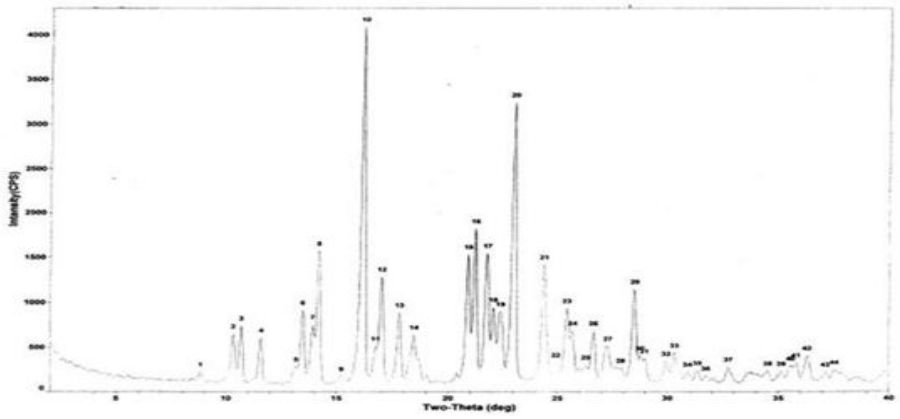
(a) (b)

(c)

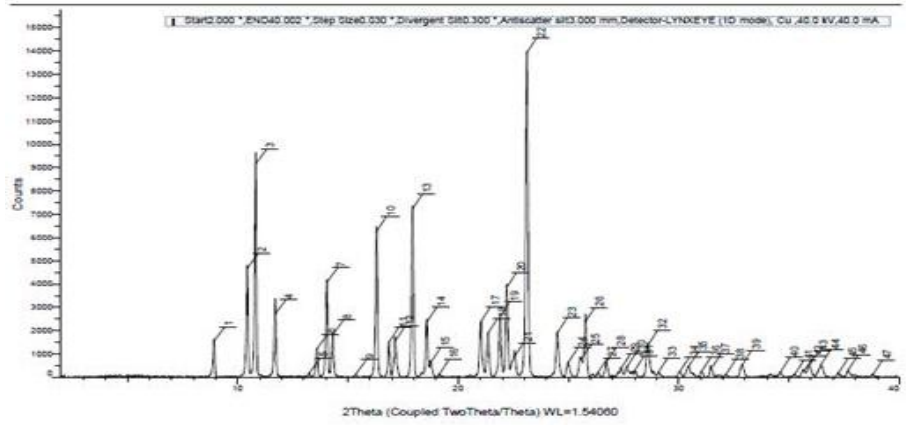
Figure 10: Crystal morphology as seen using SEM of (a) compound (1B), (b) with PEG 300 & (c) PEG 4000

3.2.2 X-ray diffractogram

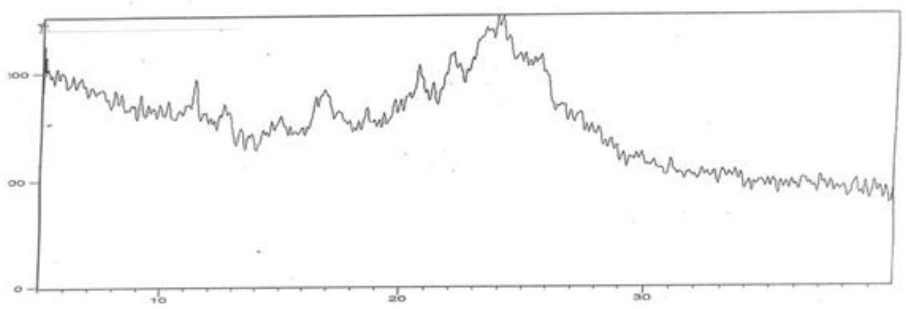
The X-ray diffractogram of the compound (1B) is given in Figure 11(a). The XRD pattern of compound (1B) with PEG-300 is given in Figure 11(b). It can be seen that there is a change in the XRD pattern indicating modification in the crystal lattice. With PEG-200, PEG 4000, PVA and PVPK-30, the XRD patterns were amorphous in nature [(Figures 11 (c) to 11(f)].



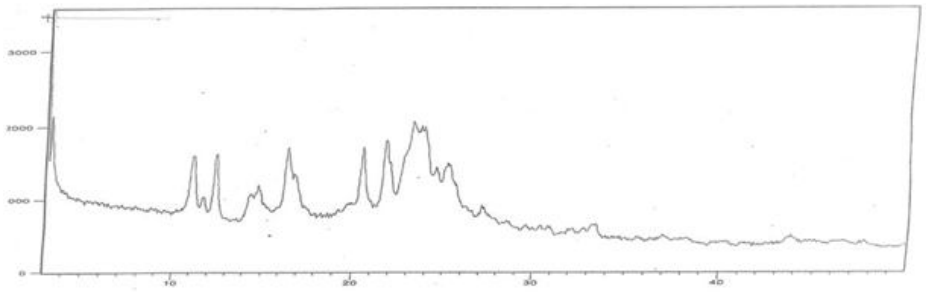
(a)



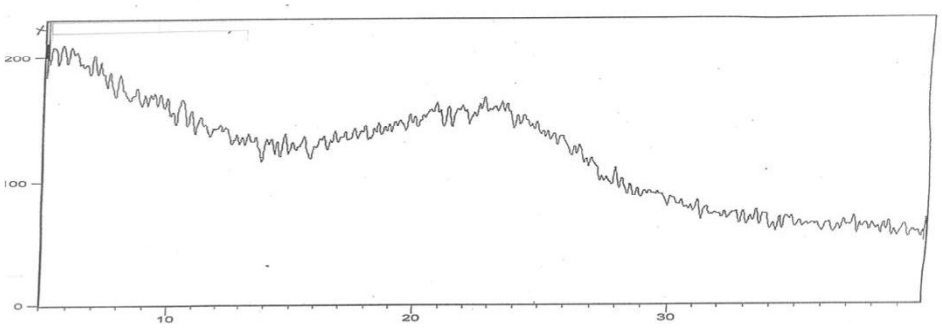
(b)



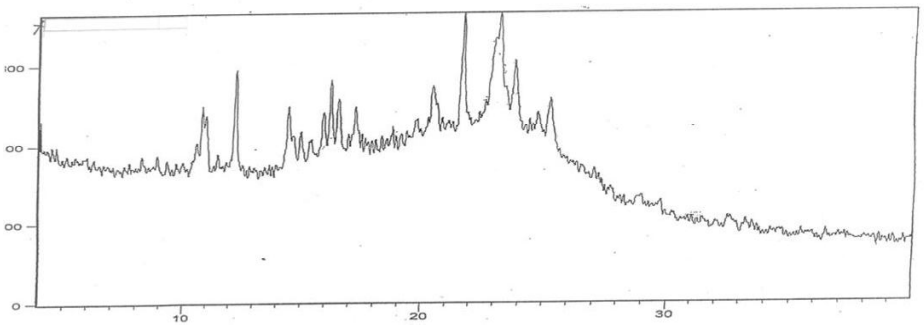
(c)



(d)



(e)

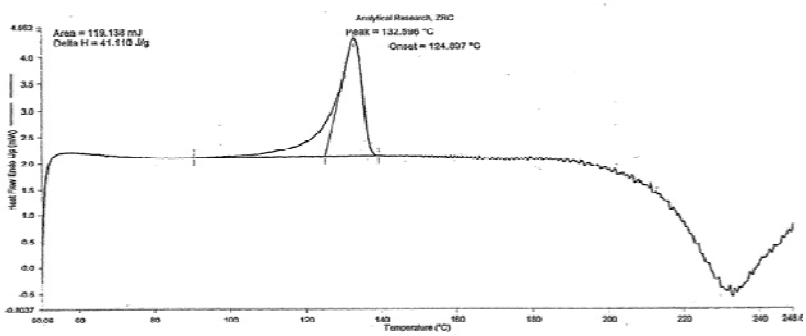


(f)

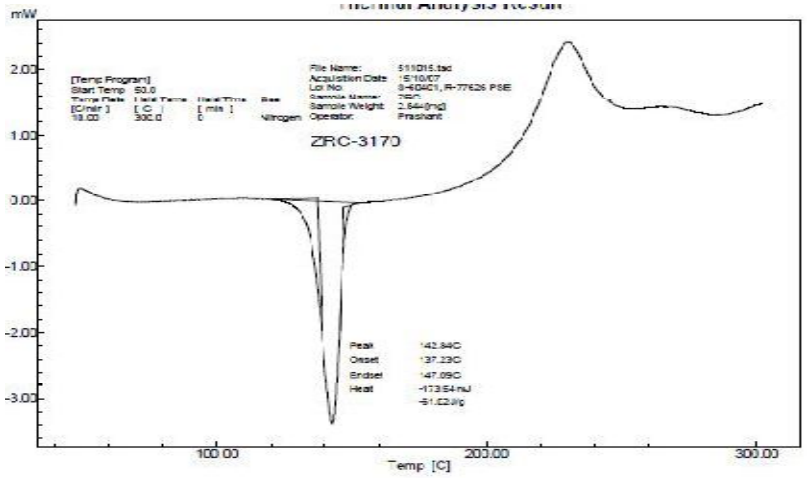
Figure 11: XRD pattern of (a) compound (1B); (b) Compound (1B) crystallized from PEG-300; (c) crystallized from PEG-200; (d) crystallized from PEG-4000; (e) crystallized from PVA and (f) crystallized from PVPK.

3.2.3 DSC thermogram

Since when each of PEG-200, PEG 4000, PVA and PVPK-30 were used, the compound turned amorphous, no further studies were undertaken. This is because the aim was to obtain crystalline salt. The DSC thermograms of compound (1B) and when crystallized from PEG-300 are provided in Figure 12(a) and (b) respectively. The compound (1B) showed an endothermic peak at 132 °C with an onset at 124.9 °C whereas when the compound is crystallized from PEG-300, the DSC peak shifted to 142.9 with an onset at 137.2 °C. Such a shift is also indicative of a change in the crystalline state.



(a)



(b)

Figure 12: DSC pattern of (a) compound (1B) and (b) with PEG 300

3.2.4 Solubility studies:

The solubility of compound (1B) and when it is crystallized using PEG-300 were studied. The results are provided in the following table (Table 2):

Table 3:

Sample ID	Solubility (24 hour at Room Temperature)
Compound 1(B)	0.68 mg/ml
Compound 1 (B) with PEG 300	Not Detectable (ND)

It was found that when the compound 1(B) is crystallized from PEG 300, there is a decrease in solubility.

3.2.5 *In Vivo* Efficacy

Study objective:

Effect of test compounds on inhibition of ADP-induced platelet aggregation in Wistar rats.

This study was performed to evaluate the efficacy of compound (1A) and (1A) modified with PEG-300, after 2, 24 and 48 hours of single intravenous dose in male Wistar rats. The results are provided in Table 4.

Table 4: Effect of Comp. 1(B) and Comp. 1(B) -PEG-300 on % platelet aggregation induced by 20 µM ADP after 2, 24 and 48 hours of intravenous administration in rats. Results are expressed as mean ± SEM (n = 6)

Time (h)	Vehicle			Comp. 1(B) (10mg/kg)			Comp. 1(B) -PEG-300 (10mg/kg)		
02 h	75.8	±	4.8	56.8	±	5.3	63.7	±	5.4
24 h	78.0	±	8.4	64.7	±	10.5	63.3	±	1.5
48 h	73.5	±	10.8	65.3	±	15.8	57.8	±	10.7

Similarly, the % Inhibition of platelet aggregation (%IPA) of Comp. 1(B) and Comp. 1(B) -PEG-300 Vs vehicle control induced by 20 µM ADP after 2, 24

and 48 hours of intravenous administration in rats was also studied. The results are provided in Table 5 below. Results are expressed as mean ± SEM (n = 6).

Table 5:

Time (h)	Comp. 1(B) (10mg/kg)			Comp. 1(B) -PEG-300 (10mg/kg)		
02 h	25.1	±	7.0	16.0	±	7.1
24 h	17.1	±	13.5	18.8	±	1.9
48 h	11.1	±	21.5	21.3	±	14.6

It can be seen that use of PEG-300 decreased the efficacy compared to that of compound (1B) alone.

4 Conclusion

The various conformers belonging to the GRAS class of compounds can be used to change the crystal morphology as well as the crystal lattice of pharmaceutical compounds. Since these conformers are generally considered to be safe, use of these compounds can be an important tool to alter the crystal behavior of important pharmaceutical compounds and may be used to change the physico-chemical properties of difficult to handle therapeutically active compounds. From the solubility studies it can be inferred that use of high molecular polymers may cause changes in solubilities of the compounds. Similarly, the results of the efficacy studies for the compounds (1A) and (1B) indicates that the efficacy may also change when various conformers are used. More work therefore needs to be done to better understand the coformer behavior towards compounds having different physical properties as well as different pKa values.

5 Experimental section:

5.1 Materials and methods:

5.1.1 DSC (Differential Scanning Calorimeter):

The DSC of the compounds were measured as follows:

Instrument Detail:

Make: Perkin Elmer

Model: Pyris 1

Software: Pyris 1

Weigh accurately 2 mg to 3 mg of sample in a clean aluminum pan, place the lid and seal it with the help of sealing process. The heating was done from an initial temperature of 50°C and the temperature was gradually raised at a heating rate of 10°C/min till 300°C. Nitrogen gas was used at a flow rate of 20ml/min.

Blank Run: A blank sample was run for baseline correction by placing empty aluminum pans in both sample and reference compartments of the DSC furnace and running a scan using the temperature range and at a heating rate mentioned in Instrumental parameters.

Sample Run: Place the sample preparation in the sample compartment and blank aluminum pan in the reference compartment of the DSC furnace and run a scan using the temperature range and heating rate mentioned as in instrumental parameters using the base line file obtained in the blank run above for the baseline correction.

5.1.2 XRPD Method

Sample Preparation: Place a Sufficient quantity of sample to be analyzed on the sample holder plate and flatten it with the help of another plate to achieve a smooth surface. The diffraction patterns are recorded as per below instrumental parameters

Instrument used : 2k W XRD

Model : MF2100

Make : Rigaku

Instrument Parameters:

1. X-ray	:	Cu/40kV/30mA
2. Diversion slit	:	1°
3. Scattering slit	:	1°
4. Receiving slit	:	0.15mm
5. Filter	:	Ni-kß filter
6. Counter	:	Scintillation counter
7. Scan mode	:	Continuous
8. Scan speed	:	4.000°/minute
9. Sampling width	:	0.010°
10. Scan axis	:	2theta\theta
11. Scan range	:	2.0° to 40.0°
12. Theta offset	:	0.000°

5.1.3 Scanning electron microscopy (SEM)

The surface morphology of the respective samples was examined under ZEISS, EVO 18, scanning electron microscopy (SEM). Solid samples were evenly dispersed on small segment of microscopic glass slide. Then they were kept in vacuum desiccators for ~1.5 h and the samples were attached to aluminum sample stubs using double-sided carbon tape and were coated with gold in a sputter coater and observed under SEM at an accelerating voltage of 15 kV. Average diameters were measured on at least 25 randomly chosen crystals at several regions of the samples.

5.2 Synthesis:

The compound of formula (1A) and (1B) were prepared in-house as described in the earlier chapters. The co-formers were purchased commercially from

Sigma-Aldrich. Analytical grade solvents were used for the crystallization experiments.

5.2.1 Preparation of crystals of compound (1A) with PEG-200

Placed 0.500 g (1.017 mmol) of compound (1A) into round bottom flask, to it was added 5 ml acetone. The suspension was warmed upto 45-50°C in a water bath to get clear solution. To it was added a solution of 20% PEG 200 in acetone (5 ml). The mixture was cooled and left to ambient conditions. Crystals were obtained as colourless blocks after two days which were separated by filtration.

5.2.2 Preparation of crystals of compound (1A) with PEG-300

Placed 0.500 g (1.017 mmol) of compound (1A) into round bottom flask, to it was added 5 ml acetone. The suspension was warmed upto 45-50°C in a water bath to get clear solution. To it was added a solution of 20% PEG 300 in acetone (10 ml). The mixture was cooled and left to ambient conditions. Crystals were obtained as colourless blocks after four days which were separated by filtration.

5.2.3 Preparation of crystals of compound (1A) with PEG-4000

A 1:1 mixture of compound (1A) and PEG 4000 were ground manually in an agate mortar at room temperature for 5 min together with the addition of a few drops of acetone. However, upon removal of the solvent, the components remained as mixtures.

Subsequently, a 1:1 mixture of compound (1A) and PEG 4000 were taken in a round bottom flask, to it to it was added 30 ml was dissolved in30 mL of acetone under constant stirring at 50 ◦C. The solution was filtered and cooled at room temperature. The resulting crystals obtained after two days were filtered and dried in a desiccator over silica gels at room temperature.

5.2.4 Preparation of crystals of compound (1A) with PVA

A 1: 1 mixture of compound (1A) and PVA were suspended in 30 ml of acetone and the solution was heated at 45 °C and left for evaporation of the solvent at ambient conditions. Crystals of the compound (1A) and PVA were obtained as colorless blocks in 5 days.

5.2.5 Preparation of crystals of compound (1A) with PVPK

A 1: 1 mixture of compound (1A) and PVPK were suspended in 40 ml of acetone and the solution was heated at 45 °C and left for evaporation of the solvent at ambient conditions. Crystals of the compound (1A) and PVPK were obtained as colorless blocks in 3 days.

5.2.6 Preparation of crystals of compound (1B) with PEG-200

Placed 0.500 g of compound (1B) into round bottom flask, to it was added 10 ml methanol. The suspension was warmed upto 30°C in a water bath to get clear solution. To it was added a solution of 20% PEG 200 in methanol (5 ml). The mixture was cooled and left to ambient conditions. An amorphous mass was obtained.

5.2.7 Preparation of crystals of compound (1B) with PEG-300

Placed 0.500 g of compound (1B) into round bottom flask, to it was added 5 ml methanol. The suspension was warmed upto60°C in a water bath to get clear solution. To it was added a solution of 20% PEG 300 in methanol (10 ml). The mixture was cooled and left to ambient conditions. The solution was scratched on the sides repeatedly. Crystals were obtained as colourless blocks after seven days which were separated by filtration.

Attempts to obtain crystals of compound (1B) with PEG 4000, PVA and PVPK in different solvents and techniques similar to those described above did not yield any crystalline substance. Only amorphous forms were obtained.

5.3 Solubility studies:

Procedure: Excess amount of sample was dissolved in 1 ml. of water and kept at room temperature for 24 hours with occasional shaking.

Standard solutions of the samples were prepared in methanol at 0.1 mg/ml concentration.

Standard and samples were filtered through 0.2 micron series filters and injected in liquid chromatography (HPLC). Peak response was detected in UV detector at 220 nm. Amount of soluble content was calculated using the following formula:

$$\text{Solubility [mg/ml]} = \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times \text{standard concentration (mg/ml)}$$

5.4 Biological studies:

5.4.1 In Vivo efficacy studies of compound (1A) and its modified crystals based on their effect on inhibition of fasting induced food intake in C57 mice

The animals (C57 mice) were trained for fasting induced food intake. In this procedure, they were allowed access to chow diet from 11 a.m. to 3 p.m. every day, and fasted for rest 20 hours of the day. They were randomized based on their intake, when their daily four hour intake was consistent for two consecutive days. On the day of experiment, the mice were administered the test compounds formulated in saline by intraperitoneal route. The food intake was measured for next four hours, and for subsequent 20 hours. The percent inhibition in the food intake was calculated based on the vehicle control group.

5.4.2 Effect of compounds (1B) and (1B)-PEG300 on inhibition of ADP-induced platelet aggregation in Wistar rats

This study was performed to evaluate the efficacy of the compounds after 2, 24 and 48 hours of single intravenous dose in male wistar rats. For each time point new set of animals were used. Wistar rats were randomly

assigned into three groups for each time point i.e. saline (vehicle), and treatment groups. Each group comprised of six animals. Platelet rich plasma was collected from each group after mentioned time interval and ADP (Adenosine diphosphate)-induced platelet aggregation was estimated using microtitre plate reader method.

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