# A stable hydrocortisone nanosuspension for improved dissolution: Preparation, characterization and *in vitro* evaluation

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**Abstract**: Drug nanosuspensions have gained tremendous attraction as a platform in drug delivery. In the present work, a nanosuspension was prepared by a wet milling approach in order to increase saturation solubility and dissolution of the water insoluble drug, hydrocortisone. Size of the generated particeles was 290 nm ± 9 nm having a zeta potential of -1.9 mV ± 0.6 mV. Nanosized particles were found to have a rod shape with a narrow particle size distribution (PDI =0.17). Results of differential scanning calorimetry and X-ray diffraction analyses revealed minor modifications of crystallinity of hydrocortisone following the milling process. Solubility of hydrocortisone was enhanced by nanonization to 875µg/ml ±2.5, an almost 2.9-fold compared to the raw hydrocortisone. Moreover, the nanosuspension formulation substabtially enhanced the dissolution rate of hydrocortisone where >97% of the hydrocortisone was dissolved within 10 minutes opposed to 22.3% for the raw 50% for the raw hydrocortisone and the commercial tablet, respectively. The bioavailability study resulted in AUC $_{0\text{-}9h}$ for HC nanosuspensions (31.50±2.50), which is significantly ($p<0.05$) higher compared to the AUC $_{0\text{-}9h}$ (14.85±3.25) resulted for HC solution. The nanosuspension was physically stable at room temperature for 24 months.

**Keywords**: Nanosuspension; Hydrocortisone; Milling; Solubility; Dissolution; Stability

## INTRODUCTION

A range of APIs (active pharmaceutical ingredients) are water insoluble and to address this important issue has become a hard challenge for drug delivery researchers both from academia and industrial sectors (De Waard *et al*. 2008). Approximately 70% of the developed drug candidates have low aquoues solubility and 40% of the marketed APIs designed in oral dosage forms are practically insoluble in water (Kawabata *et al*. 2011; Ku and Dulin 2012). One of the main problems linked to drugs with of poor solubility is the little and inconsistent bioavailability based on slow dissolution and irregular absorption (Plakkot *et al*. 2011; Kesisoglou *et al*. 2007). A number of strategies have been utilized to alter the dissolution and solubility characteristics of these drug compounds. The adopted techniques include incorporation of surface active agents, complexation, co-solvency or modification of the drug molecules (prodrugs or formation of salts). However applications of these methods are limited, because the drugs are required to must possess some special features which include ionizable group to form salts, definite solubility in some of organic solvents or having appropriate molecular structure with soluble carriers such as cyclodextrins). The probable toxicity from solubility enhancers or solvents residues may add other disadvantages. Therefore, alternative approaches of solubililization are still strongly demanded (Blagden *et al*. 2007; Qiao *et al*. 2011).

Nanosuspension platform has quickly gained a proven record to formulate and improve the bioavailability of hydrophobic drugs (Ghosh *et al*. 2011). Nanosuspension is a dispersion of submicron (<1000nm) drug particles stabilized by small amount of surfactants and polymers (Letchford and Burt 2007). Nanosuspensions can be fabricated using two well-known methods including top down and bottom up (Wang *et al*. 2012). In Top-down methods the particles are broken down by mechanical and attrition forces. Such techniques often necessitate lengthy processing periods, consume energy and yield heterogeneous products (Kakran *et al*. 2010). In "bottom-up" approach, the nanoparticles are formed by nucleation of drug molecules in solutions followed by precipitation or by quick removal of the solvent. A major drawback of bottom up technologies such as such as precipitation from supercritical fluids using antisolvents is the uncontrolled particle growth primarily through Ostwald ripening

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(Kakran *et al*. 2010). The presence of residual solvents can however also provide a barrier to adoption of these technologies, whilst challenges in scale up and high production costs can also reduce the attractiveness of these methods.

A key issue associated with the preparation approaches is the possible changes in of crystalline state of the prepared nanoparticles. Partial or complete amorphization of drug particles can be induced during the progress of nanonization. Such energetic particles are not stable and tend to convert to crystalline state of low energy eventually (Wu *et al*. 2011). Moreover, changes of crystalline integrity of drug particles are crucial as it could induce potential changes in physical properties as solubility and impacts drug bioavailability. Crystalline nanoparticles are therefore paramount important and preferable to be fabricated because of the long term stability compared to the amorphous counterpart (Ghosh *et al*. 2011; Junghanns *et al*. 2008).

This designed study aimed to fabricate a stable hydrocortisone nanosuspensions (crystalline) formulation of model hydrophobic compound hydrocortisone (Ali *et al*. 2011; El Maghraby *et al* 2008) using a top down technology media milling, Lena DM 100 (Plakkot *et al*. 2011). The imperative physicochemical characterization of the produced nanosuspension which includes XRD, DSC SEM, TEM and solubilities studies demonstrated the special characteristics of nanocrystals in nanosuspension. DSC and XRD analyses were performed to assess crystallinity of the raw and milled hydrocortisone. Furthermore to the effect of nanonization on solubility and dissolution profiles of hydrocortisone was investigated to confirm the hypothesis of the advantage of surface area enlargement.

## MATERIALS AND METHODS

### *Materials*

Hydrocortison (B.No: 1000786475 with 98%, purity), SLS (sodium lauryl sulphate) and methyl paraben were purchased from Sigma-Aldrich (USA). Absolute ethanol, acetonitrile HPLC, methanol HPLC were obtained from Fisher Scientific Ltd, (UK). PVP K30 was purchased from BASF (Germany). Analytical and pure lab grade water was obtained from Purelab$^{TM}$, ELGA (UK). HPMC (6cps) (Hydroxypropylmethylcellulose), was kindly provided by Shin-Etsu chemical Ltd (Tokyo, Japan)

### *Methods*

*Preparation of nanosuspensions*

Nanosuspension of HC was prepared through two main steps: the first one involves dispersing the drug in a medium of the stabilizers and second one is particle size reduction in milling chamber. HC (2%, w/v) was added to a solution of 0.1% (w/v) SDS, 0.5%, (w/v) HPMC and 0.2% (w/v) PVP and sonicated for 10min. Drug nanonization was achieved using a wet milling machine (Dena Ltd, UK) loaded with a beads of zirconium having size =0.5mm. Reduction of size of particles was evaluated by analyzing samples at different processing times.

### *HPLC method for quantification of hydrocortisone nanosuspension*

The produced nanosuspension was quantified for HC content using a USP modified method (El Maghraby 2008). HPLC (the system specification is: Model 2695 Waters with a UV detector). The C18 5µm 250×4.6mm column with a constant temperature of 30°C was used. The mobile phase consisting of, acetonitrile, methanol and water (10:50:40) was set at constant flow of 1ml/minute. The running time interval was kept constant at 15 minutes for each run and the eluent was monitored at 238nm. Six dilutions from the standard solution consisting of 100, 200, 300, 600 and 1000 microgram/ml of HC and 100 microgram/ml of methyl paraben (internal standard) were prepared to construct the standard curve. All samples were analyzed in triplicate.

### *Particle size measurement*

The particle size measurements in the produced nanosuspension was carried out using Malvern Zetasizer (Malvern Instruments, UK). All the samples without dilution were analyzed in triplicate.

### *Scanning electron microscopy*

The particles morphology of raw HC was visualized using Scanning electron microscopy (SEM) (Quanta 400, Company FEI, Cambridge, UK). The particles of Hydrocartisone were sputter coated with gold prior to examination.

### *Transmission electron microscopy*

The images of HC nanoparticles from the suspension were taken at different magnifications by transmission electron microscopy using a TEM (JEM-1200EX). The samples for TEM analysis were prepared by applying a drop of the HC nanosuspension on the copper grid, coated with carbon followed by drying at ambient temperature. Owing to poor conductivity all the samples were stained using uranyl acetate (2% aqueous) two minutes before the TEM analysis.

### *Differential scanning calorimetery (DSC)*

The DSC Thermo grams of HC were obtained by a DSC instrument (TA Q2000, USA). The samples of raw and HC nanocrystals were heated at a scanning rate (10°C/min) in atmosphere of nitrogen. The range of scanning temperature from 20 to 250°C was used to scan all the samples. The solid HC was obtained by centrifugation (Beckmann, USA) of HC nanosuspensions for 30 minutes using 15000rpm. Solid HC was collected from nanosuspension through centrifugation at 15000 for

30min. The residue of HC was dried in a vacuum desiccator (40◦C and -25 Inch Mercury).

***X-ray powder diffraction (XRPD)***

The X-rays diffract grams of raw and nanocrystals of HC were obtained using X-ray Diffractometer (D500-Simen-Germany). All the samples including raw and milled HC were scanned in triplicate using the scanning parameter (of 2-50° 2θ.

***Solubility Studies***

Solubility study of HC nanosuspension was carried out using the centrifugation method which has been reported previously (Gao *et al*. 2011). For the nanosuspension, approximately 1.5ml of the HC nanosuspension was filled in centrifugation tube (1.5ml) and kept for 24hrs. After that, it was centrifuged using a sigma centrifuge (Scientific Lab supplier, Model: Sigma 0II5982IIII) at 14800 RPM for 1hr. The supernatant layer was taken and filtered through 0.02µm filter (Syringe Filter: 20nm, Whatman anotop, Germany) to make sure the solubilized contents of the nanosuspension. The filtrate was analyzed for the HC contents using HPLC as previously described. Solubility of unprocessed HC was also measured using a similar manner.

***Dissolution studies***

Dissolution studies The dissolution behaviours of HC nanosuspension, raw HC, and commercial Hydrocortisone tablets (B.No: HYD516B, 10mg, AAH pharmaceutical Ltd, UK) were studied using USP II apparatus with paddles. 500mL of distilled water at a temperature of 37 °C±0.3 was used as the dissolution medium. Equivalent amount of HC nanosuspension (10mg) put into the dissolution vessel while get it stirred using 50 rpm. The samples (5mL) were withdrawn from the dissolution vessels at the time intervals including 0, 2, 6, 10, 15, 30, 45 and 60 min. Equivalent fresh volumes of the buffer were added to dissolution media. The collected samples were then centrifuged at 14800 rpm for 15min and filtered using Syringe Filter of 20nm (Whatman anotop, Germany) to separate the undissolved HC. Experiments were performed in triplicates and drug concentrations were quantified by HPLC.

## STATISTICAL ANALYSIS

The paramount statistical analysis including one way ANOVA with subsequent least significant difference test (LSD) were performed on the obtained results. SPSS ® (USA) software was used for this analysis. Results are considered significant where $P<0.05$.

***Physical stability study***

Physical stability studies on the fabricated Hydrocartisone nanosuspensions was carried out at 25°C for two years. In addition to monitor visually the settling and agglomeration process in nanouspension, the particle size measurements were carried out at regular time of interval using Zetasizer.

***Bioavailability Study***

The produced HC nanosuspensions was subjected to ocular bioavailability study using adult male albino rabbits. Five rabbits having weight between 2-2.5kg, were used for the designed studies. *In vivo*, studies were carried on the approval of Ethical committee, University of Malakand which follows animals Bye-Laws 2008. The protocol for *in vivo* studies, reported by Hany Ali was employed (Ali *et al* 2011). The rabbits were kept at a room where the temperature was properly controlled (25C), while providing them food and water. One drop of a Tetracaine (local anaesthetic), instilled into the eye of a rabbit which specified to be tested. After that, IOP (Intra ocular pressure) was measured by The Tonometer. The bioavailability of HC nanosuspensions was compared with HC solution. The dose of 50ul both from HC nanosuspension and HC solution was injected into the lower sac of Conjunctiva. Measurements of IOP, before and after administration of the drug was carried at different time of intervals for 09 hours. The Tonometer was calibrated and for all measurements, the same tonometer was used by the same person. A washout period of 03 days, between administration of HC nanosuspensions and HC solution was allowed. Also the normal base line for each rabbit was established before administration of the new formulations. The following equation was used for monitoring the change in IOP (ΔIOP).

$$\%\Delta IOP = (IOP_{post\ dosing} - IOP_{predosing} / IOP_{predosing} \times 100$$

The paramount bioavailability parameters which were considered for evaluation of efficacy of different formulations include; Tmax for peak ΔIOP, ΔIOPmax, $AUC_{0\text{-}9h}$ and MRT (mean residence time)

## RESULTS

***Preparation of Nanosuspension***

Fig. 1 shows the communition of raw hydrocortisone particles versus milling time. Continuous milling of HC for 90 minutes of was sufficient to convert the raw HC into a nanosuspension of 290 nm sized particles (fig. 1). A noticeable reduction in PCS diameter (to 370 nm) was observed in the first 60 minutes however, increasing processing time from 60 to 90 minutes did not reduce the particle size markedly (fig. 1). Size of HC particles remained nearly constant from 90 to 120 minutes.

***Characterisation of Nanosuspension***

***Morphology studies (SEM&TEM)***

Fig. 2 shows SEM and TEM micrograph of unprocessed and processed hydrocatisone respectively. The HC

nanoparticles appear as dark stained nanoparticles predominantly of rod morphology and seem to be crystalline in nature. In contrast, the SEM micrograph shows the unprocessed HC as heterogeneous irregularly shaped particles with many of the imaged particulates being greater than 10µm.

***DSC studies***

DSC thermograms of the powder of unmilled and milled HC are presented in fig. 3. The unmilled HC exhibited a sharp melt process with an onset temperature of 219°C and a sharp peak at 224°C. Whereas a slight decrease in the melting point of HC nanocrystals (221°C) was observed compared to the raw counterpart.

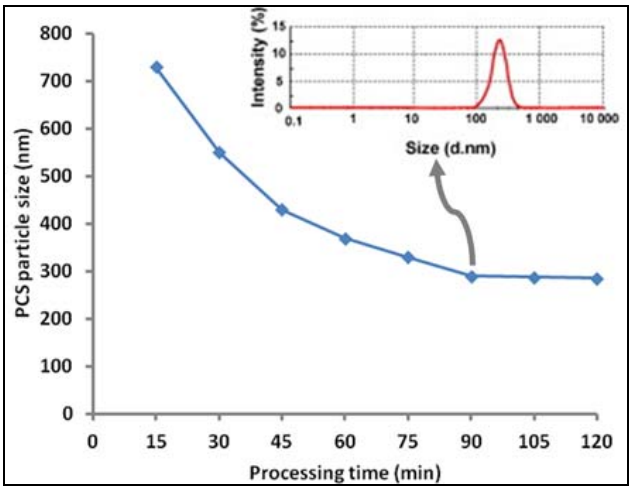


**Fig. 1**: Changes of PCS diameter of hydrocortisone as a result of milling time. The insert is a PCS diagram of the final nanosuspension.

***PXRD studies***

In addition to DSC studies PXRD studies was performed on fabricated HC nanosuspensions to assess the amorphous and crystalline state of the nanoparticles. The diffractograms of unprocessed (unmilled) and milled hydrocortisone are shown in fig. 4.

***Solubility Studies***

Solubility profile of HC nanosuspension and unmodified HC has been shown in fig. 5. in distilled water was found to be 875µg/ml ±2.5, an almost 2.9-fold increase compared to solubility of the as-received HC (fig. 5).

***Dissolution studies***

Dissolution studies were performed in order to assess the impact of nanosizing on dissolution of HC. The content of HC in the prepared nanosuspension quantified by HPLC is found to be 17mg/ml or 85% of the initially added amount of HC (table 1). Accordingly 0.59ml was found to be equivalent to 10 mg of HC.

The results of *in vitro* dissolution of different HC samples are shown in fig. 6. The experiment revealed higher dissolution rate for the nanosuspension compared to the bulk drug and the commercial tablets. Dissolution of raw drug was very low since only 22.3% of HC was dissolved in the first 10min and approximately 52.7% was dissolved after 60min. Formulating HC as a nano sized suspension significantly improved the dissolution rate with almost 97% of the drug dissolved over the first 10 minutes opposed to 50% for the commercial tablet formulation.

***Physical stability***

The growth in the size of the particles and variations in PDI (polydispersity index) values with respect to time have been shown in fig. 7. PSC size and PDI of the prepared HC nanososuspension remains close the initial values over a period of 24 months.

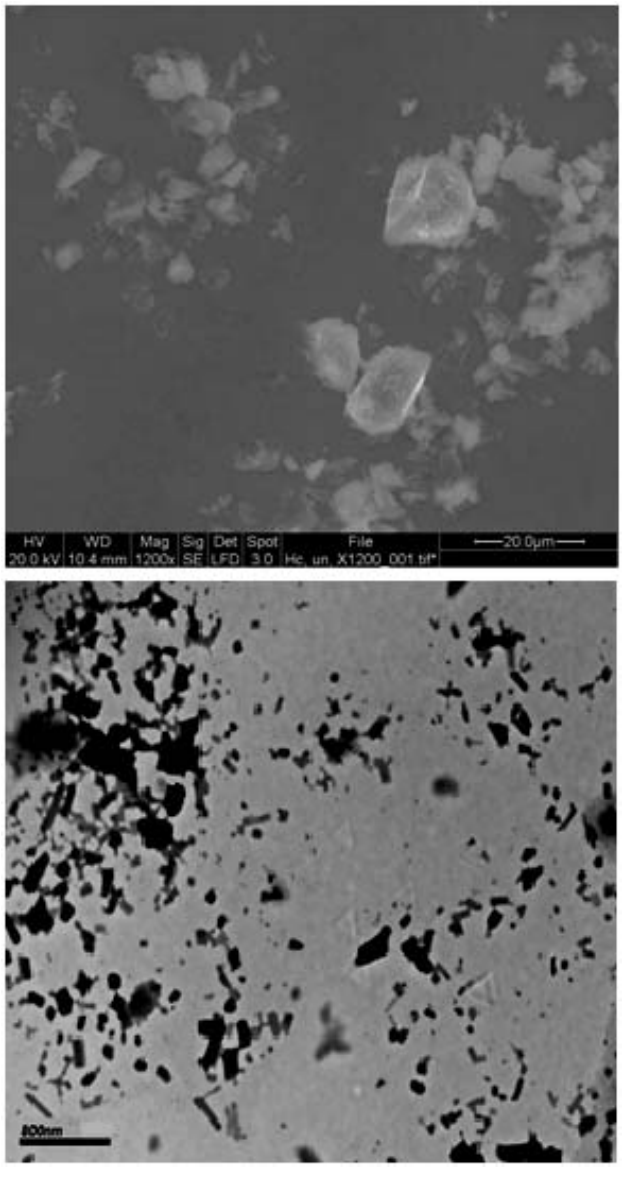

**Fig. 2**: A. SEM micrograph of unprocessed hydrocortisone and B. TEM micrograph of processed hydrocortisone

***Bioavailability Study***

Fig. 8 shows the comparative bioavailability results of HC nanocrystals and solution. The base line intra ocular pressure (IOP) used for the subjected rabbits were 12.2 mm Hg. The comparative bioavailability study demonstrated that both HC solution and nanosuspension remarkably increased the IOP to maximum level and then slowly returned to the normal pre-treated values. The maximum increase in IOP observed for HC solution in 0.5h was 53.25±5.5%. Whereas in 0.5h for HC nanocrystals, the IOP increased to 60.40±7.5%. Furthermore, the effect of HC solution remained for 4-5 hrs, however; for HC nanosuspensions the effect was rather sustained for longer time (8-9hr). Additionally, the mean residential time (MRT) calculations also demonstrated that the action of HC nanosuspensions sustained for longer time compared to HC solution. There was observed a significant difference in MRT for HC nanosuspensions and solution. The MRT for HC nanosuspensions was found to be 2.8±0.05 h, compared to the HC solution (1.50±0.04 h). The AUC calculations also showed that HC nanosuspension exhibited strong bioavailability compared to the HC solutions. The $AUC_{0-9h}$ for HC nanosuspensions was found to be 31.50±2.50, which is significantly higher compared to the $AUC_{0-9h}$ (14.85± 3.25) resulted for HC solution.

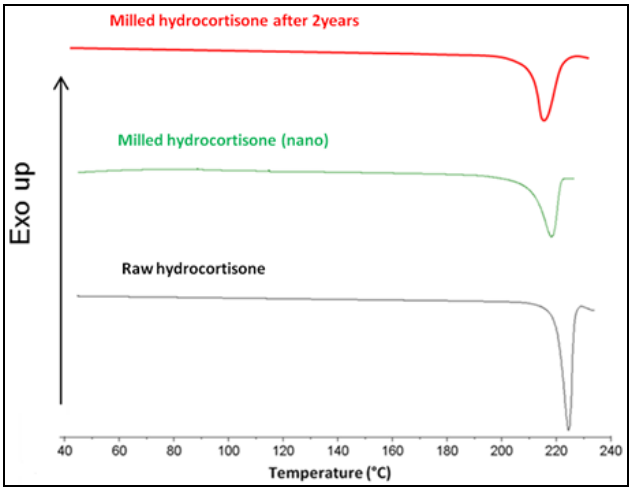


**Fig. 3**: DSC thermo grams of raw and milled hydrocortisone.

## DISCUSSION

Screening studies showed that combination of HPMC 3 cps, PVP K30 and SLS was effective in preparation of HC nanosuspension. The used polymers can adsorb onto HC particles due to hydrophobic interaction. On the other hand in nanosuspensions, where the particles are surrounded by hydrophilic groups, aggregation is prevented by steric hindrance effect (Verma *et al*., 2009). SLS is small molecule ionic surfactants which stabilize the HC nanosuspension via electrostatic stabilization (Verma *et al*., 2011). Such a slight change in particle size reveals that size reduction at the end of milling process is not achieved by fragmentation but occurs due to abrasion (Plakkot *et al*., 2011). Furthermore longer milling time was not important because a clear decrease both in particle sizes and PDI was not observed. It should be noted here that milling for longer time can cause slight increase in particles average size in nanosuspensions. For example in case of indomethacin nanosuspensions when stabilized by 6 pluronic F127 (Liu *et al*., 2011), there was observed increase in particle size at longer milling time. The final polydispersity index, PDI of HC nanosuspension was 0.17, a value representing a narrow size distribution of the size reduced HC nanosuspensions (Patravale *et al*., 2004). There is no evidence of large unmilled HC particles or particles agglomeration. The size of nanoparticles in TEM micrographs appears below 290nm (i.e. the PSC size). Minor difference in nanoparticle size measurement between PSC and TEM is because of different principles of measuring the particle sizes. In PCS the average particle sizes are calculated along the solvation layers which could be slightly larger compared to the sizes obtained by TEM (Aboofazeli *et al*., 2000).

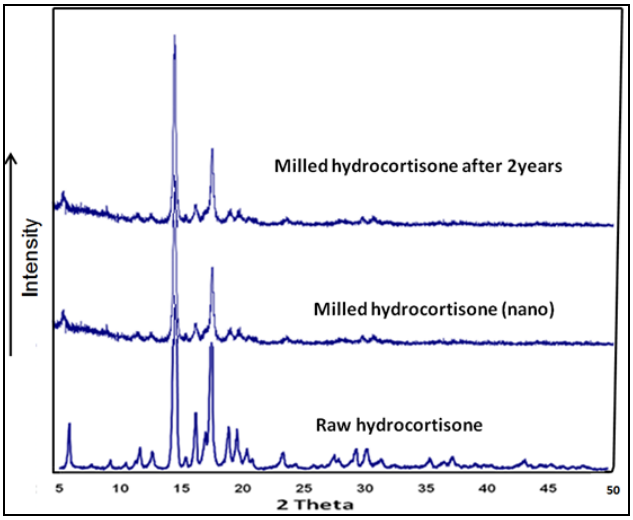


**Fig. 4**: PXRD patterns of raw and milled hydrocortisone

Generation of heat and/or energy during the milling process may affect the crystalline structure of the drug substance or even convert it into an amorphous state (Kayaert *et al*. 2012). This change in state can potentially increase the molecular mobilization leading to aggregation issues (Bose *et al*., 2012).

The decrease in melting point of HC nanocrystals as a result of milling process is indictive of the reduction in particles size to nanometer range (Khan *et al*., 2013). The nanosized particles have lower packing density compared to the micro particles (Schmidt *et al*., 1998; Lai *et al*. 1996). Overall, DSC showed that no marked change in

the physical form of HC occurred following size reduction.

The high intense peaks in the diffractogram of the unmilled HC (fig. 4) exhibits highly crystalline nature of the unmilled HC. The nanosuspension also produced consistent diffraction peaks. Nonetheless for the HC nanocrystals there was observed slight reduction and some broadening in peaks intensity compared to unmilled HC which are clear in the pattern (fig. 4). This level of peaks broadening in case of nanocrystals has previously been reported which is because of the size reduction (to less than 1µm) (Jenkins R & Snyder RL 1996).

Generally, saturation solubility of drug compounds increase by reducing drug particle size or by achieving the amorphous state of particles (Van Eerdenbrugh *et al*. 2010). However, results of PXRD and DSC studies exclude any changes of Hydrocartisone crystallinity. The impact of drug nanonization on solubility has been explained by Frendulich where it has been concluded that the solubility and particle radius has got inversely relationship. The higher the radius of the particles the smaller solubility is achieved and vice versa. According to this equation the drug shows a higher solubility when the particle radius is decreased.

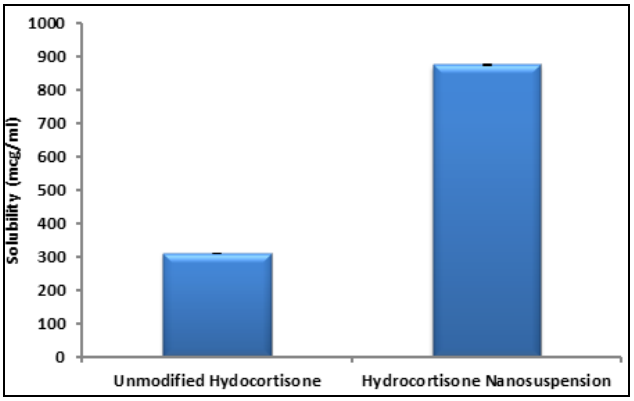


**Fig. 5**: Saturation solubility of unmodified and milled hydrocortisone (nanosuspension) in distilled water

Dissolution of raw drug was very low since only 22.3% of HC was dissolved in the first 10min and approximately 52.7% was dissolved after 60min. Formulating HC as a nano sized suspension significantly improved the dissolution rate with almost 97% of the drug dissolved over the first 10 minutes opposed to 50% for the commercial tablet formulation.

The enormous surface area coupled with the nano-sized particles results in high interfacial tension and increased free energy. Accordingly, nanosuspension is essentially a thermodynamically unstable system (Verma *et al*., 2011, Rabinow, 2004). The generated nanoparticles can potentially agglomerate quickly because of high surface free energies. Large particles may also grow further because of migration of the smaller partiocles towards their surfaces (Ostwald ripening). The used stabilizers, HPMC, PVP K30 and SLS in our case, are sufficiently adsorbed onto the HC surfaces with subsequent steric stablisation. In case of insufficient adsorption the stabilizers onto the drug surfaces, the particles grow faster which results in agglomeration and settling of the particles. (Xia *et al*., 2010). The zeta potential value calculated by zetasizer was found to be -1.9 mV ± 0.6 mV. Adsorption of steric stabilizers layer may reduce in the measured zeta potential, but this is not an indication of a reduced stability (Kakran *et al*., 2012, Verma *et al*., 2011). The low value of zeta potential indicates that stabilization of HC nanosuspension is mainly maintained by increase in osmotic pressure in the particles rather than electrostatic stablisation (Lindfors *et al*., 2008).

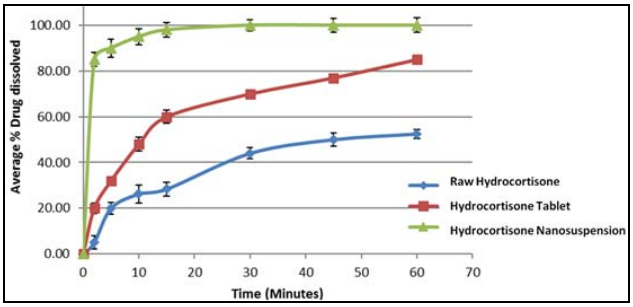


**Fig. 6**: *In vitro* dissolution profile of raw hydrocortisone, (diamond), hydrocortisone tablets (square) and hydrocortisone nanosuspension (triangle).

No change in zeta potential was observed for the HC nanosuspension after storage. The narrow particle size distribution during the entire storage period shows outstanding stability of the fabricated nanosuspensions. As Ostwald ripening is mainly because of the broader size distrbution in nanosuspensions and in our case having a polydispersity index of 0.17, the prepared HC nanosuspension demonstrates homogenous particle size distribution with reduced risk of Ostwald ripening (Xu *et al*., 2012). Furthermore, the crystalline state of HC nanosized particles enhanced stability of the nanosuspension. Despite of the increased solubility and dissolution of amorphous nanoparticles, they exhibit a tendency for recrystallization (Shegokar and Mueller, 2010).

Results of DSC and PXRD analyses confirmed that the suspended nanoparticles in HC nanosuspensions were in crystalline form. The PXRD studies conducted after two years, demonstrated that the fabricated HC nanocrystals were pretty much similar as was observed initially. (figs. 3 and 4).

The ocular bioavailability study on rabbit eyes demonstrated that HC nanosuspensions have quick onset

of action and sustained for long time compared to the HC solution. HC nanosuspensions remarkably increased IOP of rabbit eyes and then slowly returned to the normal pre-treated values. Owing to large surface area, enhanced dissolution rate and saturation solubility the bioavailability of nanocrystals/nanoparticles are markedly increased (Elvin Blanco *et al*, 2015, Rabinow, 2004). This increase in bioavailability for HC has been observed because of the nanoparticulate nature of the API. The overall of performance of HC nanosuspensions showed higher bioavailability and sustained action. These two properties are because of high affinity of nanoparticles to corneal membrane and crystalline nature of the drug particles, which could potentially trigger both enhanced bioavailability and longer time of action (R. Ravichandran, 2009). In general, crystalline nanoparticles have been reported to demonstrate longer action of time compared to the amorphous counterparts (Yang *et al*, 2010) On the other hand, for HC solution the quick removal of HC from corneal surface is resulted in reduced drug action.

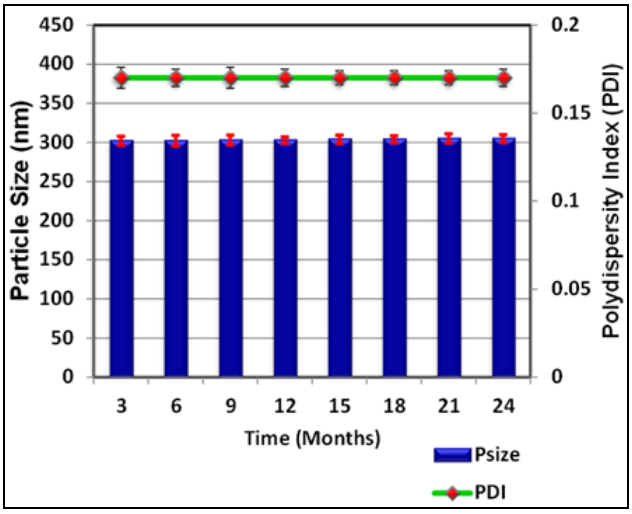


**Fig. 7**: Changes of particle size and polydispersity index with storage time at room temperature.

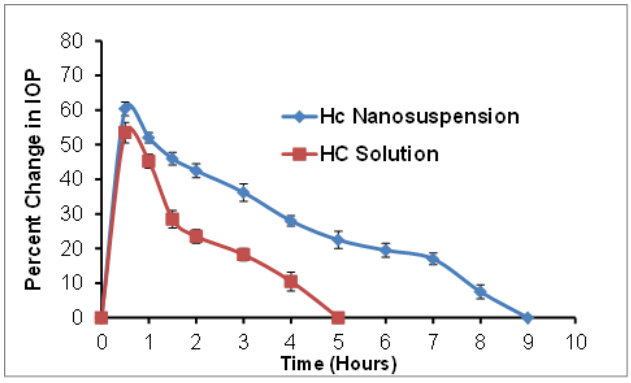


**Fig. 8**: Effect of Hydrocortisone (Hc) Nanosuspension and Solution on IOP of rabbit eyes as a function of administration time.

## CONCLUSION

In the present investigation, the hydrocortisone nanosuspension with narrow sized distribution was successfully fabricated using a wet milling approach. This method for preparation of nanosuspensions using DENNA machine was found quite reproducible, simple, fast and has scale-up feasibility. Saturation solubility was increased significantly compared to that of raw hydrocortisone. Owing to increased surface area and higher solubility the nanosized particles enhance the dissolution rate of hydrocortisone relative to the unmilled or the commercial tablet formulation. Drug crystallinity was maintained after milling as investigated by PXRD and DSC analysis. Crystalline nature of the particles plays a crucial role in enhancing nanosuspension stability. Hydrocortisone crystalline nanosuspension showed spectacular stability which was stored at room temperature for 24 months. It became evident from the ocular bioavailability study, that HC nanocrystals produced by top down method have strong bioavailability and longer action of time which can potentially lead to safe and economical ophthalmic preparation. These results categorically lead towards the exciting conclusion that nanosuspensions seem to be an unprecedented and the most successful technique to address the major issue of poor water solubility and erratic bioavailability associated with a plethora of drug compounds.

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