Research Article

Investigation of Effect of Non-Ionic Stabilizers on the Physical Stability of Drug Nanosuspension Prepared By Bottom Up Approach

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ABSTRACT
In this research work, the effects of nonionic stabilizers on the physical stability of drug nanosuspensions were investigated. For this purpose five nonionic polymers (hydroxypropylmethyl cellulose (HPMC), Hydroxypropyl cellulose (HPC), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP) and pluronic F68) and esomeprazole were selected as stabilizers and drug candidate, respectively. All the nanosuspensions were prepared using bottom up approach. The potential of Ostwald ripening for the nanosuspensions was investigated by subjecting them to various stress conditions such as storage at various temperature conditions (15°C, 25°C, 35°C, 45°C), mechanically shaking for 72 hours and fluctuation in storage temperature. All the polyvinylpyrrolidone and hydroxypropyl cellulose based formulations that were stored under different stress conditions exhibited the increase in particle size. In other cases the highest increase in mean particle size was observed at 45°C, followed by 35°C. Samples stored at 15°C and 25°C did not exhibit the significant changes in particle size. The HPMC 1 formulation stored at 45°C, exhibited a steep increase in particle size, probably due to desolvation of the HPMC molecules at this temperature and subsequent loss of stabilization of the nanoparticles. However, in case of HPMC 2 and HPMC 3 formulations (stored at 45°C), the gradual increase in particles size was obtained. This trend of increase in particle size was attributed to presentation of excess amount of HPMC. Powder X-ray diffraction analysis confirmed that all the prepared nanosuspensions were in crystalline state. Hence, physical treatments and other factors did not change the crystalline state of nanosuspensions. To Confirm the crystalline state of those samples which were undergo for 3 cycles of temperature fluctuation, the DSC (Differential scanning calorimetry) analysis was performed, and compare with raw drug. Esomeprazole exhibited the melting endotherm at an onset temperature of 178.1°C and a peak temperature at 185.31°C. The thermogram revealed that crystalline state of raw drug was not changes but the melting peak drifted slightly due to presence of stabilizers.

Keywords: Stability, Stabilizers, Polymeric nonionic stabilizers, Stress conditions, Fluctuation in temperature, Desolvation.

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INTRODUCTION
In recent years, about 40% to 60% of new APIs have a high degree of hydrophobicity which makes them poorly water soluble. [1] This data generate the necessity to develop the formulation strategies that can address the issue of low solubility and help to improve the rate

of dissolution to enhance the bioavailability of these compounds. There are many well known strategies including use of co-solvent, complexation with cyclodextrin, solid dispersion, mixed micelles, microemulsions and in-situ salt formation have been widely used in the pharmaceutical industry to solve the solubility problem. [2] Nowadays, nanosuspension is another rapidly emerging technology, used to overcome these solubility problems. It consists of drug substance particles in the sub-micron range that are dispersed in liquid phase, and stabilized by ionic or non-ionic stabilizers. [3] There are mainly two approaches used to prepare the nanosuspensions: 'top down' and 'bottom up' technologies. The top down approach basically depends on mechanical attrition to change large crystalline particles into nanoparticles. The bottom up approach depends on controlled precipitation or crystallization. This process involves the dissolution of drug in a solvent and then precipitating it in a control manner to prepare the nanoparticles with the addition of an anti-solvent. [4-6]

The small particle size of nanosuspensions, which is main reason of their success, is also associated for their physical instability. The large surface area associated with the small particles of nanosuspension results in high interfacial tension, which leads to an increase in free energy of the system. This increases the tendency of the system to reverse to its lower energy state via flocculation, aggregation or crystal growth; thus, affecting the physical stability of the nanosuspension. This physical instability can be overcome by using the stabilizers. The approaches used to stabilize the nanosuspensions include steric stabilization and electrostatic stabilization or a combination of both. In steric stabilization mechanism, non ionic surfactants (stabilizers) are usually used. These stabilizers provide the steric stabilization via the solvation effect. [7] Non ionic stabilizers have multiple anchoring sites which provide the strong binding via cooperative multi-site attachment. Thus non ionic stabilizers adsorb on the surface of drug particles through these anchor sites, while other tail part which is solvated part extends into the bulk medium. Stabilizing moiety should be sufficiently long and dense for maintaining the steric barrier that is capable of minimizing the interaction between particles to a level that the van der wall attractive forces are less than the repulsive forces. Electrostatic stabilization is achieved by adsorbing the charged molecules onto the particle surface. These charged molecules may be ionic surfactants or charged molecules. [8-10]

Stabilizers constitute an integral part of nanosuspensions and it is very important to understand their role on physical stability of nanosuspensions. Stabilizers may be surfactants, polymers or a combination of both. [11] Commonly used excipients that are suitable for polymeric stabilization include the cellulosics, such as hydroxypropylcellulose

(HPC), hydroxypropylmethylcellulose (HPMC), povidone (PVP), and pluronics. The non-ionic surfactants are also uses as stabilizers such as polysorbate. Sometime sodium laurylsulphate (SLS) and docusate sodium (DOSS) are uses as the anionic stabilizers. Generally, cationic surfactants are not uses as stabilizers for oral formulations due to their antiseptic properties. Smaller surfactant molecules are also helping the nanoparticles to stabilize, but are usually more prone for Ostwald ripening. [12-13]

Crystal growth or Ostwald ripening is a very common problem associated with colloidal suspension. It is also responsible for particle size changes and their distribution. Ostwald ripening effect is initiated due to unequal solubility profile of different particles, dependent on their size. Small particles show higher saturation solubility in comparison of larger one. The difference in saturation solubility creates a concentration gradient between small and large particles, resulting in diffusion of molecules from higher concentration (surrounded with small particles) to lower concentration (surrounded with large particles). Thus supersaturated solution surrounds the large particles, leading to drug crystallization onto the large particles. Due to this diffusion process, the unsaturated solution surrounds the small particles and forces them to dissolve quickly in bulk medium. This diffusion process continues until all the small particles are dissolved. Ultimately Ostwald ripening effect leads to variation in particle size and size distribution throughout colloidal suspension. [14-17]

Particles of uniform size, present in the medium, can minimize the saturation solubility difference and drug concentration gradient. Thus, helps in inhibiting the Ostwald ripening. Surfactants play a very important role in inhibiting the occurrence of Ostwald ripening, because after adsorbing on the surface of nano size particles, surfactants reduces the interfacial tension between solid particles and liquid medium. Thus prevent the Ostwald ripening. [15, 18, 19] There are a number of literature reports that shows the importance of Ostwald ripening on the physical stability of nanosuspensions, but detailed studies on the physical stability of nanosuspensions including the role of Ostwald ripening have not been reported. Thus, in this study Ostwald ripening of nanosuspensions has been investigated in detail. The main aspect of this study deals with investigation of the effect of the non ionic stabilizers on Ostwald ripening. For this purpose, esomeprazole was used as a drug candidate and nanosuspensions were prepared at three different drug stabilizer ratios (low, medium and high) with five selected non-ionic stabilizers (hydroxy propylcellulose, hydroxy propylmethylcellulose, pluronic F68, polyvinylpyrrolidone, polyvinyl alcohol). All the selected stabilizers are polymer and widely use to prevent the crystal growth. Therefore, this study also deals with the effect of the characteristics of the

interfacial layer on Ostwald ripening. To determine the effect of some stress conditions on Ostwald ripening, the prepared nanosuspensions were followed for three months under certain stress conditions (storage at different temperature, mechanical shaking and fluctuation in storage temperature).

Fig. 1: Appearance comparison between esomeprazole nanosuspension and HPLC grade water (A. HPLC grade water   B. Esomeprazole nanosuspension)

MATERIALS AND METHOD
Material
Esomeprazole magnesium trihydrate was used as a model drug, gifted from Unichem laboratory, Ghaziabad, India. Pluronic F-68 was purchased from Sigma Aldrich Company, St. Louis, USA. PVA, PVP, HPC and HPMC (HPMC E4M) were purchased from CDH (P) Ltd, New Delhi, India. Methanol of HPLC grade was obtained from Rankem, New Delhi, India. HPLC grade water was also used throughout study.

Methods
Preparation of nanosuspensions
Esomeprazole nanosuspension was produced by bottom up approach using anti-solvent precipitation-ultrasonication method. A probe sonicator (Bandelin Sonoplus, Berlin) consisting the probe of 8 mm diameter was employed to provide the source of the ultrasound. In this method, 35 mg esomeprazole was dissolved in 10 ml of methanol to form the organic solution containing 3.5 mg/ml of drug. Selected non-ionic stabilizers (PVP, PVA, HPC, HPMC, Pluronic F-68) were dissolved in water, separately, to obtain a series of anti-solvents with the concentrations of 0.1%, 0.4%, 0.8% (w/v). Both solutions (solvent and anti-solvent) were passed through a 0.45μm syringe filter (RanDisc nylon syringe filter). The anti-solvent was cooled to below 3°C in an ice-water bath. Then, organic solution (10 ml) was introduced into 20 ml of the precooled anti solvent at a stirring speed of 1000 rpm, slowly and drop wise. The mixture was stirred continuously using the mechanical stirrer (Remi digital stirrer, Mumbai) at fixed speed of 1000 rpm for 2.5 h. and temperature was also kept constant below 3°C during the experiment. After the anti-solvent precipitation, the volatile solvent was evaporated by subsequently stirred the mixture at 300 rpm for next 4 h. Finally, the samples were transferred to a test tube having 2 cm diameter and 20 cm in length, and treated with ultrasound at fixed power input of 60%W for fixed time length (20 min). The frequency of waves was also set at 5x10% cycles throughout the experiment. This process resulted in the formation of yellowish transparent nanosuspension (Fig. 1).

Characterization of nanosuspensions
Particle size analysis
The particle size distribution of the nanosuspensions was determined via dynamic light scattering using Malvern zetasizer (Malvern, UK) at 25°C, which allows the sample measurement in the range of 0.1 to 10000 nm. Samples were diluted with water before measuring particle size. Viscosity of the diluted samples was measured using a Brookfield viscometer. Three dilutions for each sample were prepared and their average particle size and standard deviations are reported.

Zeta potential measurement
The zeta potential values of nanosuspensions were determined using Malvern zetasizer (Malvern, UK) at 25°C. Samples were diluted with the respective original dispersion medium and placed in the electrophoretic cell. All the measurements were made in triplicate and the average value and standard deviation are reported.

Light microscopy
Suspensions were diluted appropriately with water and observed using a photomicroscope (Radicle, Japan) at magnification 100 x. Instrument also equipped with a digital camera to determine the presence of any large crystals or aggregate that were generated during the storage of nanosuspensions.

Scanning Electron microscopy (SEM)
The size, shape and morphology were examined using Scanning electron microscopy (SEM, FEI Quanta 200F with oxford-EDS system IE 250 X Max 80, Netherlands). SEM of the initial formulations and formulations after storage were performed to determine the morphological behavior of the particles initially and also to evaluate any changes after storage at different stress conditions. For this purpose, samples were prepared by transferring the one drop of nanosuspension on a metal grid and dried under vacuum pressure for 1 hr. After this nanoparticles were coated with gold palladium and then morphological characteristics were observed at an acceleration voltage of 10 KV with suitable magnification.

Physical stability of nanosuspensions at stress conditions
Physical stability of the nanosuspensions was evaluated under certain stress conditions such as storage at different temperatures, fluctuation in storage temperature and mechanical shaking.

Storage at different temperatures
Prepared nanosuspensions were stored at different temperature conditions (15°C, 25°C, 35°C and 45°C) for

three months, and the samples were withdrawn on days 5, 7, 10, 14, 20, 30, 45, 60, 90. All the samples were characterized for particle size, size distribution, zeta potential and physical form.

Fluctuation in storage temperature

3.0 ml of nanosuspensions were filled in 10 ml vials and subjected to storage with fluctuation in temperature to evaluate the formulation robustness with respect to different temperature conditions. One cycle has been completed, when nanoformulations were stored at 4°C for 24 h followed by 40°C for 24 h. After three such cycles, nanoformulations were evaluated for particle size, size distribution, and zeta potential.

Mechanical shaking

For determining the effect of mechanical stress on the physical stability of nanosuspensions, 3 ml of nanosuspension was transferred in 5 ml vial and maintained on a wrist mechanical shaker (Hicon, India). Nanosuspensions were shaken at a rate of 150 oscillations per minute for 72 hours at room temperature. Samples were characterized for particle size distribution.

Crystalline state evaluation

The crystalline state of drug and prepared nanoformulations were investigated using the DSC and powder x-ray diffraction technique. With using of these two methods, one can yield the enough information to confirm the crystalline state of test sample. For this purpose, the 5 ml of the prepared nanoformulations were centrifuged at 10,000 rpm using the REMI digital centrifuge (RM 12 C, Mumbai, India) for 8-10 min to separate the solids. Supernatant liquid were discarded and solid particles were dried in presence of air.

Differential Scanning Calorimetry (DSC)

DSC (Perkin Elmer DSC 7, CT-USA) with a mettler M3 analytical balance was used to analyze the thermal behavior of different samples. The instrument was calibrated with Indium (3-5 mg, purity 99.999%, onset 156.6°C, heat of fusion of 107.5 J/g) for melting point and heat of fusion. 1.0 to 2.0 mg (approx.) samples were weighed into 40μl aluminum pans. Analysis was performed under a nitrogen purge (20 ml/min). A heating rate of 10°C/min was employed. The thermograms were recorded over a temperature of 10 to 250°C.

Powder X-ray diffraction (PXRD)

The drug crystalline state was also evaluated by X-ray diffraction pattern using X-ray diffractometer (WAXD Philips, Amedo, Netherland). PXRD studies were performed on the samples by exposing them to CuKα radiation (45KV, 40mA). The instrument was operated in the continuous scanning speed of 4°/min over a 2θ range of 5° to 40°.

Statistical analysis

Statistical analysis was performed with the software package SYSTAT®12 software (SYSTAT Software, Inc., San Jose, CA, USA) using student's t-test and a *p* value <0.05 was considered statistically significant. All the tests were run in triplicate (n=3) and final results were given as mean ± standard deviation (SD).

RESULTS AND DISCUSSIONS

Nanosuspensions of esomeprazole were prepared with anti-solvent precipitation ultrasonication technique using five non-ionic polymeric stabilizers; HPMC, HPC, PVA, Pluronic F68 and PVP K-30. These polymeric stabilizers provide the stability via steric stabilization. To interpret the windup of stabilizer concentration on Ostwald ripening, nanosuspensions were prepared using the three different concentrations of the stabilizers. All the prepared nanosuspensions were characterized for particle size determination, as mention in Table 1. The competent particle size of nanosuspensions was varied from 132 to 698 nm. Generally, the initial particle size of nanoformulations with medium ratio of drug to stabilizers was lower than those with high and lower ratio. The effect of Ostwald ripening in the respective nanoformulations is given below.

Table 1: Formulae of esomeprazole nanosuspensions prepared with five different polymeric nonionic stabilizers

Formulations code	Stabilizer type	Stabilizer Concentration % (w/v)	Ratio of drug to stabilizer	Average particle size (Mean ± SD, n = 3)	Zeta potential (mV) (Mean ± SD, n =3)
HPC 1	HPC	0.1	High (0.35)	556 ± 13	15.2 ± 1.8
HPC 2	HPC	0.4	Medium (0.087)	438 ± 17	18.7 ± 2.1
HPC 3	HPC	0.8	Low (0.043)	456 ± 13	20.5 ± 1.9
HPMC 1	HPMC	0.1	High (0.35)	421 ± 12	20.8 ± 1.8
HPMC 2	HPMC	0.4	Medium (0.087)	342 ± 8	22.5 ± 2.4
HPMC 3	HPMC	0.8	Low (0.043)	365 ± 11	22.8 ± 2.2
PVP 1	PVA	0.1	High (0.35)	435 ± 8	17.9 ± 2.3
PVP 2	PVA	0.4	Medium (0.087)	268 ± 18	18.5 ± 2.1
PVP 3	PVA	0.8	Low (0.043)	316 ± 15	21.2 ± 2.5
PluronicF68- 1	Pluronic F68	0.1	High (0.35)	428 ± 12	22.5 ± 1.7
PluronicF68- 2	Pluronic F68	0.4	Medium (0.087)	132 ± 20	25.1 ± 2.9
PluronicF68- 3	Pluronic F68	0.8	Low (0.043)	427 ± 20	23.1 ± 1.9
PVA 1	PVP	0.1	High (0.35)	698 ± 13	19.1 ± 1.8
PVA 2	PVP	0.4	Medium(0.087)	264 ± 9	21.4 ± 2.5
PVA 3	PVP	0.8	Low (0.043)	356 ± 19	21.1 ± 2.2

HPMC and HPC formulations

All the samples of HPC, HPMC, PVA, Pluronic F68, PVP formulations stored at 15°C and 25°C did not show any remarkable changes in mean particle size. The HPC 1 and HPMC 1 sample stored at 45°C exhibited a unexpected increase in mean particle size

up to day 20 followed by a slower rate of ripening until the end of the study period (Fig. 2 & 3). The mean particle size of sample HPC 1 and HPMC 1 was approximately double compared to the initial particle size (Table 2).
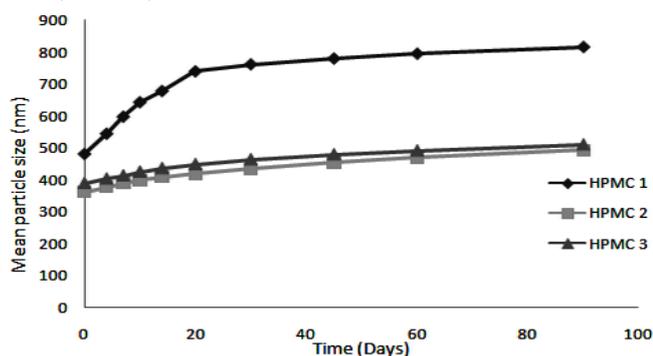

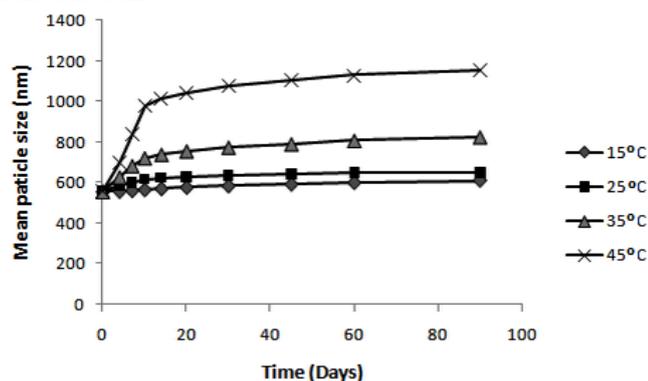**Fig. 2: Mean particle size of HPMC formulations at 45°C as a function of time**

Fig. 3: Mean particle size of HPC 1 formulation as a function of temperature and time

In correlate to the sample HPC 1 and HPMC 1, the other samples stored at 45°C, showed a gradual increase in particle size. The steep increase in particle size of HPMC 1, probably due to desolvation of the HPMC molecules at higher temperature of 45°C and subsequently loss in steric stabilization of the nano-size particles. HPMC shows the temperature dependent solubility. Accounting, at higher temperature, the chains of the HPMC molecules become dehydrate and lead to the phase separation and gelation. HPMC 1 formulation that was stored at 45°C may lead to the partial dehydration of the HPMC molecules, resulting in changes in the arrangement of the HPMC molecules in the interfacial film absorbed on the nanoparticles, so reduced the surface coverage of the nanoparticles. The reduction in surface coverage may be result in an increase in particle size due to Ostwald ripening. HPMC 1 formulation has a higher drug: stabilizer ratio (0.35) in comparison of HPMC 2 and HPMC 3 formulations, where the drug: stabilizer ratio are 0.087, 0.043, respectively. Thus, a higher concentration of HPMC is present in the HPMC 2 and HPMC 3 formulations, compared to the HPMC 1 formulation. When dehydration was occurred in all the formulations of HPMC, the excess amount of HPMC present in HPMC 2 and HPMC 3 formulation, filled the gaps in the surface coverage, and protect the particles to undergo the Ostwald ripening effect. Howsoever, HPMC 1 formulation does not have excess quantity of HPMC for achieving the adequate surface coverage; thus, favour the dehydration.

To represent the kinetics of increase in particle size of the HPMC formulations, the particle size was plotted against time (Fig. 2). For the HPMC 1 formulation, the plot exhibited the continuous increase in the particle size for the three months of study period. A non linear increase in particle size was obtained with time. The piling process was linear up to day 20, after which the ripening process slowed down. HPMC 2 and HPMC 3 formulation showed the same observations as of HPMC 1 formulation except that the linearity was obtained earlier in comparison of HPMC 1 formulation.

At different temperature (15°C, 25°C, 35°C, 45°C), the effect of drug: stabilizer ratio for HPC formulations were also tested, and the result was shown in Table 2. The highest increase in size was observed in the HPC 1 formulation which exhibited the relative particle size was 110% at 15°C, and this size was increased to 207%, relatively, at 45°C. Probably, the reason behind this may be the higher temperature at which the molecular solubility of esomeprazole in the stabilizer solution was increased, therefore, reducing the level of supersaturation, which in turn lower the rate of nucleation, and less particle nuclei may be available for particle growth; and thus, the potential for large particle size; additionally, higher temperature increased diffusion and growth kinetics at the particle boundary layer interface. Thus, a larger percentage of smaller particles of esomeprazole was dissolved at higher temperature which shifted the mean particle size of the formulation (HPC 1) stored at 45°C to a relatively large size as compared to those at 15°C. The kinetics of increase in particle size of the HPC formulation, stored at 45°C, clearly shows that most of the particles growth was obtained by day 10, with no remarkable increase after that (Fig. 3). Alike trend was observed for all HPC formulations at other storage temperature, however a gradual increase in size was observed at 15°C.

PVA and PVP formulations

The mean particle size of the PVA formulations as a function of storage temperature and time was evaluated. The particle size of PVA 1 formulation was increased initially from day 0 to day 7 at all the temperature conditions (15°C, 25°C, 35°C, and 45°C). However, the least and highest increase in size was observed at 15°C and 45°C temperature (Fig. 4), respectively. After day 7, no remarkable changes in particle size was observed in PVA 1 sample stored at 25°C and 35°C temperature conditions. But, samples stored at 15°C showed the increase in size until day 20 and after that no remarkable increase in particle size was found over the three month of study period. The mean particle size of the PVA 2 and PVA 3 formulations with respect to temperature and time was also determined. These formulations followed the

similar trends to those observed with the PVA 1 formulation. Samples of PVA 2 and PVA 3 formulations exhibited the appreciable increase in particle size between day 0 and day 7 for all temperature conditions with the exception of the 15°C.

The mean particle size of the samples of formulation PVA 2 and PVA 3 stored at 15°C increased gradually over the three month study period. The mean size of other samples, stored at other storage conditions, did not exhibit the significant changes in their particle size.

Table 2: Relative particle size of prepared nanosuspensions after different stress conditions

Formulation code	Relative size (%) after 3 months of storage (Mean ± SD, n = 3)				Relative size in % after 3 cycles of temperature fluctuation (Mean ± SD)	Relative size in % (Mean ± SD, n = 3)	
	Storage Temperature				(n= 3)	Storage at 25°C for 72 hour	Shaking at 25°C for 72 hour
	15°C	25°C	35°C	45°C			
HPC 1	110 ± 2	117 ± 1	148 ± 2	207 ± 5	115 ± 9	110 ± 4	113 ± 4
HPC 2	105 ± 2	112 ± 2	127 ± 2	151 ± 4	112 ± 6	108 ± 3	110 ± 3
HPC 3	105 ± 4	114 ± 4	136 ± 6	157 ± 5	114 ± 5	111 ± 3	116 ± 3
HPMC 1	100 ± 1	101 ± 5	139 ± 4	194 ± 3	102 ± 8	100 ± 2	101 ± 1
HPMC 2	100 ± 2	105 ± 2	124 ± 4	144 ± 1	104 ± 3	101 ± 3	100 ± 2
HPMC 3	102 ± 2	106 ± 2	121 ± 3	140 ± 2	103 ± 8	103 ± 3	104 ± 2
PVP 1	104 ± 4	112 ± 4	132 ± 5	146 ± 4	124 ± 12	107 ± 6	109 ± 3
PVP 2	102 ± 1	109 ± 3	127 ± 2	149 ± 4	110 ± 10	107 ± 5	110 ± 5
PVP 3	102 ± 3	111 ± 3	130 ± 3	148 ± 5	114 ± 11	104 ± 5	108 ± 3
Pluronic F68- 1	102 ± 3	108 ± 2	116 ± 3	148 ± 2	101 ± 9	105 ± 3	108 ± 2
Pluronic F68- 2	100 ± 2	102 ± 1	106 ± 2	112 ± 3	100 ± 4	100 ± 4	99 ± 3
Pluronic F68- 3	100 ± 5	105 ± 3	109 ± 3	114 ± 3	101 ± 9	100 ± 3	102 ± 2
PVA 1	105 ± 3	111 ± 5	121 ± 1	129 ± 5	104 ± 7	102 ± 2	102 ± 3
PVA 2	102 ± 2	104 ± 4	112 ± 3	124 ± 4	101 ± 6	100 ± 3	100 ± 1
PVA 3	100 ± 3	108 ± 3	114 ± 3	128 ± 5	102 ± 7	101 ± 2	103 ± 2

The effect of temperature and time on the particle size of the PVP 1 formulation was determined. At all the storage temperature, the mean particle size of this formulation was increased. The relative increase in particle size on day 7 compared to the initial particle size and found that those samples were stored at 45°C, exhibited the highest increase in particle size, followed by 35°C and 25°C. However, no appreciable increase in particle size was found on day 7 for samples stored at 15°C. After day 7, the mean particle size of formulation PVP 1 increased up to day 14 for the samples stored at 45°C; then finally, a plateau region was reached, where the particle size no longer changed (Fig. 4). Similar trends were found for samples stored at 25°C, 35°C and 15°C. However, samples stored at 15°C showed a more gradual increase in particle size. Formulation PVP 2 and PVP 3 exhibited a rapid increase in particle size up to day 7 followed by a decrease in rate up to day 14, under all storage temperature. After this, the particle size was also increased, but at a very slow rate over the three month study period for sample stored at 35°C and 45°C. Those sample which were stored at 15°C, achieved the plateau region around day14.

The increase in particle size of PVA and PVP nanoformulations for all storage temperature are mentioned in Table 2. Among all the storage conditions observed, the relative increase in particle size on day 7 compared to that on the initial day was the highest for samples stored at 45°C. This result can be attributed to the increased molecular solubility of esomeprazole in the stabilizer solution at higher temperature. As a result, a large no of smaller particles were dissolved at elevated temperature which changes the mean particle size of the nanoformulation samples stored at higher temperature (45°C) to a relatively large size as compared to those at lower temperature (15°C).

Kinetics of increase in particle size in PVP formulations follows the similar trends to that of the PVA formulations with some exceptions. These formulations exhibited the highest rate of Ostwald ripening for the period between day 0 to day 7 for samples stored at 35°C and 45°C. These samples also showed the continuous growth with a reduced rate up to day 14. The more time taken to reach a plateau region for PVA formulations stored at 25°C, 35°C as compared to PVP formulations, probably due to superior characteristics of the PVA interfacial film which inhibited the Ostwald ripening of these formulations.

When comparison was made in term of relative % increase in mean particle size, it has been found that the rate of Ostwald ripening was almost same in all PVP formulations. However, in the case of the PVA formulations, the PVA 2 and PVA 3 formulations showed a lower percentage increase in size in comparison of PVA 1 formulations, at all the storage temperature. Thus, PVA 2 and PVA 3 formulations exhibited a lower Ostwald ripening rate. The PVP 1 and PVA 1 formulation had low stabilizer concentration (0.1% w/v), whereas PVP 2 and PVA 2 had a comparatively higher stabilizer concentration (0.4% w/v). Despite of having higher concentration of stabilizer in the PVP 2 and PVP 3 formulations, the rate of Ostwald ripening in these formulations was almost same as of PVP 1 formulation (Fig. 5). PVP 3 and PVA 3 also had similar stabilizer concentration (0.8% v/w), but PVA 3 formulations exhibited the slower rate of Ostwald ripening in comparison of PVP 3 formulations at all the storage temperature. The slower ripening rate in the PVA 3 formulations can be attributed to the superior interfacial film that reduces the rate of attachment and detachment of the esomeprazole molecules at the nanoparticle surface.
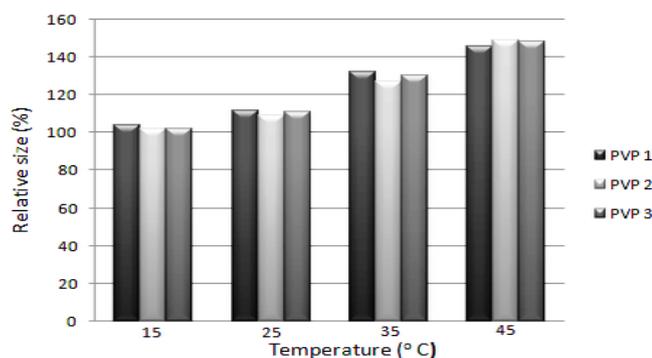
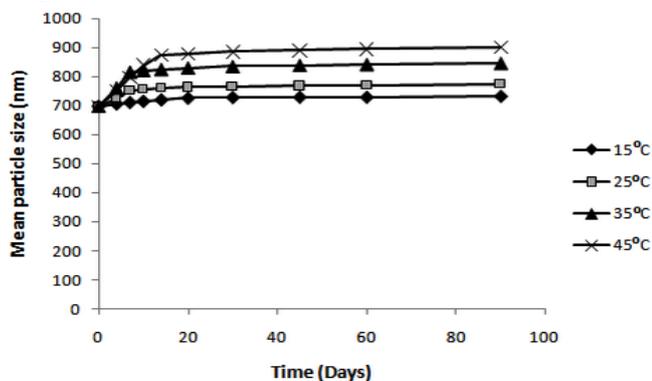**Fig. 4: Mean particle size of PVA 1 formulation as a fucntion of temperature and time**

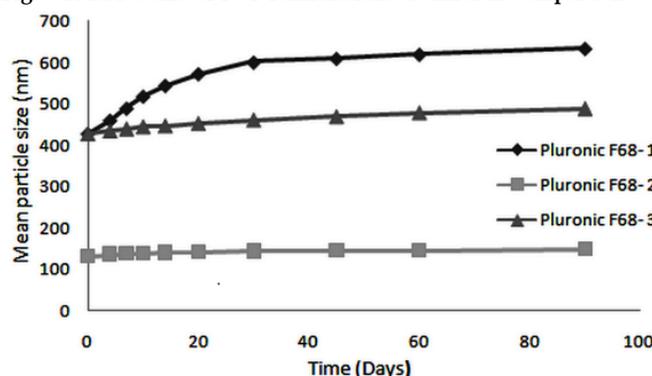**Fig. 5: Relative size of PVP formulations at different temperature**

Fig. 6: Mean particle size of pluronic F68 formulations at 45°C as a function of time

Pluronic F68 formulations

Samples of pluronic F68- 1, pluronic F68- 2 and pluronic F68- 3 formulations stored at 15°C and 25°C did not show any significant increase in particle size. The pluronic F68- 1 sample stored at 45°C exhibited the highest increase in particle size in comparison of other pluronic samples stored at 45°C. This sample showed a linear increase in particle size at 45°C up to day 30 followed by a slow rate of ripening over the three month study period (Fig. 6). The mean size of this sample was almost one and half time compared to sample stored at 15°C. In compare to pluronic F68- 1 sample, the pluronic F68- 2 and pluronic F68- 3 formulation stored at 45°C, showed a lesser and gradual increase in particle size.

Pluronic F68 is a triblock copolymer of nonionin nature. They comprise of a central block of relatively hydrophobic polypropylene oxide surrounded on both side by the blocks of relatively hydrophilic polyethylene oxide. When these molecules are immersed into the aqueous solvents, they form micellar structures above critical micellar concentration. Micellar behavior of pluronic F68 is found to be dependent upon temperature. With increasing temperature, desolvation of the hydrophilic chains occurs as the result of breakage of hydrogen bonds. Thus, as the temperature increases, the proportion of dehydrated methyl group of pluronic F68 increases. Higher proportion of dehydrated methyl group, more hydrophobic the polymer will be and this can lead the particle aggregation.

When the pluronic F68- 1 formulation was stored at elevated temperature (45°C), it is possible that the hydrogen bond present in hydrophilic chain (polyethylene oxide) of pluronic F68 become break and this may be result in desolvation of the hydrophilic chains. Thus, at 45°C, the proportion of dehydrated methyl group of pluronic F68 was increased and which led the polymer (pluronic F68) more hydrophobic. And higher the hydrophobic nature of polymer present in pluronic F68- 1 formulation can lead the particle aggregation in aqueous media. This mechanism was followed in all pluronic based formulations. But, pluronic F68- 2 and pluronic F68-3 based formulations had stabilizer concentration of 0.4, 0.8% w/v, respectively. This concentration of stabilizer is much higher compared to present in pluronic F68- 1 formulations (0.1% w/v). In pluronic F68- 2 and pluronic F68- 3 formulation (stored at 45°C), the dehydration of methyl group also occurred but not in sufficient quantity that can change the nature of polymeric stabilizer. Thus, in pluronic F68- 2 and pluronic F68- 3 based formulations, pluronic F68 maintain their integrity and nature, and ultimately protect the particles to undergo the Ostwald ripening effect. However, pluronic F68- 1 formulation stored at 45°C does not have sufficient quantity of pluronic F68 that can maintain the nature of formulation, and follows the Ostwald ripening.

Zeta potential

The zeta potential of all the formulations was measured in aqueous dispersion media. Prior to measuring the zeta potential, all the formulations were under go for sufficient dilution with aqueous phase. All the formulations prepared by nonionic stabilizer exhibited the zeta potential values ranging from -15 mV to -25 mV. No significant difference was observed in the medium and low drug: stabilizer ratio formulations for all stabilizers. These indicate that the stabilizer concentration was sufficient in both conditions that can properly cover the surface of the nanoparticles. All the nanoformulations that have high ratio of drug to stabilizers, exhibited the slightly lower value of zeta potential in comparison of other formulations having medium and low ratio. However, statistically there was no significant difference ($P > 0.05$) in zeta potential value of nanosuspensions having high drug stabilizer

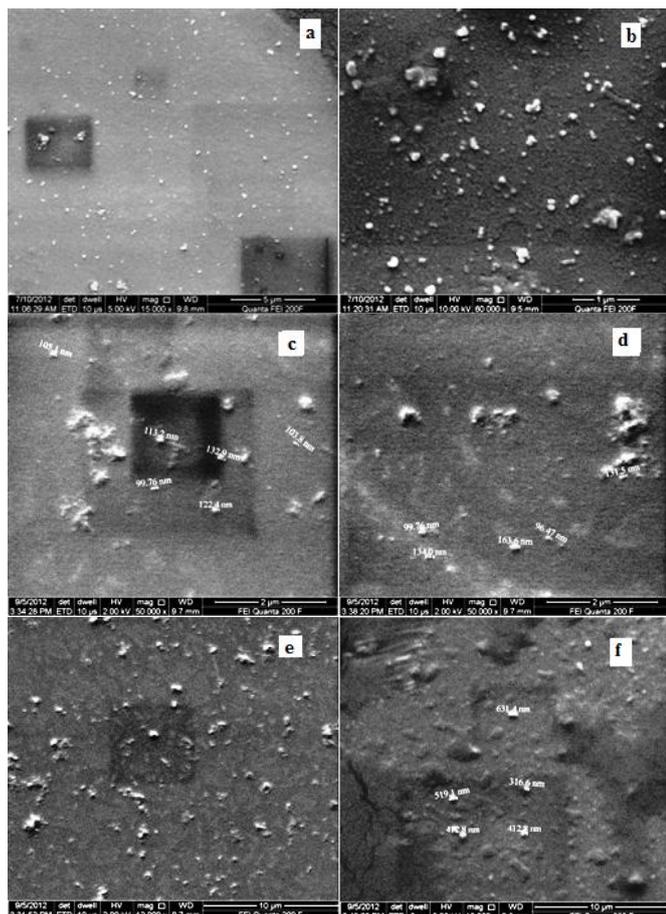ratio compared with the formulations that have medium and low drug: stabilizer ratio.

Fig. 7: **SEM image of esomeprazole nanosuspension using the different stabilizers at acceleration voltage of 10 kv (a) Freshly prepared nanosuspension using PVP (batch PVP 1); (b) Nanosuspension after thermal cycling (batch PVP 1); (c) Freshly prepared nanosuspension using Pluronic F68 (batch F68-2); (d) Nanosuspension after thermal cycling (batch F68-2); (e) Freshly prepared nanosuspension using HPC (batch HPC 1); (f) Nnaosuspension after thermal cycling (batch HPC 1)**

Fluctuation in temperature

Nanosuspension is a saturated solution of the drug which contains a lot of small particles in equilibrium state. A change in temperature can alter the equilibrium state of nanoparticles and thus affected the solubility of the particles, present in nanosuspension. This alteration in the solubility can lead to crystal growth and thus potential for large particle size. Increase in particle size can enhance the settling which may be result in agglomeration and destabilization of the nanosuspension. Fluctuations in temperature can also change the characteristics of the stabilizers, adsorbed on the particles, and this result may lead to destabilization of nanosuspension. The effect of fluctuated temperature on the prepared nanosuspensions was shown in Table 2. Increase in particle size was observed in the formulations made with HPC and PVP, while other formulations which were prepared by HPMC, PVA and pluronic F-68 did not exhibit the significant increase in particle size. This result is quite unusual but may be attributed to

differences in stabilizer characteristics. The data revealed that the ratio of drug to stabilizer did not affect the HPMC, PVA and pluronic F-68 based formulations. In the case of PVP, a relatively greater increase in particle size was obtained in the PVP 1 formulation (Fig. 7) which has higher ratio of drug to stabilizer, as compared to that having medium (PVP 2) and low (PVP 3) drug stabilizer ratio. Another interesting observation was obtained in case of HPC formulations. All the HPC based formulations (HPC 1, HPC 2, HPC 3) exhibited the increase in particle size after three such cycles but their relative size was not significantly different. Thus, no effect of drug: stabilizer ratio was observed in HPC formulations.

Mechanical shaking

To determine the cause of physical instability of the nanosuspension is a difficult task. Mechanical stress is also a factor responsible for increasing the particle size of nanosuspensions, thus leading the physical instability. So, an effort was made to determine the effect of mechanical stress on the physical stability of prepared nanosuspensions at room temperature (25°C). For this purpose the samples of all prepared nanosuspensions were subjected for storage at 25°C for 72 hour. Similarly, all the samples were also undergo for mechanical shaking at 25°C for 72 hour. The relative particle size of the formulations obtained after 72 hour of storage and shaking at 25°C is shown in Table 2. No effect of mechanical stress was observed in all prepared formulations. The tabulated data also indicated that this stress condition was not significantly affected the prepared nanosuspensions. Although, relative particle size increased in PVP and HPC formulations, but the increase was almost same to the samples stored at 25°C without shaking. Also statistically there was no significant difference ($P > 0.05$) in relative particle size of PVP 1 formulation compared to PVP 2 and PVP 3 formulation. In case of HPC formulations, similar statistical result was obtained as described for PVP formulations. This result indicated that the increase in size was not due to mechanical shaking but was a result of the storage temperature.

Differential Scanning Calorimetry

Thermal analysis of nanocrystal powder of all formulations was performed after the 3 cycles of temperature fluctuation, and compared to the raw material (esomeprazole). The temperature of fusion of all types of nanocrysals was investigated by DSC. To study the crystalline state alteration, the melting point of all formulations was compared to raw material. The crystalline state of drug can be transformed after some physical treatment such as ultrasonication, or due to formulational and environmental factor. Therefore thermal analysis was performed in this research work.

All the formulations exhibited almost similar thermogram. Thus, does not show any changes in their crystalline state. The result of thermal analysis of some formulations (HPMC 2, Pluronic F68-2) can be seen in Figure **8**. From the overlay of DSC thermograms, it has

been observed that esomeprazole is crystalline in nature. It exhibited a melting endotherm at an onset temperature of 178.1°C, a peak temperature of 185.31°C and a heat of fusion of 119.7 J/gm. The diagram reveals a similarity in the melting temperatures. According to these results, neither HPMC 2 nor Pluronic F68-2 nanocrystals modified the crystallinity of the drug after the physical treatment of ultrasonication and the subsequent drying process. As it is shown in the DSC thermograms, the melting points of esomeprazole (raw material and both nanocrystal powders) are similar. The only difference observed was a slight shift in melting temperature (from 185°C to 187°C). This modification was attributed to the presence of stabilizer.
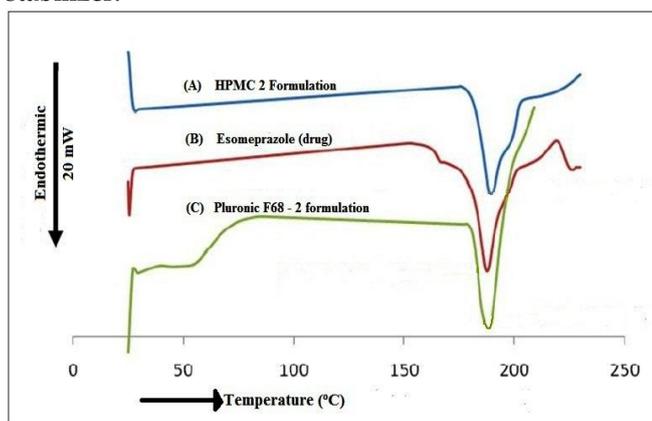

Fig. 8: DSC curves of esomeprazole after three thermal cycling. A. HPMC 2 formulation, B. Raw drug, C. Pluronic F68-2 formulation
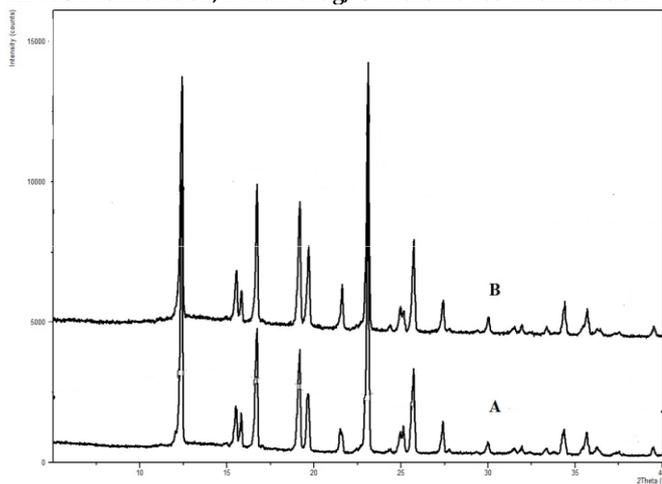

Fig. 9: Powder X-ray diffraction pattern of esomeprazole. A. Raw drug, B. Pluronic F68-2 formulation

Powder X-ray diffraction

To confirm the crystalline state of the dried esomeprazole nanocrystals, x-ray diffraction was performed for all of the air dried nanocrystals stabilized with the various stabilizers. XRD pattern of formulation F 68-2 and raw material (esomeprazole) can be visualized in Fig. 9. The diffractograms reveal that there are no different peaks for all types of nanocrystals. All the peaks were confirmed as finger print of esomeprazole . In addition, the diffractogram showed a similarity of the peak intensities for all esomeprazole formulations. This result inferred that all

nanocrystals were still in the same crystalline state as the raw material. Thus, process treatment and different type of other factors did not transform the esomeprazole nanocrystals into amorphous form.

Esomeprazole nanosuspensions were prepared using five polymeric nonionic stabilizers, and these nanosuspensions were investigated for their physical stability under different stress conditions. In this research work, the increase in particle size was observed in the nanosuspensions due to their physical instability (Ostwald ripening). Sometime, increase in the amount of stabilizer did not exhibit a significant effect on the ripening rate. However, in most cases, relatively lower rate of particle size increase was observed in this study with higher concentration of stabilizers. This result was attributed to interference of the stabilizer layer at the interface with both dissolution and growth at the nanoparticle interface. All the formulations exhibited the increase in particle by less than 60% of the original size under the most stressful conditions (45°C for three months) with the exception of HPMC 1 and HPC 1 formulations. In HPMC 1 formulation, the increase in particle size was almost double to its original size, and this was attributed to desolvation of the HPMC molecules at 45°C resulting in loss of protection of the suspended particles. HPC 1 formulation stored at 45°C also exhibited a steep increase in particle size. This observation was attributed to the unsaturation of the esomeprazole nanosuspensions at higher temperature and subsequent lowering the rate of nucleation led the increase in particle size. All the formulations showed a non linear relationship between the increase in particle size and time. Probably the reason behind this may be the presence of stabilizer layer which interfered with the growth and dissolution of the nanoparticles.

The changes in the crystalline state of esomeprazole in the nanosuspensions were confirmed by powder X-ray diffraction (PXRD) and Differential scanning calorimetry (DSC) analysis. Diffractograms exhibited that there are no changes in crystalline state of different prepared nanosuspensions compared with raw drug. Similarly, thermograms of all samples (undergone for 3 thermal cycles) indicated that crystalline state of drug was not changed. Thus, different physical process and other factors were not affected the crystalline nature of drug.

The issues of physical stability related to nanosuspensions are generally solved by using the different type of stabilizers. However, the screening process used for selecting the proper stabilizer arise a very critical, demanding and challenging issue. This is mainly due to lack of suitable technique used for selecting the proper stabilizers. Nowadays, generally hit and trial method is using for screening the stabilizers. Thus, this untouched field demands a needful work in future.

ACKNOWLEDGEMENT

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