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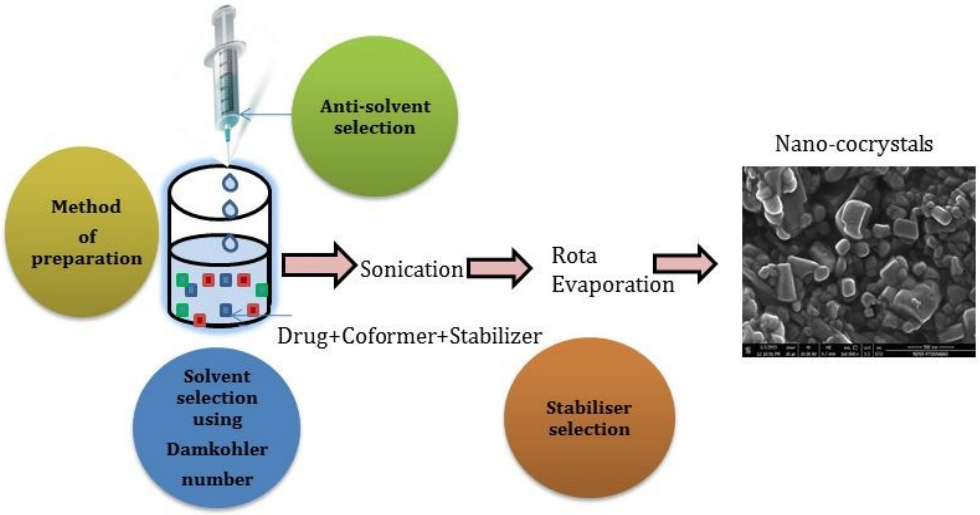
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Nano-cocrystals were optimized using L-18 Hunter's design. Selection of solvent and antisolvent was made using Damkohler number.

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Article

Preparation and optimization of Nano-sized cocrystals using quality by design approach

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The present investigation was aimed at developing a laboratory scale preparation technique for nano-cocrystals comprised of a water insoluble drug and a water soluble coformer. The challenge was to prepare a nano sized system while maintaining the integrity of the cocrystals. Carbamazepine-nicotinamide was selected as a model cocrystal for this study. Anti-solvent precipitation technique was investigated utilizing various solvents (ethanol, ethyl acetate, and acetone), anti-solvents (water, n-hexane) and stabilizers (span-80, PVPVA-64, poloxamer-407) for producing nano-cocrystals. Preliminary screening was performed based on solubility and Damkohler number which resulted in selection of acetone as a solvent and n-hexane as an anti-solvent. L-18 Hunter design was applied for studying the effect of five independent variables (type of stabilizer, concentration of stabilizer, sonication time, temperature and stirring speed) on the particle size (response variable). The experimental outcome indicated that the type of stabilizer and its concentration significantly affects the particle size of the nano-cocrystal formulation. Formulation containing 0.3% of PVPVA-64 was found to be stable with the lowest particle size i.e. D10=68.9±9.5 nm, D50=138.2±16.6 nm, and D90=260.3±17.96 nm. Further, the optimized formulation was characterized by differential scanning calorimetry, powder X-ray diffraction, and ATR-FTIR spectroscopy. A decision tree to aid in selection of solvent, anti-solvent and stabilizers for different nano-cocrystals is also presented which can be applicable for a wide range of drug and coformers with different solubility.

Keywords: L-18 Hunter design, cocrystals, nanocrystals, nano-cocrystals, precipitation, surfactants

1. Introduction

Nanocrystals are pure solid particles, crystalline in nature, with a mean diameter <1 µm. In the past two decades, nanocrystals have garnered interest in the pharmaceutical nanotechnology[1], mainly due to their unique properties such as, saturation solubility by virtue of significant increase in surface area, enhancement of dissolution rate and improvement of intracellular penetration.[2, 3] Currently, many nanocrystal formulations, mostly organic nanocrystals, have been approved for human use, such as Megase®, Rapamune®, Cesamet®, Tricor®, Emend® and few are in clinical trials.[4] Similarly, cocrystals are also massively explored to modify the solubility, dissolution rate, bioavailability, tableting, physical stability and physicochemical properties of active pharmaceutical ingredients (APIs).[5-8] Cocrystals are the components that co-exist in the cocrystal lattice with a defined stoichiometry interacting non-ionically.[9] Nano-cocrystals are cocrystals that are nanosized. The nano-cocrystals hence are generally composed of two or more different components in a single nanocrystal, that are reported to exhibit better features than nanocrystals.[10,11] On integration of nanocrystal and cocrystal technologies, improvement in properties such as thermo-sensitivity,[12] photochemistry, dissolution rates, mechanical behaviour[13] and reduced side effects of APIs through the combination of the benefits from both cocrystals and nanocrystals have been reported.[14, 15] Nano-cocrystals can be prepared by top-down approach i.e. ball milling[14, 16-18] and by bottom-up approach i.e. precipitation[14]. Karashima *et al.,* reported preparation of nano-cocrystal using wet ball milling, however, dissociation of cocrystal was observed when one component was water soluble and other was water insoluble.[16] This indicated a challenge in preparation of nano-cocrystals by ball milling for differential solubility components. Alternatively, reported nano-cocrystals prepared by precipitation are phenazopyridine-phthalimide (both water insoluble)[10], paclitaxel-disulfiram (both

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water insoluble)[19] and caffeine-p-hydroxy benzoic acid (both water soluble).[20]

However, to the best of our knowledge, no report is available for the preparation of nano-cocrystals by precipitation technique when one component is water soluble and other is water insoluble, which is generally the most common combination in cocrystal synthesis. In addition, development of nano-cocrystals is more challenging than developing simple nanocrystals by precipitation, due to differential solubility of cocrystal components and the dissociation tendency or phase instability of cocrystals in the aqueous medium.[15] Well-judged selection of the solvent, the anti-solvent, the stabilizer and the process parameters are decisive for obtaining stable nano-cocrystals with the desired particle size profile. There exists a high probability that one component in the cocrystal will precipitate out when an anti-solvent is added. This may be due to the differential solubility of the cocrystal components in the solvent or the anti-solvent.[21] Lack of an efficient nano-cocrystal preparation method would certainly limit the application of nano-cocrystals in the medical field. Therefore, there is a need of a systematic study in the field of preparation method for nano-cocrystals and criteria for formulation parameters optimization, especially, the type or amount of solvent or anti-solvent.

The objective of the present investigation was to develop a method for nano-cocrystal synthesis by precipitation technique for a water insoluble drug and water soluble coformer. Carbamazepine nicotinamide was used as a model cocrystal for this study.[22] L-18 Hunter screening design was employed to optimize the formulation and process variables to obtain the nano-cocrystals of desired particle size. The organic solvent used in the preparation was removed for the long-term stability of nano-cocrystals by rotary evaporation to obtain dry nano-cocrystals.

2. Experimental section

2.1 Materials

Carbamazepine anhydrate was purchased from Yarrow Chem Pvt Ltd., Mumbai, India. Nicotinamide was procured from Alfa Aesar, Mumbai, India. Poloxamer-407 was purchased from Sigma Aldrich, Mumbai. HPMC-E5 (hydroxyl propyl methyl cellulose) was purchased from Colorcon Asia Pvt Ltd and span-80 was obtained from SD Fine Chemicals, Mumbai, India. PVPVA-64 (Kollidon® VA 64) was purchased from BASF, Mumbai. Sodium lauryl sulfate (SLS) was purchased from Sisco Research Laboratories Pvt Ltd. Mumbai, India. All the solvents and chemicals were of analytical grade and were used as received. Distilled water was generated in-house.

2.2 Preparation of cocrystal

A carbamazepine nicotinamide model cocrystal was prepared by liquid assisted grinding and solvent evaporation. Carbamazepine and nicotinamide (1:1 mole ratio) were taken in an agate mortar and grinding was performed for 30 min with the addition of 100 µL of methanol. Single crystals were also obtained by recrystallization from the solvent system DMSO-methanol (1:1).[22]

2.2.1 Selection of solvent and anti-solvent for nano-cocrystals

Preliminary screening was performed for the selection of solvent and anti-solvent on the basis of solubility of the drug and coformer in different solvents and Damkohler number (Da) using solvents (acetone, ethyl acetate, and ethanol) and anti-solvents (water and n-hexane). Damkohler number provides a basic platform for the selection of solvents and antisolvents. Damkohler number is the ratio of mixing time for solvent and antisolvent to the precipitation time. Da was determined for the selected solvent and anti-solvent combination which provides the importance of mixing parameter in preparation of nano-cocrystals. Da is the dimensionless number which gives ratio between T_{mix} (mixing time) to $T_{precipitation}$ (precipitation time). Target value of Da for the formation of nanoparticles should be less than 1. If Da is less than 1, immediate homogeneous mixing of the solvent and anti-solvent occurs, resulting in immediate supersaturation and formation of small particles.[23, 24] If Da is greater than 1, the entire process is mixing controlled. In this case, the metastable zone (the zone in which particle growth is possible, but no nucleation occurs) is crossed slowly, which promotes particle growth instead of nucleation, leading to the formation of large crystals.[23, 24]

2.2.2 Damkohler number (Da) determination

For determination of Da, 50 mg of carbamazepine and 25.84 mg of nicotinamide in 1:1 molar ratio were dissolved in 4 mL of solvent under stirring at 250 rpm, and anti-solvent was added drop-wise. The time point at which the solution showed first sign of turbidity was considered as T_{mix}. The % yield of the precipitate was determined by isolating the precipitate, drying and weighing at various time points on addition of anti-solvent and was noted. A plot of yield vs time was obtained and the time with maximum yield was recorded as $T_{precipitation}$ (Figure S1).[25]

2.2.3 Preparation of nano-cocrystals

Carbamazepine and nicotinamide (1:1 mole ratio) were dissolved in 40% v/v of solvent containing the stabilizer under magnetic stirring, followed by dropwise addition of 60% v/v of anti-solvent to precipitate the cocrystals. The precipitated solution was sonicated by probe sonicator (Sonics and Materials, Inc. US). The resultant nano-cocrystals were recovered by rotary evaporator (Heidolph, Germany).

2.2.4 Optimization of nano-cocrystals by L-18 Hunter design

Preliminary screening trials were carried out for evaluating the process parameters of carbamazepine-nicotinamide nano-cocrystals. Based on the outcome, factors such as, stabilizers and its concentration (0-0.4% w/v), sonication time (0-2 min), stirring speed (250-750 rpm) and rota evaporation temperature (40-60°C), were identified as critical to obtain a product in the desired nano-range with the required stability. The interaction between these variables is complex resulting in a time-consuming and laborious process for optimization of significant factors.[26] Hence, Hunter L18 screening design was constructed using JMP software (version 10, SAS inc., NC, USA) to find an optimized formulation using minimum

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number of experiments. The independent variables selected were stabilizer type (A), surfactant concentration (B), sonication time (0 min or 1 min at 40% amplitude, 10 sec on / 5 sec off cycle) (C), temperature (D) and stirring speed (E) at different levels as shown in Table 1. Out of these five independent variables, A and C were the categorical variables and the rest were continuous. Three levels were investigated for A and B, and two levels for C, D, and E. Eighteen formulations were prepared and the response (particle size) was measured in terms of signal to noise (S/N) ratio (Table 2). ANOVA (P<0.5) was conducted to estimate the significance of each factor using the software. S/N ratio was plotted against each level to determine the significance of each factor by their rank and to establish their optimum levels for the product preparation (Table S1).

Table 1: L18-Hunter design factors and their levels

Code	Independent variables	Levels		
		1	**2**	**3**
A	Stabilizers	Poloxamer-407	PVPVA-64	Span-80
B	Stabilizer concentration (% w/v)	0.1	0.2	0.3
C	Sonication time (min)	0	1	-
D	Temperature (°C)	40	60	-
E	Stirring speed (rpm)	250	500	-

2.3 Characterization of nano-cocrystals

2.3.1 Scanning electron microscopy (SEM)

Particle size and morphology of prepared nano-cocrystals were characterized by scanning electron microscope (Quanta 250, FEI, USA) in high vacuum mode at an accelerating voltage of 5 kV. Solid samples were directly placed on the copper grid. As the sample was non-conductive, it was coated with gold for 10 min prior to analysis. Sample analysis was carried out using backscatter electron detector.

2.3.2 Differential scanning calorimetry (DSC)

Thermal analysis of the samples was performed using Mettler Toledo DSC (Star software). High purity indium standard was used to calibrate the DSC instrument for temperature and heat flow. Accurately weighed samples were placed in hermetically sealed aluminium crimped pans. Samples were heated at rate 10°C/min and scanned from 20°C to 200°C during which dry nitrogen gas was purged at a rate of 60 mL/min.

2.3.3 Powder X-ray Diffraction (PXRD)

PXRD patterns of samples were recorded by using x-ray diffractometer (Philips X'pert MPD System). Samples were illuminated using Ni-filtered Cu-K at the voltage of 40 kV and current of 30 mA. Data were obtained over 2θ scan ranging from 2^0 to 40^0 with a step time of 0.013 steps per 0.5 sec.

2.3.4 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

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IR spectra of samples were measured using Perkin Elmer IR spectrophotometer. Around 5 to 10 mg samples were directly placed on the diamond crystal surface for surface analysis. The IR spectra data was recorded over the range of 500 to 4000 cm^{-1} by the accumulation of 16 scans.

2.4 Evaluation of nano-cocrystals

2.4.1 Solubility study

Solubility studies (n=3) were carried out for the drug, corresponding physical mixture, cocrystal and nano-cocrystal in 1 % w/v aqueous solution of sodium lauryl sulphate (SLS) by adding in excess to the 5mL vial. These vials were kept in orbital shaker at 37°C and 100 rpm for 72h. Concentration of drug was determined by measuring the absorbance at wavelength of 305 nm in UV spectrophotometer. A calibration curve for carbamazepine was linear in the range of 5 to 20 µg/ml (Y=0.0314x-0.004; R^2 = 0.9996; n=3). Nicotinamide did not interfere with the analysis of carbamazepine at 305 nm (Figure S2).

2.4.2 Dissolution study

Dissolution studies (n=3) were carried out by using USP apparatus II at 37°C, 75 rpm in official dissolution media of 1 % w/v aqueous solution of SLS.[27] Carbamazepine, cocrystal, nano-cocrystal and physical mixture equivalent to 100 mg of carbamazepine were added to the 900 ml of dissolution medium. Samples, 5mL each, were withdrawn at predetermined time point and were substituted with same quantity of fresh media warmed at 37°C. Samples were filtered using 0.22 µ filter and were analysed for carbamazepine by UV spectrophotometer at 305 nm. DDsolver software was used for determination of drug released at 15 minute (Q_{15}), % dissolution efficiency (DE_{15}) at 15 minute and similarity factor (f_2) for drug, cocrystal and nano-cocrystal.[28]

3. Results and discussion

3.1 Screening of solvents and anti-solvents

One of the challenging tasks in preparation of nano-cocrystals is the selection of suitable solvent and anti-solvent. The solvent selected should have adequate solubility of drug and coformer to permit sufficient interaction during crystallization. If high amount of organic solvent is required to dissolve the drug and coformer, more time is required for evaporation and the particles will remain in contact with the organic solvent for longer time, which may lead to increase in particle size. Whereas, solvents and anti-solvents with differential solubility for drug or coformer will lead to precipitation of either drug or coformer with no co-crystal formation.

Three different solvents such as ethanol, ethyl acetate and acetone and two different anti-solvents such as water and n-hexane were selected for the preparation of carbamazepine-nicotinamide nano-cocrystals. The drug and the coformer had high solubility in solvent systems selected whereas the anti-solvents were selected based on

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their miscibility with the solvents. When water was used as anti-solvent, carbamazepine precipitated alone without forming a co-crystal, due to the high solubility of nicotinamide in water. This was confirmed by DSC and ATR-FTIR which showed the characteristic peaks for drug and coformer instead of cocrystal. It can thus be concluded that water may not be a suitable anti-solvent for nano-cocrystal system that has one component water soluble and another component water insoluble. In contrast, systems with n-hexane as anti-solvent did not result in drug and coformer separation. This clearly indicated the importance of anti-solvent selection in the preparation of such nano-cocystals.

Da the ratio of T_{mix} to $T_{precipitation}$ plays an important role in selection of solvent. Mixing time implies the time taken for the mixing process of solvent and antisolvent. On complete mixing, dissolved molecules starts to nucleate which result in hazy appearance i.e. turbidity starts to appear. This time point was noted as the mixing time. This first sign of turbidity concluded that drug and coformer interacted with each other at molecular level and started to nucleate as cocrystal. Precipitation time starts from appearance of turbidity and ends as when the product yield remains constant. The time gap from appearance of turbidity to completion of precipitation was considered as precipitation time.[25] Additionally this concept was supported by the work of IC Wang *et al*., wherein they had calculated the amount of carbamazepine-saccharine cocrystal formed during antisolvent co crystallization by ATR-FTIR spectroscopy in which carbamazepine-saccharine cocrystal nuecleated in 2-3 minutes as hazy appearance and complete constant cocrystal yield was observed around after 30 minutes.[29]

Nano-cocrystal preparation includes two key process, T_{mix} and $T_{precipitation}$.[30] If T_{mix} is more as compared to the $T_{precipitation}$, reactants will take time to contact with each other. Therefore, supersaturation will achieve slowly resulting in formation of larger sized particles. If mixing time is small, then the reactants come in contact quickly with the anti-solvent, attaining rapid supersaturation and faster formation of nuclei which ultimately leads to the formation of nano-size particles.[23, 24] Therefore, T_{mix} is required to be small as compared to $T_{precipitation}$ for the formation of nanoparticles. T_{mix} of ethanol with n-hexane was more compared to acetone n-hexane system, as a result, it produced micron size particles. Therefore, ethanol was not a suitable solvent for nano-cocrystal formation. Da for ethanol n-hexane system was greater than 1, whereas, Da for acetone and ethyl acetate was less than 1 (Figure 1). The solubility of carbamazepine in solvents was observed in the given order i.e. ethanol = acetone > ethyl acetate. Due to the low solubility of carbamazepine in ethyl acetate, this system gave low yield, hence was not selected as a solvent for preparation of nano-cocrystal. As a result, acetone was selected as a solvent and n-hexane as an anti-solvent**.** Results thus indicated that the solvent selection is not only dependent on the solubility parameter but also on mixing parameter with anti-solvent.

Preliminary trials namely trial A (33%: 67%), trial B (40%: 60%), trial C (45%: 55%) and trial D (50%: 50%) for selection of solvent to antisolvent ratios were performed. An optimum antisolvent ratio is

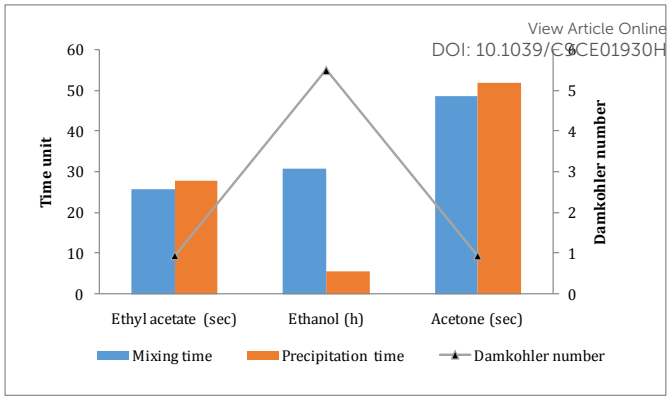


Figure 1: Damkohler number for different solvents with n-hexane as antisolvent

desired to result in appropriate supersaturation so that the nuclei generation dominates over the crystal growth phase. This results in smaller size crystals. However, further decrease in antisolvent decreases the supersaturation resulting in increase in particle size. Similar outcome was reported in literature[31]. Trial B with solvent: antisolvent ratio of 40%: 60% showed smaller particle size as compared to other ratios (Figure S3). Hence this ratio was used for further optimization process.

3.2 Selection of stabilizers based on solubility in anti-solvent systems

Selection of surfactant is also a critical parameter for stabilization of nano-cocrystals.[32] Surfactants are required to decrease the surface area of the particle by reducing surface tension and thus avoiding agglomeration. Immediate precipitation was observed on addition of anti-solvent when only drug and coformer were present in the solution that led to increase in particle size due to agglomeration. In n-hexane based anti-solvent system, SLS and HPMC are not soluble therefore, PVPVA-64, span-80 and poloxamer-407 were screened as stabilizers.

3.3 Preliminary screening

Results from the preliminary screening trials suggested that micron size particles were formed in absence of stabilizer. Addition of stabilizer reduced the particle size with nano-range particles being formed in the range of 0.3% to 0.4% w/v stabilizer concentration (Figure S4). Stirring speed also had a significant impact on the particle size. Particle size obtained at 500 rpm was smaller when compared to 250 rpm. (Figure S5) When the stirring rate is increased, the energy transferred to the reaction medium is also increased and the reaction solution can be dispersed into smaller size droplets.[33] Similarly, anomalous diffusion of the particles at higher degree of agitation reduces the growth kinetics of the particles, resulting in smaller sized particles. Above 500 rpm, the particle size increased due to agglomeration. Therefore, stirring speed of 250 and 500 rpm were selected for the optimization studies. Particle size obtained at 40 °C and 60 °C was relatively smaller as compared to other lower temperature studied, as the system remained in contact with the organic solvent for longer time encouraging an increase in crystal dimensions (Figure S6).

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3.4 L-18 Hunter design response treatment

Various factors affect nano-cocrystal formulation. Hence, it is imperative to know which of the factors affect the formation of nano-cocrystal significantly. A screening design was hence used to determine the significant factors and their optimum levels to prepare an acceptable formulation with desired quality attributes. L-18 Hunter design is a screening design when factors with different levels are used in experiments and alternative designs like Taguchi screening design are not suitable.[34] Hence, by using L-18 Hunter design, 18 experiments were built to screen the influence of five parameters at their lowest and highest factor levels. The input factor conditions and the resulting experimental response data for 18 screening experimental runs are shown in Table 2. For better accuracy of the measured response, signal to noise (S/N) ratio was considered. S/N ratio determines how the response varies relative to target value under different noise condition. Minitab software was employed for calculation of S/N ratio by using the equation: $S/N=-1*\log(\sum(1/Y^2)/n$, Where, n= Number of observation on the particular product and Y is the variable. This value helps to establish the significance of each factor and aids in determining their optimum levels for the formulation.

3.5 Particle size determination

Particle size determination by commonly employed technique of dynamic light scattering method is challenging for water insoluble and water soluble component combination due to dissociation of nano-cocrystal upon dilution with water.[35] Hence, particle size and morphology of the prepared carbamazepine-nicotinamide nano-cocrystals with different surfactants in acetone: n-hexane system was determined by SEM as other methods like zeta sizing involved dilutions with media. Nano-cocrystals prepared using span-80, PVPVA-64 and poloxamer-407 produced platy shaped crystal, irregular lamellar shaped crystal and rod shaped crystal, respectively (Figure 2). More than 160 particles in different fields were selected and the particle size distribution of nano-cocrystals was expressed in terms of D10, D50, and D90 (Table 2).

3.6 Effect of stabilizers

Particle sizes (D90) of the formulations obtained with different stabilizers were found to be 8583.5 nm (poloxamer-407), 29805 nm (span-80) and 308.15 nm (PVPVA-64) at 0.1 % w/v concentration (Table 2). These results highlighted that PVPVA-64 was more effective in reducing the particle size as compared to poloxamer-407 and span-80 (Figure 3A). This effect was mainly attributed to superior crystal growth inhibition property of PVPVA-64 during solvent evaporation.

3.7 Effect of stabilizer concentration

A decrease in particle size increases the surface area and surface free energy of the particle leading to a tendency to increase in the apparent particle size. The concentration of stabilizer here plays a decisive role in maintaining the particle size of the nano-cocrystals. Coating of stabilizers on the surface of particles reduces the surface free energy of particles. However, inadequate amount of surfactant may result in the formation of unstable particles due to insufficient coating of the particles. A remarkable decrease in the particle size was noticed with increase in the concentration of stabilizers which was confirmed from S/N ratio that revealed a direct relationship to the particle size of the nano-cocrystals (Figure 3B). Formulations prepared using 0.1 % w/v span-80 and poloxamer-407 produced micron size particles probably due to inadequate coating of the particles, leading to larger particle size (F17 & F3). In contrast, formulations prepared using 0.1% w/v PVPVA-64 produced nanosized particles (F2). Increase in stabilizer concentration to 0.2 % w/v produced mixed results wherein particles of both nano and micron size were obtained due to agglomeration. Further increase in concentration to 0.3 % w/v demonstrated significant reduction in particle size for all stabilizers; span-80 (26145 to 766.65 nm), poloxamer-407 (4658 to 572.5 nm) and PVPVA-64 (308.15 to 260 nm) which was attributed to significant decrease in surface free energy of particles and effective coating of particles.

3.8 Effect of sonication time

The formulations were either not sonicated (0 min) or subjected to sonication for 1 min. According to the results obtained, sonication showed a positive impact in the reduction of the particle size of the formulation which can be concluded by the low value of S/N ratio when sonication was performed (Figure 3C). This may have occurred due to high energy released in the ultrasonic process and which leads to a rapid breakage of particles into nanodroplets of small size with uniform distribution.[36] Hence, 1 min sonication was considered optimum for preparation of nano-cocrystals.

3.9 Effect of temperature

Particles obtained at 60°C were smaller as compared to the particles formed at 40°C. The influence of temperature on formulations were studied on the basis of S/N ratio which gave direct relationship to the particle size of the formulation. S/N ratio decreased with an increase in the temperature (Figure 3D). The particle size at 60°C decreased from 343.9 to 260.3 nm (PVPVA-64), 2428 to 572.5 nm (poloxamer-407) and 2585 to 766.65 nm (span-80). As mentioned above, at higher temperature, the solvent evaporated rapidly thus decreasing the residence time of the particles in the solvent media leading to smaller particles.[37]

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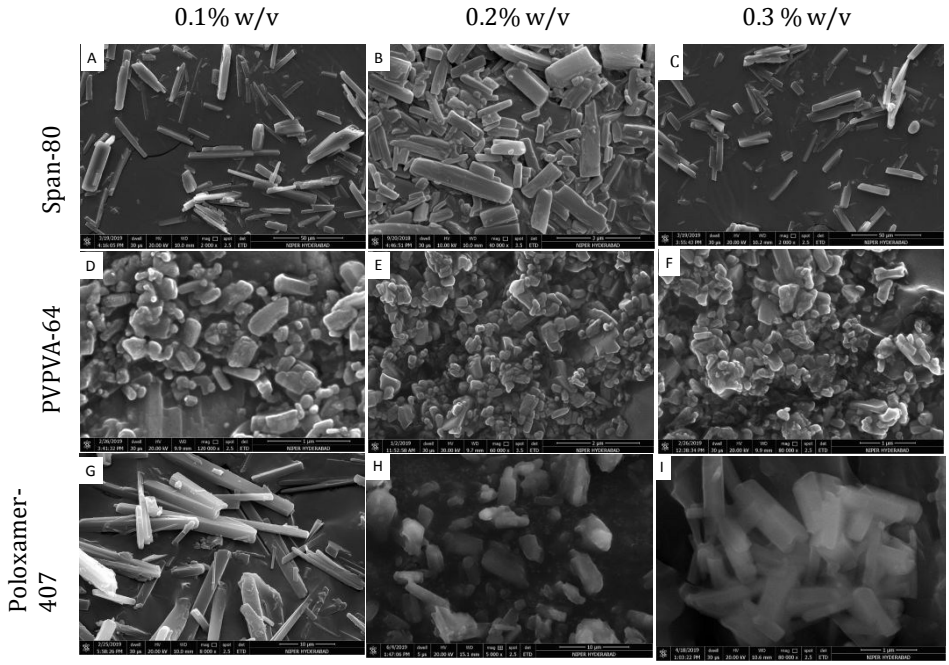


Figure 2: SEM micrographs of nano-cocrytsals [A] F17 (50 µm scale) [B] F6 (3 µm scale) [C] F16 (2 µm scale), [D] F9 (1µm scale)[E] F7 (4 µm scale) [F] F2 (1 µm scale), [G] F3 (30 µm scale) [H] F1 (10 µm scale) and [I] F5 (1 µm scale)

Table 2: L-18 Hunter design experiments and their responses

Formulation	Independent variables					Response			S/N ratio
Codes	A	B	C	D	E	D10	D50	D90	
F1	Poloxamer-407	0.2	0	60	500	1462±172.5	2760±204.5	4719±540.2	73.47
F2	PVPVA-64	03	1	60	250	68.9±9.5	138.2±16.6	260.3±17.96	48.30
F3	Poloxamer-407	0.1	0	40	250	1026±61.7	3082±212.8391	8583.5±702.1	78.67
F4	Span-80	0.2	1	40	500	1358.5±51.6	2867±458.2	6336.5±1588.2	76.03
F5	Poloxamer-407	0.2	1	60	250	380.1±25.5	670.8±22.7	1263.5±0.707	62.03
F6	Span-80	0.2	1	60	250	366.5±6.3	711±8.4	1274±2.1	62.10
F7	PVPVA-64	0.3	0	60	500	107.45±6.85	181±11.31	343.9±15.55	50.72
F8	Poloxamer-407	0.3	1	40	500	757±132.2	1326±202.3	2428±146.3	67.70
F9	PVPVA-64	0.1	1	60	250	77.65±0.34	148.7±19.37	308.15±29.2	49.77
F10	PVPVA-64	0.1	1	40	500	78.535±2.1	191.95±40.9	357.85±32	51.07
F11	Span-80	0.3	0	40	250	505.4±30.4	1230±131.5	2585±190	68.24
F12	Span-80	0.3	0	60	500	211.25±53.38	388.45±43.2	766.65±96.6	57.69
F13	Poloxamer-407	0.3	1	60	250	125.6±28.56	232.5±55.6	572.5±35.7	55.15
F14	PVPVA-64	0.2	0	60	500	129.95±12.65	263±1.5	487.8±22.4	53.76
F15	Span-80	0.1	0	60	500	2925±251.7	10750±2913	26145±5000	88.34
F16	Poloxamer-407	0.1	0	60	500	865.5±36	2092±504	4658±320.3	73.36
F17	Span-80	0.1	1	60	250	6495±144.9	15080±516.8	29805±1675.8	89.48
F18	PVPVA-64	0.2	0	40	250	205.05±6.15	368.75±18.03	668.3±4.5	56.49

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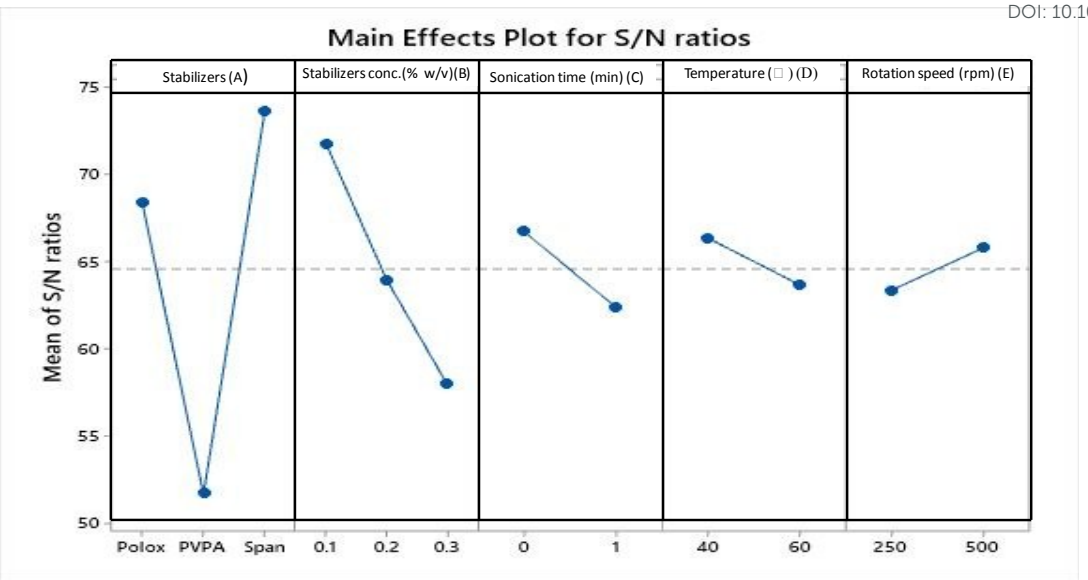


Figure 3: Effect of (A) stabilizers (B) % w/v of stabilizers (C) sonication time (D) temperature (E) Stirring speed on response

3.10 Effect of stirring speed

Stirring speed and particle size were found to be inversely related to each other. At higher stirring speed, higher shear energy and turbulence are produced, which is generally beneficial in reducing the particle size. However, in the present case, S/N ratios for 250 rpm and 500 rpm were statistically similar as shown in Figure 3E. Therefore, it was concluded that nano-cocrystals prepared at a stirring speed of 500 rpm would be quite similar in size as compared to the nano-cocrystal prepared at 250 rpm indicating negligible effect of stirring speed on particle size.

3.11 Optimization

The analysis of mean S/N ratio were further confirmed with ANOVA The outcome for individual factors (A–E) at different levels and the influences of factors were predicted and summarized in Table S1 and Table S2. Further, the influence of each factor on the response variables were also evaluated with the help of Pareto charts. Pareto chart (Figure 4) explains the impact of stabilizers, its concentration, stirring speed, sonication time and temperature on particle size. The Pareto chart clearly indicated that the type of stabilizers and its concentrations were the significant factors. Whereas, sonication time, temperature and stirring speed were found to be insignificant in the preparation of nano-cocrystals. These findings were similar to the results of the S/N ratio response. Based on the optimization studies, the factors and their optimized levels were selected at A2B3C2D2E1 (formulation F2) for the development of carbamazepine-nicotinamide nano-cocrystals.

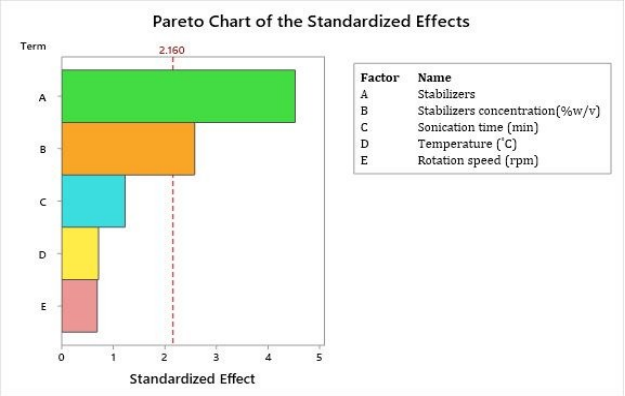


Figure 4: Pareto chart of standardized effects

Schematic flow of the selection of solvent and anti-solvent for the preparation of nano-cocrystals is represented in the following flow chart (Figure 5) for easy understanding.

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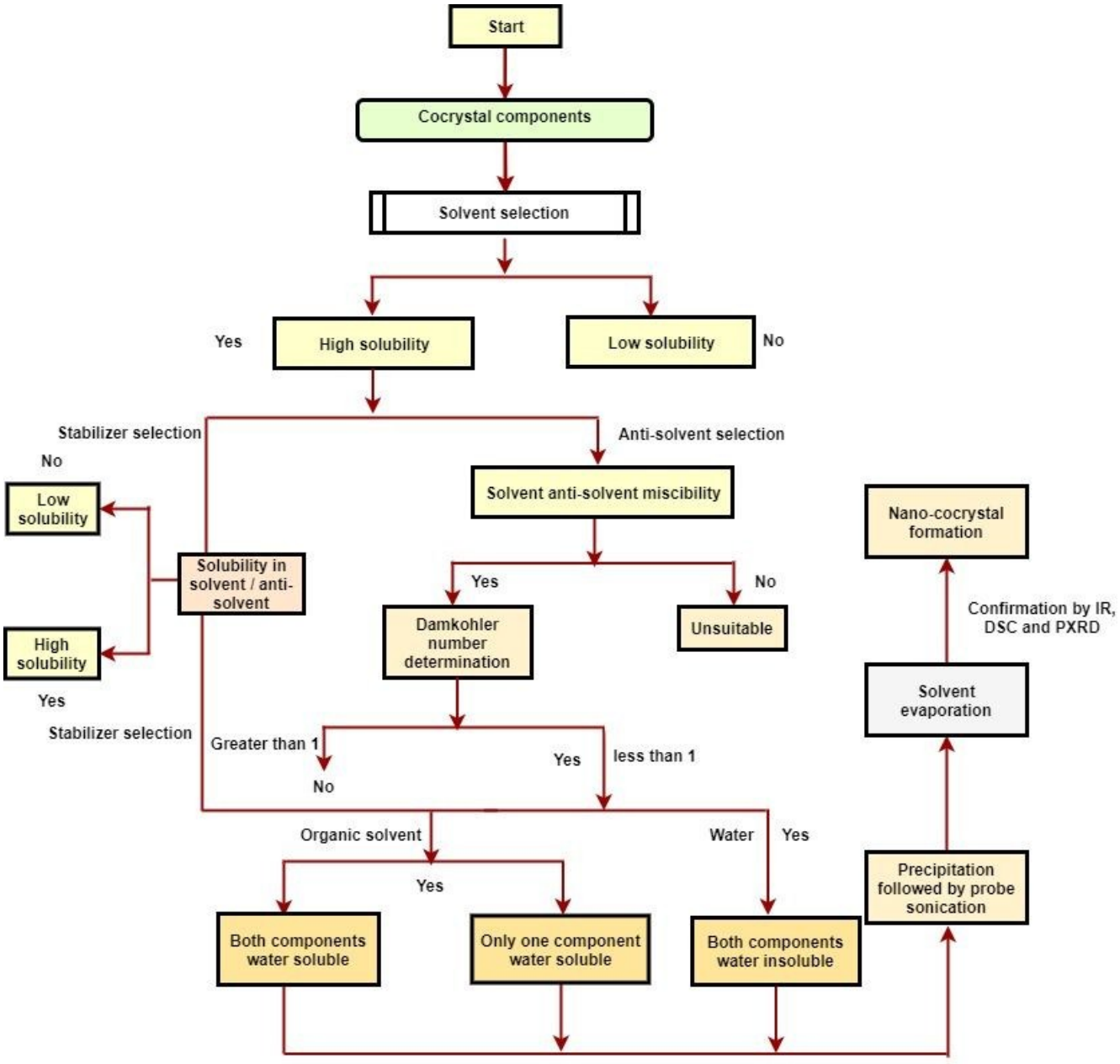


Figure 5: Decision tree for the selection of solvent and anti-solvent in preparation of nano-size cocrystals

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3.12 DSC

The melting endotherms of carbamazepine, nicotinamide and cocrystal were observed at 191°C, 131°C and 159°C respectively (Figure 6). The thermogram of carbamazepine showed endotherm at 165.4°C followed immediately by a weak exotherm and an another endotherm at 191°C (Figure 6A) due to the polymorphic transformation of form III to form I.[38] As shown in Figure 6C, melting endotherm of nano-cocrystal with anti-solvent n-hexane was observed at 159°C which matched with the melting endotherm of the cocrystal (Figure 6B). When water was used as an anti-solvent, carbamazepine dihydrate (Figure 6E) was formed which can be confirmed by DSC thermogram of carbamazepine dihydrate.[39] Therefore water was not selected as an anti-solvent in nano-cocrystals preparation.

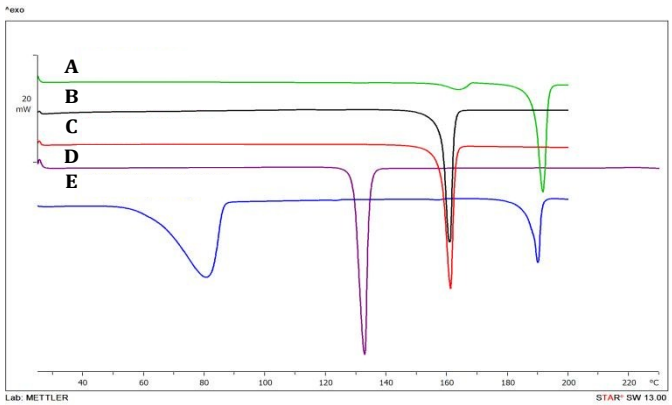


Figure 6: DSC thermogram of [A] Carbamazepine [B] Carbamazepine-nicotinamide cocrystal [C] Carbamazepine-nicotinamide nano-cocrystal with hexane (F2) [D] Nicotinamide [E] Carbamazepine-nicotinamide formulation with water.

3.13 PXRD

PXRD patterns depicted in Figure 7 showed sharp diffraction peaks demonstrating that samples were crystalline in nature. Carbamazepine exhibited sharp characteristic peaks at 2θ value of 13.11°, 14.19°, 15.11°, 17.1°, 19.5° and 25.03° that matched with form III of carbamazepine.[40] Nicotinamide showed sharp characteristic peaks at 2θ value of 11.3°, 14.8°, 22.25°, 25.8°, 27.3° and 36.95° that matched with form I polymorph of nicotinamide.[41] Carbamazepine-nicotinamide cocrystal exhibited characteristic peaks which showed 2θ value of 6.85°, 9.23°, 10.52°, 20.85°, 26.85°, 27.2° which are in accordance to the reported cocrystal by neat grinding.[22] Carbamazepine-nicotinamide nano-cocrystal showed characteristic peaks at the 2θ value of 6.88°, 9.18°, 10.52°, 20.81°, 26.80°, 27.2°. PXRD pattern of nano-cocrystal is identical to PXRD patterns of that of cocrystal which demonstrated the successful formation of nano-cocrystals using n-hexane as an anti-solvent and acetone as a solvent. Additionally, the formation of nano-cocrystal with span-80 and poloxamer-407 was confirmed by PXRD (Figure S7).

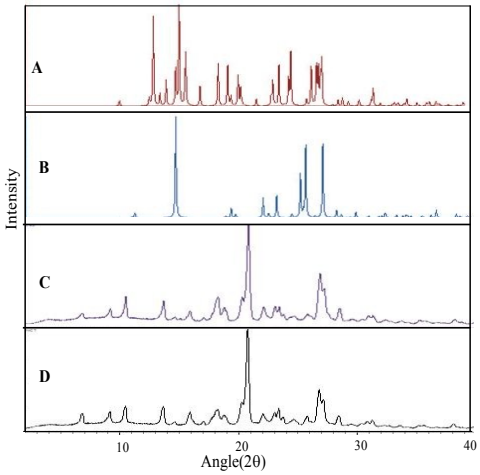


Figure 7: PXRD patterns of [A] Carbamazepine [B] Nicotinamide [C] Carbamazepine-nicotinamide cocrystal [D] Carbamazepine-nicotinamide nano-cocrystal

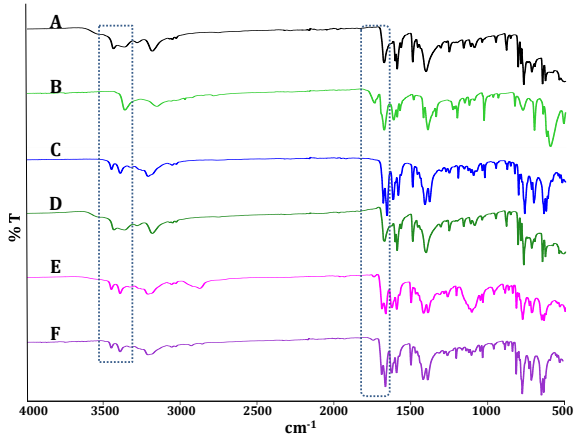


Figure 8: IR spectra of [A] Carbamazepine [B] Nicotinamide [C] Carbamazepine-nicotinamide cocrystal [D] Carbamazepine-nicotinamide formulation in water [E] Carbamazepine-nicotinamide nano-cocrystal in ethyl acetate:n-hexane [F] Carbamazepine-nicotinamide nano-cocrystal in acetone:n-hexane with PVPVA-64

3.14 ATR-FTIR

ATR-FTIR analysis was performed to detect the interaction between drugs and coformers. Plain carbamazepine showed characteristic N-H stretching at 3433 cm^{-1} which display the presence of amine group in carbamazepine. Apart from this, a peak at 3145 cm^{-1} and 1680 cm^{-1} indicated the presence of aromatic stretching of C-H and C=O stretching of CO-NH_2 respectively (Figure 8A). Presence of peak at 1591 cm^{-1} and 1487 cm^{-1} was an indication of C=C aromatic

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stretching. Changes in the position and intensity of IR stretching and bending vibrations confirm the formation of new hydrogen bonds. As shown in Figure 8C, E and F, a significant shift in –NH stretching and C=O stretching was observed at 3484 cm^{-1} and 1684 cm^{-1} respectively. This was explained by the successful formation of cocrystal. An IR spectrum of the formulation prepared by water as anti-solvent was not similar to the cocrystal IR spectrum. Instead, it showed similarities with the spectra of carbamazepine plain drug which implied that only carbamazepine was precipitated (Figure 8D). Nano-cocrystal prepared by n-hexane, had almost a similar spectrum to that of cocrystal as shown in Figure 8C. This demonstrated the successful formation of the carbamazepine-nicotinamide nano-cocrystal without separation of drug and coformer.

4.0 Evaluation of nano-cocrystal

4.1 Solubility study

Carbamazepine solubility from plain drug, cocrystal, physical mixture and nano-cocrystal in 1% w/v of aqueous solution of SLS is reported in Figure 9. Drug, physical mixture and cocrystal showed similar solubility. However, nano-cocrystal showed 1.2-fold enhancement in solubility. Improved solublization of nano-cocrystal could be due to reduced particle size and increased wetting.[42] Phase stability of all samples was determined from the obtained residue after solubility experiments. From the DSC results, it was concluded that all samples; drug, cocrystal, nano-cocrystal and physical mixture showed formation of carbamazepine dihydrate (Figure S8) that was in tune with the earlier reports of carbamazepine tendency to convert into its dihydrate form on contact with water.[43]

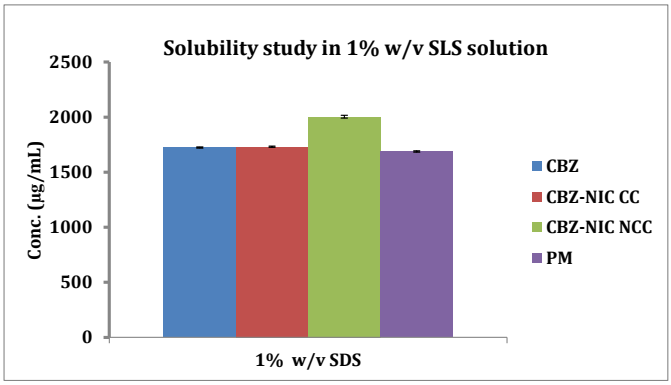


Figure 9: solubility study of CBZ (carbamazepine), CBZ-NIC CC (Carbamazepine-nicotinamide cocrystal) CBZ-NIC NCC (Carbamazepine - nicotinamide nano-cocrystal) PM (physical mixture).

4.2 Dissolution study

Drug, cocrystal, nano-cocrystal and physical mixture were subjected to dissolution studies in 1 % w/v aqueous solution of SLS media (Figure 10). Plain drug showed more than 85 % release in 60 min, whereas 85 % of the drug was released from the cocrystal, physical mixture and nano-cocrystal at 45, 90 and 5 min respectively. Nano-cocrystal showed high release rate as compared to drug, cocrystal and physical mixture. Various dissolution parameters were employed to understand the release profile of the drug, cocrystal, nano-cocrystal and physical mixture (Table S3). Drug showed Q_{15} value of 62.50 %. Q_{15} for cocrystal, physical mixture and nano-cocrystal were 64.78, 61.96 and 97.95% respectively. % DE describes the efficiency of the drug release. Drug showed % DE_{15} value of 47 %. DE_{15} for cocrystal, physical mixture and nano-cocrystal were 46, 43 and 71 % respectively. Based on these data it can be concluded that nanococrystal showed a significant 1.54 times increase in dissolution. Nano-cocrystal showed superior release profile as compared to drug and cocrystal. This could be attributed due to an increase in surface area of the nano sized cocrystal.

Two dissolution profiles can be considered as similar when f_2 values are in between 50 to 100.[44] Similarity factor f_2 value was calculated for cocrystal, physical mixture and nano-cocrystal as 84.29, 67.56 and 25.19 with drug as reference. Cocrystal and physical mixture showed similar profile with drug, however, nano-cocrystal showed dissimilar profile in comparison with drug. This justified the claim of nano-cocrystals being superior to cocrystals and physical mixture in terms of their dissolution parameters

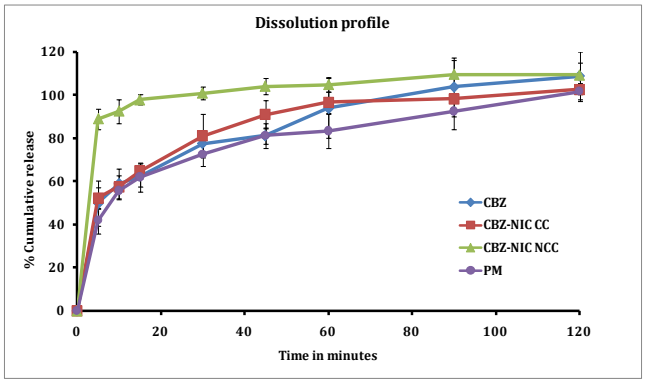


Figure 10: Dissolution profile of CBZ (carbamazepine), CBZ-NIC CC (Carbamazepine-nicotinamide cocrystal), CBZ-NIC CC (Carbamazepine - nicotinamide nano-cocrystal), and PM (physical mixture).

5. Conclusions

The current research demonstrated that the precipitation technique can be an effective method in the production of nano-cocrystals with appropriate selection of solvent, anti-solvent, and surfactant. Carbamazepine-nicotinamide nano-cocrystals were successfully formulated by integrating the cocrystal and nanocrystal preparation technologies. In the preparation method, acetone was selected as a solvent and n-hexane was selected as an anti-solvent. L-18 Hunter design was successfully used to optimize the formulation and process parameters to get the desired size of nano-cocrystal. Based on ANOVA and S/N ratio, stabilizer and its concentration were found to be the most significant factors affecting the particle size of nano-cocrystals. Formulation F2 containing 0.3 % w/v of PVPVA-64 was found to be the optimized formulation. Dissolution and solubility of carbamazepine were improved by nano-cocrystal formulation. We anticipate our findings will provide guidance for nano-cocrystal preparation of water

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insoluble drug and water soluble coformer for selection of solvent, anti-solvent, and stabilizer in the scarcely explored nano-cocrystal area. In future this methodology could be utilized for nano-sized multi-component drug delivery and spray drying method for scale-up.

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Conflict of interest

The authors report no conflicts of interest.

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