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Accelerated stability evaluation and *in vitro* antimicrobial activity of a preformed ciprofloxacin nanocrystal formulation

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Abstract: Ciprofloxacin (CIP) is frequently used to treat variety of bacterial illnesses. Drugs can lose their therapeutic efficiency due to physical and chemical deterioration. Therefore, the objective was to evaluate CIP nanocrystals stability and antibacterial activity. Nanocrystals were formulated using CIP drug (250 mg) and poloxamer 188 (2.5% w/v) using probe sonication method. Stability investigations were carried out under International Conference on Harmonization guidelines. Various stability parameters like particle size, zeta potential, and polydispersity index were examined at different storage conditions. Positive outcomes from the formulation's stability were observed and formulation was stable up to three months. The test parameters shown no discernible changes during stability. Accelerated stability testing was done to calculate shelf-life and was found 2.96 years. The antibacterial activity was evaluated by calculating the minimum inhibitory concentration (MIC) against *S. aureus* and *E. coli* and CIP nanocrystals shown lower MIC than pure drug. This study revealed that the antibacterial activity of the formulation was maintained for 3 months at both temperatures (4 and 25°C) and CIP nanocrystals have excellent potential to treat infections caused by such microorganisms. Therefore, CIP nanocrystals could significantly increases its antimicrobial activity, which may translate into a significant improvement in therapeutic outcomes.

Keywords: Ciprofloxacin; nanocyrstal; stability; shelf-life; antibacterial

INTRODUCTION

In 2015 ciprofloxacin (CIP) was included in the WHO model list of necessary medications including a list of minimal drug needs for the primary healthcare system that includes the most efficient, safest, and affordable medications for emergency conditions (WHO, 2015). It has been recommended to treat many acute bacterial illnesses, including typhoid fever, infectious diarrhea, bone and joint infections, infectious diarrhea, intra-abdominal infections and urinary tract infections (Zhanel *et al*., 2002). In addition, CIP showed synergistic antibacterial activity against clinical methicillin-resistant *S. aureus* (MRSA) when certain flavonoids were combined (Liu *et al*., 2011; Mun *et al*., 2013). Almost always, the medication CIP needs to be thoroughly dissolved in solution for the gastrointestinal tract to absorb it into the bloodstream.

Today, the healthcare sector and research labs manipulate medication candidates at the nanoscale primarily for desired solubility, bioavailability and improved patient compliance. Without increasing the administered antibiotic dose, nanoparticulate drug delivery systems may increase therapeutic efficacy by increasing the concentration of the antibiotic in the bacteria (Azhdarzadeh *et al*., 2012). Nanocrystals are submicron colloidal drug delivery systems without carriers that consist of pure drugs and the smallest amount of surfactant necessary for stability with average nanometer-sized particles, generally between 10-800 nm (Junghanns and Müller, 2008). Nanocrystals have several benefits. The possibility of giving medicine through many administration methods is one benefit (oral, intramuscular, intravenous, pulmonary, ophthalmic and dermal), the ability to create medicine in a variety of pharmaceutical dosage forms (ointments, solutions, tablets, capsules, etc.), higher solubility than typical particles (depending on the drug's solid physical state, thermodynamic or kinetic) faster rate of disintegration than traditional particles (Gigliobianco *et al*., 2018).

Pharmaceutical product stability testing comprises following a series of steps with the end goal of ensuring the production of high-quality, safe and efficient products (Tangri and Bisht, 2012). When a formulation remains stable in a given container, it means that the product continues to meet criteria while maintaining its physical, chemical, microbiological and toxicological integrity (Bajaj *et al*., 2012; Tangri and Bisht, 2012). A series of processes are used in the stability study of pharmaceutical formulations with the ultimate goal of ensuring the quality, safety, and efficacy of manufactured active products. This study tested the stability of CIP nanocrystal formulations and assessed their potential antibacterial activity against *S. aureus* as Gram-positive and *E. coli* as Gram-negative strains.

MATERIALS AND METHODS

Sigma Aldrich (St. Louis, MO, USA) provided the drug ciprofloxacin (CIP) and Poloxamer 188. We purchased Tween® 80, from S.D. Fine-Chem. Ltd. (Mumbai, India).

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The other chemicals and reagents were purchased from Merck Specialties Private Limited (Mumbai, India) and are of analytical grade.

Formulation of CIP nanoformulation

The CIP nanosuspensions were produced using the reported procedure by Mujtaba (Mujtaba *et al*., 2019). Precisely weigh the CIP (250mg) that has been added to the 50ml of poloxamer 188 (2.5%w/w) aqueous solution. The suspensions were mechanically stirred at 1000 rpm for 10 minutes to get homogeneous. Probe sonication (SONICS Vibra cell VC750, USA) was applied to the resulting suspension at an amplitude of 30% and pulsed for 30 seconds for 10 minutes, to form nanocrystals. An ice bath was used to keep the temperature at 0°C throughout the sonication process. Mannitol was used as a cryoprotectant during lyophilizing the resulting nanosuspensions. Therefore, before deep freezing, 7.5% w/v mannitol was used as a cryoprotectant to the nanosuspension. Vials containing 50mL of nanosuspension were frozen in a deep freezer for 24 hours at -20°C. To obtain the free-flowing dry powder, such frozen particles were freeze-dried in a lyophilizer (Zirbus technology, Germany) for 24 hours at a vacuum of 200 Pascal.

Characterization of nanoformulation

The formulated nanosuspensions were evaluated for their particle size (PS), polydispersity index (PDI) and zeta potential (ZP). The PS, PDI and ZP of the CIP nanosuspensions formulation were determined using a dynamic light scattering (DLS) (Malvern Zetasizer, Nano ZS, UK) method. Samples were adequately diluted with distilled water before testing to avoid the multi-scattering phenomenon. The scattering angle was maintained at 90^{o} and the temperature was kept constant at about 25±2^{o}C. Each measurement was conducted in triplicate (Mujtaba *et al*., 2022).

Morphological analysis of CIP nanosuspensions was also performed using transmission electron microscopy (TEM). To prepare the sample for TEM, phosphotungstic acid staining was used. A drop of the nano-suspension was applied to a copper grid that had carbon on it to create the TEM sample. The sample was dried before being TEM analyzed (JEOL JEM1010, Tokyo, Japan) (Kohli *et al*., 2022).

Accelerated stability and shelf-life assessment

To determine stability, an accelerated stability study was conducted for the formulation packaged in glass vials with rubber stoppers at 40±2^{o}C and 75±5% RH for 3 months. The formulation was redispersed in distilled water at intervals of 0, 30, 60 and 90 days and its stability was assessed in terms of particle size (PS), zeta potential (ZP) and polydispersity index (PDI). PS, ZP and PDI were determined following the precise procedure described by Mujtaba (Mujtaba *et al*., 2019). We also carried out stability investigations by keeping the samples at 4±0.5°C and 25±2°C/60±5%RH for three months.

To determine shelf-life, the CIP formulation was kept at three different temperatures for three months: 40±2^{o}C; 50±2^{o}C and 60±2^{o}C. Samples were taken out at the predetermined time at 0, 30, 60 and 90 days and the amount of drug present in each was calculated using a double beam UV/Visible spectrophotometer (Jenway 6850 double beam spectrophotometer, Cole-Parmer, Saint Neots, UK) operating at λ_{max} of 276 nm. Controls consisted of zero-time samples. The logarithm of the remaining drug percentage was plotted against time (days). The slope of the plots at each temperature was used to get the degradation rate constant 'k' using equation 1:

$$\text{Slope} = \frac{-K}{2.303} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Plotting the logarithm of K values versus the reciprocal of absolute temperature at various temperatures (Arrhenius plot). The graph was used to establish the K value at 25^{o}C (K_{25}), which is then substituted into equation 2 to predict shelf life.

$$T_{0.9} = \frac{0.1052}{K25} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where, $T_{0.9}$, also known as the shelf life, is the amount of time needed for a product to degrade by 10%.

Antibacterial activities

The cup plate method was employed to compare the antibacterial properties of CIP nanocrystal formulation with those of the free drug against Gram-positive *S. aureus* (ATCC29213) and Gram-negative *E. coli* (ATCC25922) (Uduma *et al*., 2017; Alarifi *et al*., 2020). After transferring molten Muller-Hinton agar to petridishes that had been sterilized, the petridishes received 0.2ml of the previously indicated strains' overnight cultures. The culture was distributed uniformly, and the medium was left to solidify. A well borer that had been sterilized in the flame was used to bore equidistant wells in the solidified media. The samples were put in the wells after different dilutions of formulation and drug had been prepared. To allow for diffusion, the plates were kept at 4°C for 30 minutes in a refrigerator. Plates were then incubated. The diameter of the inhibitory zone was measured and recorded in mm after the plates had been incubated for 24 hours at 37°C. The experiment was carried out three times. The laminar flow hood's aseptic section was used for all microbiological experiments. Before usage, all test-related glassware was autoclaved at 121°C for 15 minutes to ensure sterilization. The MIC is the lowest amount of an agent that will cause no detectable bacterial growth when compared to the control plate.

The agar well diffusion assay described above was also used to assess the antibacterial efficacy of the CIP nanoformulation against *S. aureus* and *E. coli* for up to 90

days. 50 µL samples containing CIP formulations (equal to 5 µg CIP) and 5 µg pure CIP were placed into wells made in an agar plate using a sterile pipette. The inhibitory zone diameter was measured and recorded in millimeters, and the antibacterial activity of the formulation was evaluated for samples held at 25°C (room temperature) and 4°C (in the refrigerator).

STATISTICAL ANALYSIS

Data are presented as Mean ± SD. Graph Pad InStat demo version (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze the raw data and were examined using one-way ANOVA.

RESULTS

Characterization of nanoformulation

The formulation of CIP nanosuspensions was assessed for their PS and determined to be 299.1±11.74 nm with a PDI of 0.493±0.06 (fig. 1A). The formulation's ZP was found to be 22.32±0.56 mV (table 1). TEM was used to investigate the CIP nanocrystal surface morphology (fig. 1B).

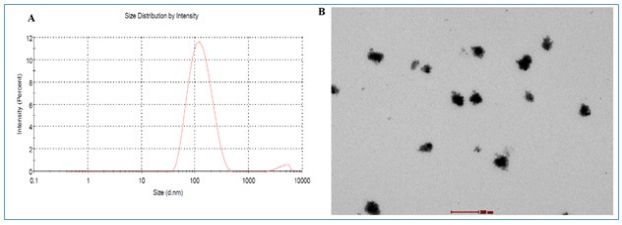

Fig. 1: Particle size and TEM image of the formulation.

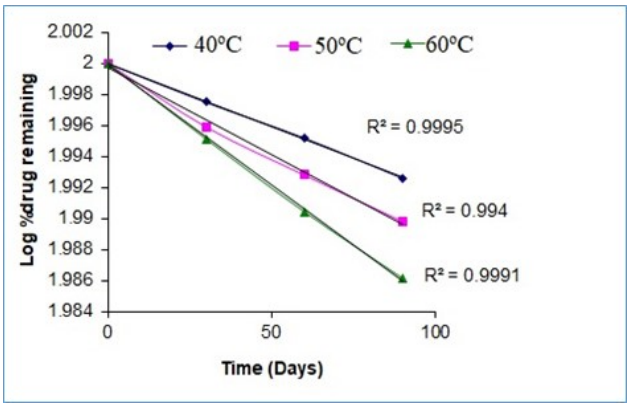

Fig. 2: Log percent drug remaining versus time plot

Accelerated stability and shelf-life assessment

A three-month accelerated stability study was performed on CIP nanocrystals according to ICH guidelines. Table 1 shows the effect of storage at 40±2°C and 75±5% RH on the properties of preformulated nanoformulations. Results showed no significant variation in selected parameters of the formulation over 90 days. Additionally, stability tests were carried out for formulation at 4°C and room temperature for 90 days (table 1).

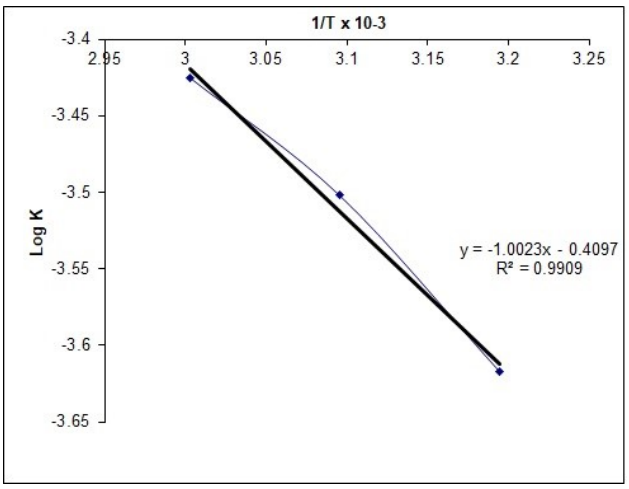

Fig. 3: Arrhenius plot

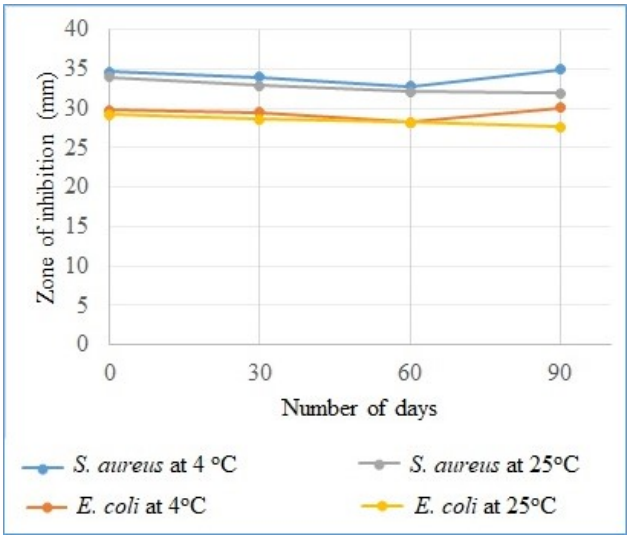

Fig. 4: Antimicrobial activity of CIP nanocrystals formulation during the stability testing against *S. aureus* and *E.coli*

Shelf-life estimation

The percentage of drug remaining in the formulation after three months of storage at 40±2°C; 50±2°C and 60±2°C was calculated; the findings are reported in table 2. The order of degradation for each temperature was established using a graphical method. The results showed that first-order degradation was taking place (fig. 2). Table 3 lists the values of K at each temperature. Fig. 3 displays the Arrhenius plots showing the formulation's stability data for each of the three storage temperatures. Extrapolating, it was determined that the formulation's K value at 25°C (K_{25}) was 0.974×10^{-4}, corresponding to a 2.96-year shelf life (table 3).

Antibacterial activities

Gram-positive and Gram-negative bacteria were used as test subjects for the antibacterial activity of CIP and its nanocrystal formulation (table 4). Fig. 4 displays the

Table 1: Stability data

CIP nanocrystals formulation (4°C ± 0.5°C)				
Parameters	0 days	30 days	60 days	90 days
Particle size (nm)	299.1 ± 11.74	302.7 ± 12.03	303.2 ± 14.32	304.6 ± 16.03
PDI	0.493 ± 0.06	0.494 ± 0.08	0.496 ± 0.07	0.498 ± 0.09
Zeta potential (mV)	22.65 ± 1.13	22.46 ± 1.21	22.71 ± 1.23	22.77 ± 1.32
CIP nanocrystals formulation (25°C ± 2°C)				
Parameters	0 days	30 days	60 days	90 days
Particle size (nm)	299.1 ± 14.74	315.4 ± 16.03	319.3 ± 18.32	321.6 ± 21.03
PDI	0.493 ± 0.06	0.501 ± 0.08	0.503 ± 0.08	0.504 ± 0.09
Zeta potential (mV)	22.65 ± 1.13	22.58 ± 1.19	22.77 ± 1.27	22.98 ± 1.33
CIP nanocrystals formulation (40°C ± 2°C)				
Parameters	0 days	30 days	60 days	90 days
Particle size (nm)	299.1 ± 14.74	318.2 ± 16.53	321.1± 19.33	324.6 ± 20.83
PDI	0.493 ± 0.06	0.499 ± 0.07	0.504 ± 0.08	0.505 ± 0.08
Zeta potential (mV)	22.65 ± 1.13	22.46 ± 1.10	22.83 ± 1.35	23.01 ± 1.41

Table 2: Accelerated stability study data

Storage Condition	Time (days)	% of drug remaining	Log % drug remaining
40 ± 2 ºC	0	100	2
	30	99.42	1.998
	60	98.89	1.995
	90	98.32	1.993
50 ± 2 ºC	0	100	2
	30	99.06	1.996
	60	98.36	1.993
	90	97.68	1.989
60 ± 2 ºC	0	100	2
	30	98.87	1.995
	60	97.82	1.990
	90	96.84	1.986

Table 3: Degradation rate data for formulation at different temperatures

Temp. (°C)	Absolute Temp. (K)	$1/T \times 10^{-3}$	slope$\times 10^{-4}$	$K \times 10^{-4}$ ($Days^{-1}$)	Log K	Shelf-life ($T_{0.9}$) at 25°C
40	313	3.1949	-0.833	2.29	-3.617	
50	323	3.0959	-1.367	3.14	-3.502	
60	333	3.0030	-1.633	3.76	-3.425	
25	298	3.3557		0.974	-4.0111	2.96 years

Table 4: Antibacterial activity

Test microorganism	MIC (µg/ml)	
	CIP	CIP nanocrystal formulation
S. aureus	0.568 ± 0.10	0.236 ± 0.09
E.coli	1.19 ± 0.13	0.489 ± 0.11

outcomes after the formulation's antibacterial activity was sustained for 90 days at both temperatures. According to the stability testing, the formulation could be kept intact for 90 days without displaying any indicators of instability. Additionally, the formulation's primary function-its antibacterial activity-remained consistent throughout the test, which is promising.

DISCUSSION

The formulations were developed using poloxamer-188 in the probe sonicator. Through a variety of processes, electrical energy is transformed into shock waves during probe sonication. These shockwaves cause bubbles to erupt violently and collide with other particles, creating

heat (Xia *et al*., 2010). The surfactant poloxamer 188 showed strong stabilizing abilities and was able to maintain the nanocrystals without a steric barrier. Polymers and surfactants are used as stabilizers to nanosuspension formulations to lower the system's free energy by reducing interfacial tension and stopping nanoparticle aggregation through steric or electrostatic stabilization (Verma *et al*., 2011). The long-term stability of colloidal nanoformulations is improved by freeze-drying.

Low values of PDI showed narrow particle size distributions and the PS distribution curves for the formulation were found to be unimodal. The stability of colloidal dispersion is greatly influenced by the surface charge. The zeta potential of nanosuspensions was found to be within acceptable limits. The TEM and DLS measurements of nanoparticle size showed a good correlation.

Stability studies indicate how a drug's quality changes over time under the effect of several factors like temperature, light, and humidity. At the commencement of a study and for the duration of the intended shelf-life, an ideal drug product needs to be adequately defined physically, chemically, and microbiologically.

Nanocrystal PS and ZP did not change significantly at 4°C, however, some statistically significant variations in PS and ZP values were seen at room temperature, indicating the susceptibility to stability issues during storage at room temperature. Due to faster redispersion on the particles at high temperatures, which led to larger particles, Ostwald ripening was more facilitated. As a result, prepared nanosuspension must be stored at 4°C to maintain stability throughout its shelf-life.

According to the evaluation of stability data specifications (ICH Q1E Step 4) for drug substances, the shelf-life was assessed at room temperature. A study on accelerated stability was also performed to give the formulation a proper shelf-life. Accelerated stability studies have shown that the CIP nanocrystal formulation has a reliable shelf-life.

When loaded with CIP nanoparticles, the MIC values decreased significantly ($p<0.05$) in both microorganism strains. As a result, CIP nanoparticles were more effective against both Gram-positive and Gram-negative strains of *E. coli* and *S. aureus.* Due to the unique existence of the cell wall in their structure, gram-negative bacteria are frequently more resistant to antibiotics than gram-positive bacteria. These specific features prevent antibiotics from penetrating the bacterial cell (Lotfipour *et al*., 2008).

In this study, the lowered MIC levels in both bacteria may be attributed to increased drug penetration by nanoparticles into the bacterial cell, which stopped the growth of the bacteria. The outcomes demonstrated that the formulation of CIP nanocrystals could significantly slow down the growth of both strains of Gram-positive and Gram-negative germs. Their MIC values were less than the MIC of the free drug.

CONCLUSION

In conclusion, CIP nanocrystal formulations have been designed and selected for their *in vitro* antibacterial activity and stability assessment. During the three months of storage at various temperatures, the PS, PDI, and ZP of the CIP nanocrystal formulation did not change significantly, indicating the physical stability of the prepared formulation. At room temperature (25°C), the formulation has a shelf life of 2.96 years. The present work produced nanoparticles with small particle sizes, which may increase medication penetration into bacterial cells and enhance antibacterial action. The outcomes demonstrated that the formulation of CIP nanocrystals could significantly slow the growth of two strains of Gram-positive and Gram-negative germs. Additionally, the formulation's antibacterial activity remained active for three months at both temperatures (4 and 25°C). Therefore, incorporating CIP into a formulation of nanocrystals greatly improves its antibacterial activity *in vitro*, which can lead to greatly improved therapeutic results.

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