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SITE SPECIFIC DRUG DELIVERY OF CIPROFLOXACIN HYDROCHLORIDE NANOPARTICLES FOR IMPROVISED TREATMENT OF PERIODONTITIS

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ABSTRACT
Periodontitis, commonly known as gum disease, is a significant infection of the gums where both the gums and the deeper structures supporting the teeth become inflamed. This condition manifests as redness, swelling, and a propensity for gums to bleed when brushing, all caused by bacteria that accumulate on the surface of the teeth. In more advanced stages, it can lead to sore and bleeding gums, difficulties in chewing, and potentially result in tooth loss. Approximately 20-50% of the global population in both developed and developing nations is affected. The prevalence of periodontitis is notably high among adolescents, adults, and older individuals, making it a pressing public health issue. The aim of this study is to develop nanoparticles of ciprofloxacin hydrochloride that can address the challenges of administering the medication systemically for periodontal disease. Targeted drug delivery of ciprofloxacin hydrochloride can ensure that the medication reaches the affected area effectively over an extended period without causing side effects. Ciprofloxacin hydrochloride nanoparticles were synthesized employing the ionotropic gelation technique with 1.5% glacial acetic acid. An orthogonal factorial design was utilized to formulate the nanoparticles. Characterization of the nanoparticles was conducted to assess particle size, zeta potential, percentage of drug loading, and drug entrapment efficiency. Scanning Electron Microscopy (SEM) was used for surface analysis of the nanoparticles. The in-vitro drug release profile of nine different batches was evaluated using the Franz Diffusion Cell method, and for the batch with the smallest particle size, an in-vitro antimicrobial assay was performed using the turbidimetric approach. Ciprofloxacin hydrochloride nanoparticles served as a superior carrier compared to other antibacterial medications, demonstrating effective and extended drug action lasting up to 168 hours.

KEYWORDS: Periodontitis, Nanoparticles, Targeted Drug Delivery, Zeta potential, Scanning Electron Microscope, Franz Diffusion Cell, Ionotropic Gelation.

INTRODUCTION
Periodontitis is a serious gum inflammation which affects the gums and deeper periodontal structures of the teeth. This gum inflammation occurs as a result of host response to microorganisms that accumulate on the tooth surface and turns the gums to swell and bleed during tooth brushing. This accumulation occurs due to the plaque build up of bacteria and food debris. This inflammatory response is responsible for serious damage to gums and spreads down below the gums to the roots of tooth and cause periodontal ligament damage that support the tooth. If no proper treatment regimen is followed, it ultimately leads tooth loosening and its potential loss. Gingivitis is the first stage of onset of periodontal inflammation with red and swollen gums along with a discoloured bacterial plaque layer. As the disease progress, chronic inflammation occurs that cause gum attatchment loss, periodontal pockets, bone of jaw to be destroyed and teeth to be lost. It generally occurs at adult age and routine examination can monitor the periodontal status with Periodontal Screening Index. Untreated periodontitis have serious effects on health such as complication during pregnancy, increase risk of heart disease and diabetes.

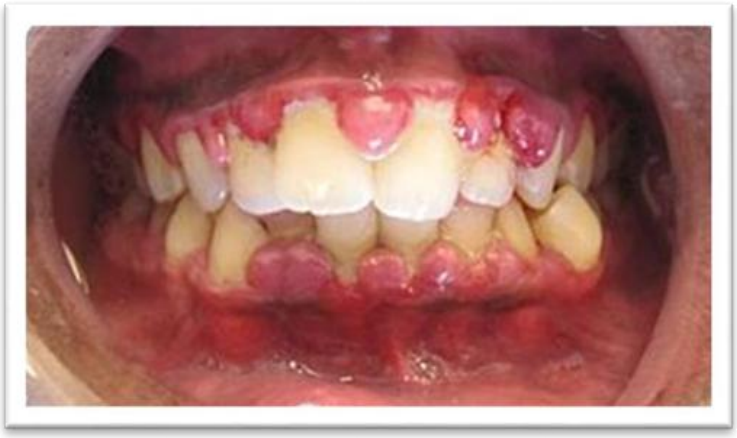
1.1.1 Sign and Symptoms of Periodontitis
- Swollen gums
- Bright red to purple gums
- Tenderness of gum while touching
- Bleeding gums
- Bad breath
- plaque or tartar build-up
- Gingival recession, teeth look longer than normal
- Pink-tinged toothbrush after brushing

- Bleeding gums after brushing or flossing
- Pus between teeth and gums
- Loose tooth and tooth loss
- Painful chewing
- Spaces between teeth
- Periodontal pockets
- Change in the tooth positioning while biting

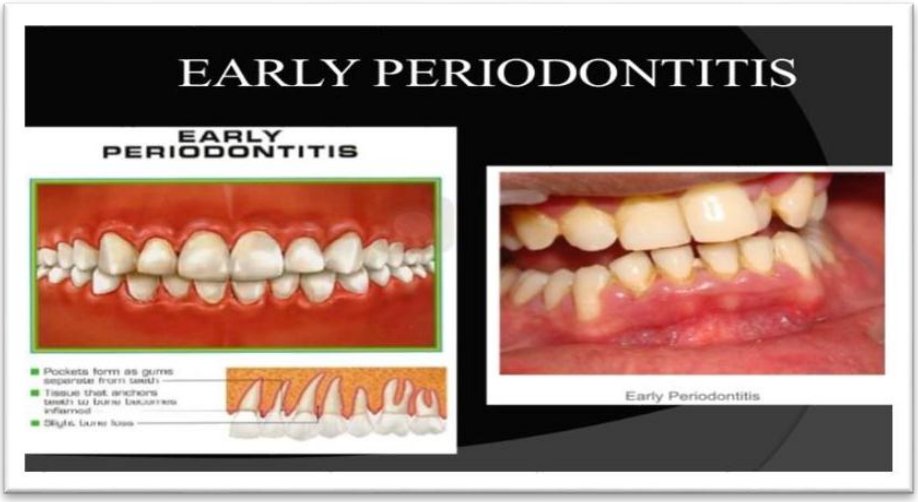
1.1.2 Stages of Periodontitis

1. Gingivitis: Gingivitis is a prevalent and mild type of periodontitis characterized by inflammation at the base of the teeth, resulting in swelling, redness, and irritation. Poor oral hygiene, smoking, dry mouth, inadequate nutrition, a lack of vitamin C, HIV/AIDS, hormonal fluctuations, genetic factors, and infections are the most common contributors to gingivitis. It can be reversed with consistent dental checkups. Severe forms of gingivitis, such as chronic gingival inflammation and necrotizing ulcerative gingivitis, necessitate proper oral health practices and regular visits to the dentist. Symtoms of this are listed below:

- Minor marginal and papillary redness and edema
- 1-3 mm pockets (4's localized to the posterior)
- Light to sensible plaque, bleeding and calculus build-up



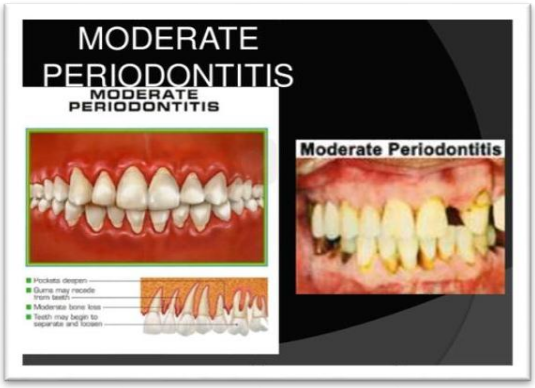
1. **Early periodontitis**: in early periodontitis, pockets are formed as gums separate from the teeth. Inflammation of the tissues that supports the teeth is visible with slight bone loss.



- It is Minor to moderate marginal papillary redness and edema
- 4-5 mm pockets (5's locallized to the posterior)
- Moderate plaque, bleeding and calculus build-up
- Minor bone loss starting.

2. **Moderate Periodontitis**: Deeper pockets with receding gums are seen in moderate periodontitis. The tooth loosens from the gum and begin to separate with moderate bone loss.

- Moderate marginal and papillary redness and edema
- 5-6 mm pockets (4's and 5's generalized in posterior)
- Moderate to heavy plaque, bleeding and calculus build-up
- Moderate bone loss
- Mobility possible



3. Advanced Periodontitis

- Severe marginal and papillary redness and edema
- 7 mm pockets or more
- Heavy plaque, bleeding and calculus build-up
- Moderate to severe bone loss
- Mobility

4. Refractory Progressive Periodontitis:

- Several unclassified types of periodontitis are considered by rapid bone and attachment loss or show but constant attachment loss, usually associated with inflammation and continued pocket development even with good plaque control.
- Frequently there are vertical and irregular bone loss, especially around the initial molars and lateral incisors.

Classification of Periodontitis

1. Chronic periodontitis:

It represents the forms of destructive periodontal disease that are generally characterized by slow progression.

2. Aggressive periodontitis:

It is a diverse group of highly destructive forms of periodontitis affecting primarily young individuals, including conditions formly known as "early onset of periodontitis" and "rapidly progressing periodontitis".

1. Periodontitis as a manifestation of systemic disease

It is a heterogeneous group of systemic pathological conditions that include periodontitis as a manifestation.

2. Necrotizing periodontal disease

It is a group of conditions that share a characteristic phenotype where necrosis of the gingival or periodontal tissue is a prominent feature.

3. Periodontal abcesses

It is a clinical entity with distinct diagnostic features and treatment requirements.

1.1.2 Pathogenesis

- As the gum inflammation increases, the leucocytes, neutrophiles, T-lymphocytes, and plasma cells gets activated and in response they secrete antibodies, lipopolysaccharides, cytokines, chemokines and proteins.
- Complex host response occurs when lipopolysaccharide present in the bacterial cell wall which act as powerful stimulants.
- At the periodontal site, initially cytokines, macrophages and cytokines are produced along with tumor necrosis factor, prostaglandins and interleukin.
- Bone resorption occurs when the matrix metalloprotiens are secreted as inflammatory response which influence the fibroblasts. They breakdown the collagen
- The inflammatory mediators such as lymphocytes activate osteoclasts that result in loss of bone.
- The periodontal inflammation is dependent on both host response and mediators of the disease.

1.1.3 Pathophysiology

- Below the gums, there is a shift in the composition of the biofilm from basically streptococcus to an actinomyces dominant plaque. Motile bacteria were also seen more often as the biofilm accumulates on the teeth near and due to this gingival inflammation occurs.
- Gingivitis which represents in the inflammation confined to the soft tissues above the bone level. Inflammation of gingiva can continue at the gingivitis level for prolong period and will not proceed to periodontitis unless in the presence of local conditions or generalized host susceptibility.
- The immune systems response occurs to plaque accumulation change from a predominantly neutrophilic mediated response to lymphocytic and plasma cell facilitated response.
- Clinically, the gingival is swelled, red and bleeding gums. This influences the bacterial environment leading to change in the composition of oral microbiota. Due to this, a predominantly gram – negative environment is established, and periodontal pathogens proliferate.

1.1.4 Bacteria responsible for periodontitis:

✓ There are around 700 species of microrganisms present in human oral cavity.

Anaerobic bacteria

1.) Gram – Positive
-Peptostreptococus spp.
-Bifidobacterium spp.
-Eubacteriaaerofaciens
-Lactobacillus fermentum
-Propionibacterium spp.
-Actinomyces naeslundii

2.) Gram – negative
-Prevotella oralis
-Bacteroides ovatus

Aerobic Bacteria

1) Gram – positive
-Streptococcus spp.
-Gemellamorbillorum
-Staphylococcus spp.
-Coagulase – negative
-Micrococcus spp.

2) Gram – negative
-Neisseria spp.
-Haemophilus parainfluenza

1.1.5 Periodontal disease causes:

- Alcohol abuse
- HIV/AIDS
- Diabetes mellitus
- Age
- Gender
- Genetics
- Gingivitis
- Cancer
- Hormonal changes
- Onging medications
- Nutrient deficiency
- Poor oral hygiene
- Smoking/Chewing tobacco
- Stress

1.1.6 Prevention of periodontitis:

To prevent periodontal disease by daily oral hygiene measures, include:

- Properly brushing on a regular basis (twice in a day)
- Using antiseptic containing mouthwash
- Using dental brushes and flossing daily
- To maintain dentist-prescribed medication use periodontal trays (In disease condition)
- As required go for regular check-up and professional teeth cleaning
- Evaluation of bio-film clinically (To regaining healthy oral flora).

Special instruments are used by dentist to interrupt growing plaque below the gum line and clean teeth below the gum line. This is standard treatment for prevention of periodontitis. Tooth loss and periodontal disease are linked with an increased risk of cancer in male patients. A high alcohol consumption is considered as contributing causes.

1.2 Treatment of chronic periodontitis

Positive conclusion of treatment is taken by quitting smoking and maintaining a superior oral hygiene.

- Scaling and root planning are a technique in which mechanical debridement of the periodontal pocket depths and interrupt the present microbial bio-film.
- Open Flap debridement is used by physician, in case of deeper pocket areas.
- In treatment of chronic periodontitis enamel matrix derivative is favoured by some physician. Researchers have resolved that after one-year application of it, probing level of attachment was improved by it compare to control.
- After debridement, common antibiotics are given to patient. Added therapy may provide change in PPD, decrease risk of additional loss of attachment and additional advantage over scaling and root planning.
- Tetracycline, metronidazole, chlorhexidine has established extensive positive result and it decrease number of pathogens at injured site of periodontal pocket.

1.2.1 Parts of Periodontium

The anatomy of periodontium consists of:

- Gingiva or the gum
- Periodontal ligament(PDL)
- Cementum
- Alveolar bone proper

These components play different role as a part of periodontium of tooth. Such as:

- **Gingiva or the gum:** The gums or gingival, consists of mucosal tissue which lies above the mandible and maxilla of the oral cavity. It is the part of soft tissue lining of mouth. It surround the teeth and provide a seal around them. They help in resistance to the friction of tooth passing over them. It provides a barrier to the barrage of periodontal insults to deeper tissue when they are healthy. It is usually coral pink or naturally darker with melanin pigmentation. Bacterial Plaque affect it and it turns to bleed. When gum tissue are not healthy, it provide a gateway for periodontal disease to advance into deeper tissue, which leads to poorer prognosis for prolong retention of teeth. The gums are anatomically divided as marginal, attached and interdental areas. The gingival cavity consists of food debris, saliva etc that support the growth of microorganisms that can be injurious to health. Improper oral care can lead to various gum infections. Gum recession occurs when the apical movement of the gum margin away from occlusal surface and inflammation such as periodontitis, pyorrhoea, dry mouth, pocket formation etc can occur. It may also lead to root sensitivity.

- **Periodontal ligaments:** it is a group of specialized connective tissue fibres that usually attach a tooth to alveolar bone. It goes into the root cementum one side and onto alveolar bone on the other. It consists of principal fibres, loose connective tissues, blast and clast cells, oxytalan fibres and Cell Rest of Malassez. It consists of 70% water to withstand stress load on tooth. It is 0.15 to 0.38mm wide. They are supportive, sensory, nutritive and remodelling. It undergoes drastic changes in chronic periodontitis leading to delayed healing of alveolar bone socket and ultimately extraction of tooth.

- **Cementum:** It is a specialized substance covering the root of the tooth. It is a part of the periodontium that attaches the teeth to the alveolar bone by anchoring the periodontal ligament. It is entrapped in cementoblasts called cementocytes.if cementum is observed on tooth, this means that roots are exposed, showing clinical crown is bigger than the anatomical crown. This occurs due to gingival recession and indicate the presence of periodontal disease.

- **Alveolar bone:** It is the thickened ridge of bone that contains the tooth socket on the jaw bones that hold teeth. The curved part of each alveolar process on the jaw is called alveolar arch. It consists of 67% inorganic matrix and33% organic matrix. Alveolar bone loss occurs due to resorptionof osteoclasts breaking down the hard tissue of the bone. Alveolar bone loss is closely associated with periodontal disease.

1.2.2 Diagnosis of Periodontitis:

The diagnosis of periodontal conditions is complex and requires a high degree of skill.

- **Clinical examination and periodontal probing:**

The examination of gingival and periodontal tissue require a sequential manner. Subjectively, visual inspection is the first step to assess gingival tissues. It shows the presence of or absence of gingival inflammation by assessing the colour and degree of swelling of the tissues. As an initial assessment of the level of oral hygiene due to plaque and calculus levels. After this, assessment of probing depth occurs. The choice of periodontal probe is the first decision to make. For epidemiological studies, Community Periodontal Index (CPI) probe is used as per World Health Organisation (WHO). Probes such as Williams, UNC PCP-15, Florida probe. In the presence of inflammation, the probe tip penetrates the base of the junctional epithelium which leads to overestimation of pocket depth while the probe tip does not reach the base of the junctional epithelium in the absence of inflammation. Probing depth can truly equate the pocket depth. The probing depth measurements are taken from mesio- buccal, mid-buccal, disto-buccal, mesio- palatal, mid-palatal and disto-palatal. The bleeding on probing defines the level of inflammation in periodontal tissues. Absence of bleeding on probing is a reasonably good indicator of periodontal health and stability of tissues.persistent bleeding on probing at successive maintenance visits is the indication of periodontal disease progression.

- **Radiographic assessment techniques:**

Radiographic assessment is essential to provide information regarding the pattern and extent of alveolar bone loss. The use of radiography is driven by, and is secondary to, the results of the clinical examination. Every effort should be made to minimise radiation dose. Radiographs of caries diagnosis should be utilized, if possible, to aid in the assessment of alveolar bone levels. Paralleling techniques should be used for intraoral periapicals, and attempts made to position sequential radiographs reproducibly over time to allow for better detection of changes in alveolar bone levels that may occur. Panoramic radiograph and periapical radiographs are also taken. The radiographs of teeth present and teeth missing, bone loss, presence of specific vertical bone defects, presence of calculus,apical pathology, caries, enamel lucencies,ledges, and other pathological defects.

- **Recession and loss of attachment:**

Probing depths alone is misleading to assess the cumulative effects of periodontal inflammation. For example- A patient with the history of periodontitis and has successfully treated have shallow probing depths yet generalised gingival recession, this shows widespread loss of periodontal tissue which cannot be detected by data of probing depth alone. Recession in localised gingival recession is also important. Measurement of recession adds to the clinical information for treatment.

Loss of attachment (mm) = Probing depth (mm) + recession(mm)

- **Tooth mobility**

Loss of attachment and loss of alveolar bone results in increased tooth mobility. Rigid instruments are used for assessment and scores are given to tooth. There are three grades commonly used:

Grade I- mobility in excess of physiological mobility <0.2mm in horizontal direction but less than 1mm in horizontal direction)

Grade II- horizontal mobility >1 mm

Grade III- mobility of crown in a vertical direction

- **Involvement of furcation:**

Progression of periodontitis around multi-rooted teeth may result in horizontal loss of attachment into furcation area. This should be assessed as part of routine periodontal assessment due to the anatomy of teeth roots. Naber's probe is used for this purpose. In maxillary molars, 3 furcations are assessed- buccal, mesio-palatal and disto-palatal. In mandibular molars, 2 furcations are assessed- buccal and lingual.

- **Plaque levels and oral hygiene**

The sublingual bio film is assessed to check the presence of inflammation- gingivitis and periodontitis. Plaque

control is the vehicle by which infection is controlled, plaque and oral hygiene is assessed and is the standard component of periodontal assessment. For research purposes, plaque index scoring system is used.

- **Sensibility testing**

Sensibility testing is a part of periodontal assessment, in suspected cases such as periodontal/ endodontic lesions. Sensibility is assessed by 2 independent methods- cold test: ethyl chloride and electric pulp testing.

- **Occlusion**

Occlusal trauma, occlusal interferences or fremitus are necessary to assess inflammation evidence. Tooth/arch relationships, developmental aspects or iatrogenic factors are responsible for occlusal interferences. Increased tooth mobility, widening of periodontal membrane space, tenderness are seen in primary occlusal trauma and secondary occlusal trauma exhibits tissue breakdown due to pre-existing periodontitis.

1.3 Drug Delivery system

1.3.1 Site specific drug delivery system:

- Now a days, biomaterials in regenerative medicine is believed to maximize the beneficial effects of cellular therapy and minimize the poor engraftment. Also, controlling the growth factor delivery for periodontal therapies.
- Using variety of polymers formulate the novel formulation for controlled drug delivery and beneficial to us as low price, higher stability, nontoxicity, biodegradability.
- The topical drug delivery system is directly applied in the periodontal pocket and give controlled release effect.
- Novel formulation which consists of fibres, strip, film, nanoparticle, nanofibres, microparticle, hydrogel.

➢ **Advantages of site specific drug delivery into periodontal pocket as follows:**

- Direct access to target disease
- Improvement of patient compliance
- Avoidance GIT-related issues due to oral drug delivery
- Bypass of first-pass metabolism by the liver
- Enhanced the therapeutic efficacy of the drug
- Reduced treatment cost
- Longer duration of action can be achieved
- Non-invasive, painless and simple application

➢ **Limitation of Site specific drug delivery in to periodontal pocket are as follows:**

- Local irritants cannot be administered.
- Dose is limited because of relatively small area.
- The enzyme like peptidase and esterase may cause pre systemic metabolism.
- Peptide administration is not predictable due to peptidase.
- This route understood the needs for high-potency drugs.
- Manufacturing cost of the patches or devices is a matter to be considered.

1.3.2 Site specific drug delivery carrier system:

- Presently, there are various drug carrier system available to carry the active anti- microbial agents. These includes,
- Fibres
- Films
- Injectable systems
- Microspheres
- Gels
- Strips and compacts
- Vesicular systems
- Nanoparticle system

➢ **Fiber**

- The fibre system consists of the thread-like devices which act as a reservoir for the active drug. These fibres are placed circumferentially into the pockets with an applicator and secured with cyanoacrylate adhesive.
- These fibres are released active drug for an extended period of time. Various materials have been used to make these fibres including poly (e-caprolactone) (PCL), polyurethane, polypropylene, cellulose acetate propionate, ethyl vinyl acetate (EVA).
- The examples of commercially available system using fibres include tetracycline fibres and chlorhexidine fibres.

➢ **Films**

- Films were matrix delivery system in which active pharmaceutical ingredient is distributed throughout the polymer and release occurs by drug diffusion and/or matrix dissolution.
- Films used as carrier for active drugs were made up of synthetic biodegradable polymer such as poly (lactide-co-glycoside), (PLGA) or cross- linked gelatine.
- The synthetic films may be prepared by solvent casting or direct milling. This was cut into the small pieces suitable for insertion into periodontal pockets. The drug is released slowly with the degradation of carrier matrix. These can be easily placed in the periodontal pocket and no additional aids for retention are required because of the adhesive nature of the carrier material.
- The film based local drug delivery systems are available for chlorhexidine diacetate, metronidazole, tetracycline, and minocycline.
- Injectable systems
- These are the comparatively easy systems for delivering the antimicrobial agent in the periodontal pocket. The drug can be easily delivered without pain.
- The drug was pushed into the periodontal pocket so

that it can reach the deepest portion of the pocket, thus getting access to the micro flora of periodontal pocket.

➢ **Microspheres**

- This system is uses biodegradable material to make microspheres containing the active drug, which is released by the degradation of the microspheres.
- Microspheres can be made up of synthetic polymers or natural polymers.

➢ **Gels**

- Various gel formulations were available for site specific drug delivery
- These systems were based on hydroxyl ethyl cellulose and Carbopol974. The gel carrying active drug is applied in the periodontal pocket with the help of a blunt syringe.
- A novel development in gel technology is – hydro gels.
- These are polymeric materials that do not dissolve in water at physiological conditions. However, they swell considerably in aqueous medium. These gels have a phase transition in response to the change in pH, ionic strength, and temperature which make them degradable. High water content and large pore sizes of most of the hydro gels often result in the relatively rapid drug release, over a few hours to a few days.
- The example of presently used gel based site specific drug delivery system include metronidazole gel and doxycycline gel.

➢ **Strips and compacts**

- Strip is made from mixing polymers, monomers and different concentration of the chemotherapeutic agents.
- The strips have flexible for easy placement and have a position securing mechanism similar to film. These are placed in the periodontal pocket where with progressive degradation of the polymer, the drug is released into the surrounding environment, maintaining a high level of chemotherapeutic agents.
- Strips containing tetracycline, metronidazole or chlorhexidine have been tested for their efficacy in the reduction of periodontal pathogens.

➢ **Vesicular system**

- These are the primary liposomal systems similar to bio-membrane in terms of structure and bio-behaviour.
- Structurally, liposomes are concentric bi-layer vesicles in which an aqueous volume is entirely enclosed by membranous lipid bi-layer, mainly composed of natural or synthetic phospholipids.
- Liposomes have distinct advantage of being both nontoxic and biodegradable because they are composed of naturally occurring substances.
- Vesicular delivery systems have been designed for metronidazole, triclosan and chlorhexidine and have been investigated for control of dental plaque and gingivitis.

➢ **Nanoparticle system**

- Various types of nanoparticles system have been designed, including biodegradable polymeric micelles, Nano capsules, Nano-gels, fullerenes, solid lipid nanoparticles (SLN), nanoliposomes, metal nanoparticles and quantum dots.
- The main advantage of this system is high dispersibility in an aqueous medium, controlled release rate, and increased stability. Due to their small size, these particles can penetrate the regions that may be increasable to other delivery systems, such as deep periodontal pockets and function areas.

1.4 Nanoparticles

- Nanoparticles were particulate dispersion or solid particles having size range from 10- 100nm.
- They were highly dispersible in aqueous medium, offers controlled release rate and improved stability. Because of their small size, nanoparticles can access sites unreachable for other devices, like the periodontal pocket region below the gum line.
- A uniform distribution for prolonged time period is obtained thus decreasing the dosage frequency.

1.4.1 Advantages of Nanoparticles:

- Controlled release rate
- High dispensability in aqueous medium
- Increased stability
- Nanoparticles have been small size, penetrate region that may be increasable to other delivery system, such as the periodontal pocket area below the gum line
- Reduced the frequency of administration and provide a uniform distribution of active agent over an extended period of time
- Film is an Economic
- Dimension and shape of films can be easily controlled
- Decrease in dose frequency
- Extend and control the drug release for number of days
- Less side effects
- Site targeted drug delivery
- Improved patient compliance
- The administration is less time consuming than mechanical debridement and a lesser amount of drug is enough to achieve effective concentration at the site of action.
- Minimize the increase in resistance of periodontal microbes.

Table 1.1: Marketed Formulation of Periodontitis.

SR.NO	ANTIMICROBIAL AGENTS	PRODUCT AVAILABLE	DOSAGE FORM
1.	Tetracycline hydrochloride	Actisite	fiber
2.	Minocycline hydrochloride	Dentomycin	ointment
3.	Mertonidazole benzoate	Elyzol	Gel
4.	Chlorhexidine/Tetracycline	Periochip	Films
5.	Chlorhexidine gluconate	Periochip	Chip
6.	Chlorhexidine	Chlo-site	Gel

MATERIALS AND METHOD

Nanoparticles of Ciprofloxacin hydrochloride (Active pharmaceutical ingredient) were prepared using Ionotropic gelation method s. Chitosan was used as polymer with Sodium tetra phosphate as cross linking agent.

Equipment used for performing the experiment are:

Table 3.2: List of Equipments used in present experiment.

Sr. No	Equipment	Purpose	Manufacture/ company with Model
1.	Fourier Transform Infrared Spectroscopy	Compatibility study of Drug-Excipients	Bruker, Germany
2.	UV Spectroscopy	For Analysis of Drug and Formulation	UV-1800 Shimadzu
3.	Single Pan Balance	For Weighing purpose	EMFC Technology Shimadzu
4.	Magnetic Stirrer	For stirring and mixing	CIS-24, REMI
5.	Centrifuge	Separation of formulation of nanoparticles	REMI Instrument
6.	Zeta sizer	Zeta size and Zeta potential measurement	Malvern ns 90
7.	Scanning electron microscope	Morphology and surface topography of nanoparticles and film	Sicart laboratory
8.	Lyophilizer	Vacuum	Alledfrost
9.	Freeze dryer	For freeze drying of nanoparticles	Blue Star, India

3.2.1 Determination of Drug Solubility:

Solubility study of ciprofloxacin hydrochloride was performed in different solvents such as water, ethanol, isopropanol and acetone by dissolving 10mg of drug in 10ml of mentioned solvents and sonicationg for 10minutes.

3.2.2 Determination of Drug Melting point:

Standard technique for the determination of melting point is Capillary Method as per pharmacopoeia. In this method, a capillary is sealed from one end and from the other end the small amount of drug is filled in it. This compact column is introduced into a liquid bath closely attatched to high accuracy thermometer. As the temperature in the heating stand gradually increases, the sample drug liquify and temperature is recorded.

3.2.3 Determination of Drug-Excipient Compatibility

- **Fourier Transform-Infrared spectroscopic study**

FT-IR spectra for drug alone and with excipients mixture were recorded using a FT- IR spectrophotometer with KBr pellets to study drug-excipients interaction and compatibility. The FTIR study was carried out using pressed pellet technique. A KBr press in which KBr was taken and kept in a hot air oven for two hours for the removal of any moisture. Then the above dried KBr was taken for the preparation of pellets of drug and the selected formulation excipients. The prepared pellets were placed in the sample holder and kept in the instrument to record the IR peaks. The result of the infra-red studies for the drug and polymer mixture was obtained.

- **Differential Scanning Calorimetry**

DSC curves of samples of drug- ciprofloxacin hydrochloride, physical mixture of polymers- chitosan and sodium tri polyphosphate and prepared nanoparticles was measured with a DSC-60(Shimadzu instrument, Japan). All the three samples were weighed accurately in DSC aluminium pan and were crimped, followed by heating under the inert atmosphere of nitrogen at a scanning rate of 10°C/min from 40°C to 400°C. Aluminium pan containing same quantity of aluminium oxide was used as reference.

3.3 Analytical Method:

3.3.1. Determination of maximum absorbance of drug

Analytical method was performed using Ultra-Violet spectroscopy. The absorption was carried out at 270 nm and the standard curve of the drug was taken in phosphate buffer pH 6.8.

- **Preparation of solvent-Phosphate buffer pH 6.8:**

28.80 gm of disodium hydrogen phosphate and 11.45 gm of potassium dihydrogen phosphate was dissolved in distilled water (1000ml) and stored in cool place.

- **Preparation of standard stock solution of ciprofloxacin hydrochloride in Phosphate buffer pH 6.8:**

Accurately weighed 100 mg ciprofloxacin hydrochloride was dissolved in 100 ml phosphate buffer pH 6.8 in 100 ml volumetric flask to obtain the stock solution of 1000µg/ml.

3.3.2 Calibration of Ciprofloxacin hydrochloride in Phosphate buffer pH 6.8:

Accurately weighed 100 mg ciprofloxacin hydrochloride was transferred into 100 ml volumetric flask and dissolved in 100 ml phosphate buffer pH 6.8. The volume was made up to the mark of flask with phosphate buffer pH 6.8 to prepare a stock solution of 1000µg/ml. Aliquots 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml, and 1.2ml from the stock solution representing 2, 4, 6, 8, 10 and 12 µg/ml drug were transfer in 10 ml volumetric flask and volume was adjusted 10 ml with the phosphate buffer pH 6.8 and was scanned for UV spectrum by using Shimadzu UV-visible double beam spectrometer.

3.3.3 Analysis of Ciprofloxacin hydrochloride in 1.5% glacial acetic acid:

100mg of ciprofloxacin hydrochloride is dissolved in 100ml 1.5% glacial acetic acid to obtain stock solution and from this aliquots of 0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml, 0.6ml, 0.7ml, 0.8ml, 0.9ml were transferred to 10ml volumetric flask and scanned at 278nm using Ultra-violet double beam spectrophotometer.

3.4 Method of preparation of Ciprofloxacin hydrochloride nanoparticles:

- **Ionotropic Gelation Method:** Ionotropic gelation method was used to prepare nanoparticles. Appropriate quantities of drug and polymer were completely dissolved in 100ml distilled water using magnetic stirrer. 100ml solution of sodium tri polyphosphate and 1.5% acetic acid aqueous solution was flushed drop wise in prepared drug and polymer solution with continuous stirring on magnetic stirrer at 30 min.

3.4.1 Applications of Factorial Design:

- Factorial designs are ideal for screening design objectives.
- They are simple and economic for small number of factors
- If chosen properly, they can be balanced and orthogonal.
- They have low number of runs compared to high information obtained.

Table 3.3: Factors and levels of orthogonal test.

Levels	Factors		
	Chitosan Concentration (%)	Stirring speed (rpm)	Temperature (°C)
1	1.5	400	40
2	1	800	60
3	0.95	1200	80

Table 3.4: Designed formulations of orthogonal experiments.

Batches	Factors		
	Chitosan Concentration (%)	Stirring speed (rpm)	Temperature (°C)
B1	1.5	400	40
B2	1	800	60
B3	0.95	1200	80
B4	1.5	400	60
B5	1	800	80
B6	0.95	1200	40
B7	1.5	400	80
B8	1	800	40
B9	0.95	1200	60

3.4.2 Characterization of trial batches of Nanoparticles

3.4.3 %Drug loading

5mg of obtained nanoparticles were weighed accurately and scanned in ultraviolet spectrophotometer with 5ml 1.5% glacial acetic acid as solvent. The drug loading was calculated as-

$$\%\text{Drug loading} = \frac{\text{Mass of ciprofloxacin hydrochloride in nanoparticles}}{\text{Mass of nanoparticles recovered}} \times 100$$

3.4.2 % Drug entrapment efficiency

The entrapment efficiency is also known as Association Efficiency. The drug loaded nanoparticles were centrifuged at a high speed of 3500-4000 rpm for 30 min and about 10mg supernant is assayed for non-bound drug concentration by UV spectrophotometer.

It is calcuated as:

$$\%\text{ Drug entrapment efficiency} = \frac{\text{Drug in supernatent liquid}}{\text{Total drug added}} \times 100$$

3.4.3 Particle size

The particle size was determined using photon correlation spectroscopy (PCS) with in-built Zeta sizer. The nanoparticles were transferred to a disposable polystyrene cuvette with the help of plastic syringe. The instrument is based on the principle of dynamic light scattering (DLS), also called as photon correlation spectroscopy elastic light scattering. It acts on principle of measurement of Brownian motion and compare with particles. Brownian motion is the zig-zag type movement of particles due to the bombardment by the solvent molecules that surrounds them.

Table 3.5: Particle size and polydispersity index of ciprofloxacin hydrochloride nanoparticles.

No.	Factors			Experimental results	
	Chitosan Concentration (%)	Stirring speed (rpm)	emperature (°C)	Particle size (nm) (mean±SD)	Polydispersity index (mean±SD)
1	1.5	400	40	178.4±119.36	0.177±1.193
2	1	800	60	164.6±115	0.344±1.107
3	0.95	1200	80	152.2±163	0.312±1.146
4	1.5	400	60	173.6±197	0.248±0.682
5	1	800	80	165.3±226.36	0.355±0.607
6	0.95	1200	40	151.6±114.69	0.378±1.99
7	1.5	400	80	167.5±241	0.283±0.614
8	1	800	40	158.7±173.69	0.164±0.602
9	0.95	1200	60	141.2±108.36	0.234±1.103

Data are prepared as means ± standard deviation (n=3)

3.4.4 Zeta potential:

The obtained nanoparticles were diluted with distilled water 10 times. Then they were measured with Malvern zetasizer Nano ZS 90 using clear disposable zeta cell. The measurement is based on the electrophoretic mobility (µm/s) of the particles, from which zeta potential of nanoparticles is obtained.

Zeta-sizer working principle: It works on Mie's theory of laser light scattering. The IHSD (Intelligent High-Speed Detectors) manages the measurements. The sample and the hold circuit capture signals from the scattered light detector. This provides a "snapshot" of the scattered light detectors. Software controls the measurement time.

Measurement criteria:

- Temperature: 25˚C
- Viscosity: 0.01 poise
- Refractive index: 1.33
- Scattering angle: 90˚C
- Run time: 30 sec
- Range: 0-3000 nm

3.5 Scanning electron microscopy (SEM):

Scanning electron microscopy (SEM) of ciprofloxacin hydrochloride nanoparticles was performed to examine the surface morphology. For this purpose, prepared nanoparticles were mounted on metal stubs and the stub was then coated with conductive gold sputter coater attatched to the instrument (pressure 0.001 torr) for 2min. The gold coated samples were observed at room temperature under the scanning electron microscope and photographs of appropriate magnification were received.

3.6 In-Vitro release study (%CDR):

In vitro drug diffusion study was performed by using prepared formulation by use of Franz diffusion cell. The prepared nanoparticles were weighed, and it was kept in donor compartment of Franz diffusion cell .The receptor compartment containing the 22ml phosphate buffer pH 6.8 and continuously stirred with 50 rpm by using magnetic stirrer. Sampling was done from receptor compartment at regular intervals of 24 hr, 48 hr, up to 168 hr and equal volume of diffusion medium was replaced in the receptor compartment. Then the amount of drug present in sample was determined by the UV-spectrophotometer.

3.7 In-vitro antimicrobial activity (Turbidimetry method for *E.coli* and *S.aureus*):

The minimum inhibitory concentration (MIC) of chitosan nanoparticles was determined by turbidimetric method. In this method, a number of test tubes each containing 5.0ml of Muller – Hinton broth were autoclaved for 15min at 121°C. Chitosan nanoparticles powder was accurately quantified and added to distilled water. To the first tube, 5ml of nanoparticles(1mg/ml) suspension was added. After mixing, 5ml of the mixture was transferred to the second tube, and similar transformations were repeated. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The tubes were inoculated under aseptic conditions with 50µl of the freshly prepared bacteria suspension. The positive control was ciprofloxacin hydrochloride and the blank control tubes were only contained Muller-Hinton broth and 1.5% acetic acid. After mixing, the tubes were incubated at 37°C for 24hours. The tubes were then studied for visible signs of growth or turbidity. The lowest concentration of nanoparticles that inhibited the growth of bacteria was considered as minimum inhibitory concentration or MIC.

RESULTS

1.1 Preformulation Studies

1.1.1 Determination of Solubility of Ciprofloxacin hydrochloride:

The solubility of drug was carried out using different solvent such as water, ethanol, isopropanol, and acetone and their result are shown in table 4.1

Table 4.1: Solubility study of ciprofloxacin hydrochloride.

SR. NO	SOLVENT	CIPROFLOXACIN HYDROCHLORIDE SOLUBILITY
1.	Water	Highly Soluble
2.	Ethanol	Soluble
3.	Isopropanol	Poorly Soluble
4.	Acetone	Poorly Soluble

1.1.2 Determination of Melting point of Ciprofloxacin hydrochloride

Capillary method is standard technique for the determination of melting point, the melting point was found to be in range of 256-257°C as reported in literature.

Figure 4.1: Melting point of Ciprofloxacin hydrochloride.

1.1.3 Determination of Drug- excipient compatibility

- **Fourier Transform Infrared Study**

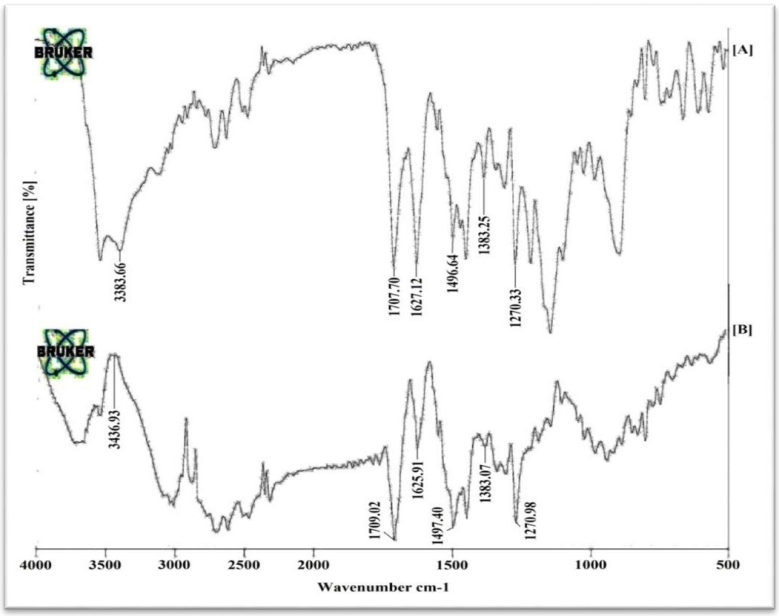


Figure 4.2: FT-IR Spectra [A] Pure Ciprofloxacin hydrochloride [B] Excipients.

Table 4.1: FT-IR interpretation of Ciprofloxacin hydrochloride and excipients.

Drug Peak	Formulation Peak	Peak Characteristics
3383.66	3436.93	-OH stretch
1707.70	1709.02	-COOH stretch
1627.12	1625.91	-C==O vibration
1496.64	1497.40	C-H stretch
1383.25	1383.07	Aromatic C=C
1270.33	1270.98	C-F stretch

- **Differential Scanning Calorimetry**

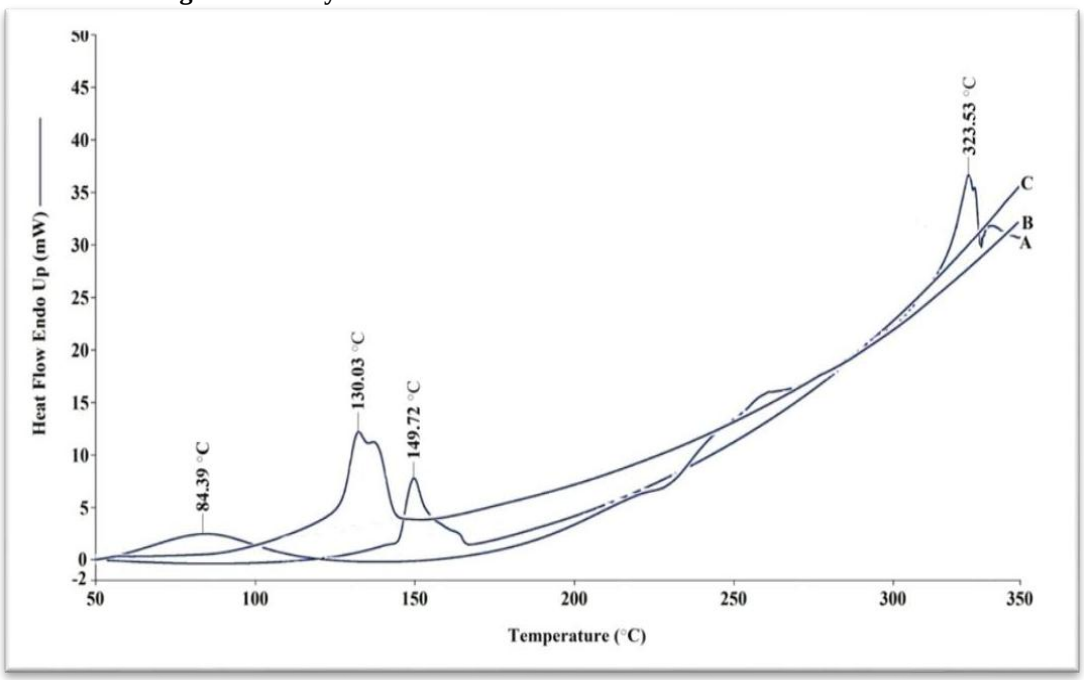


Figure 4.3: DSC Thermogram: A. Pure drug (cxiprofloxacin hydrochloride), B. Physical mixture(drug+ excipient) and C. Nanoparticles.

5.1 Analytical Method:

5.1.1 Caliberation of Ciprofloxacin hydrochloride in phosphate buffer pH 6.8:

- **Determination λmax of Ciprofloxacin hydrochloride by UV spectroscopy:**

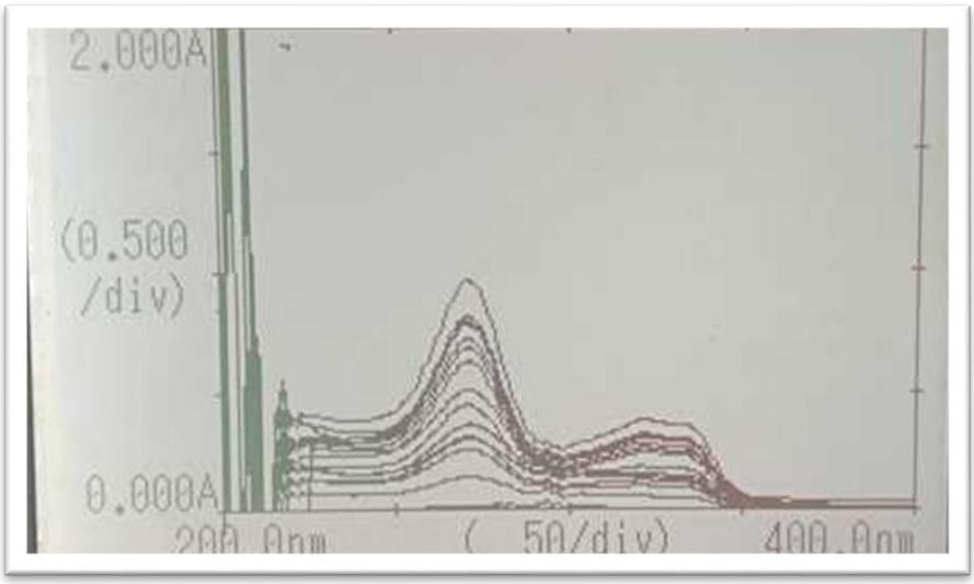


Figure 5.1: UV spectra of Ciprofloxacin hydrochloride.

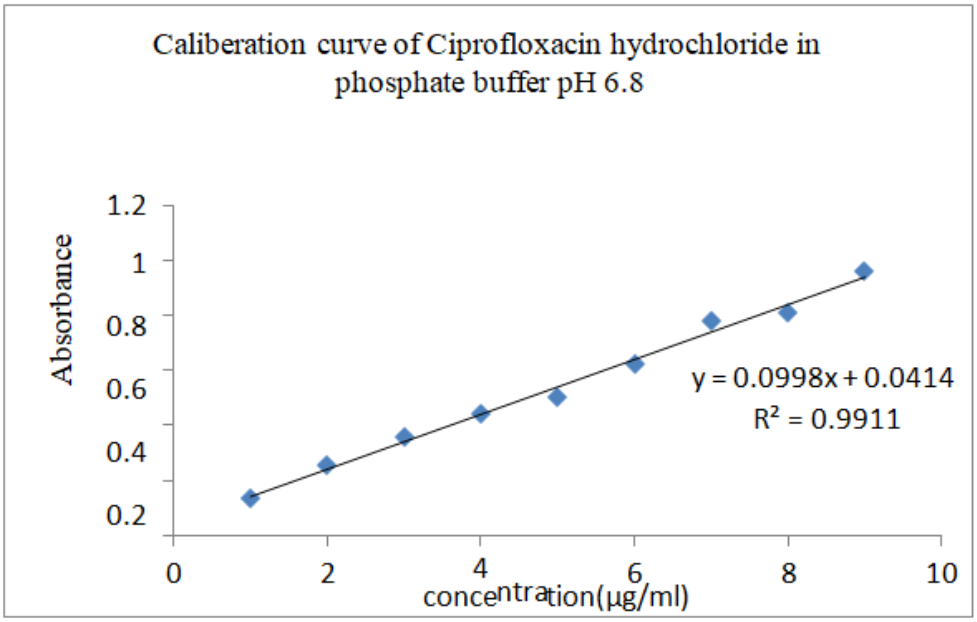
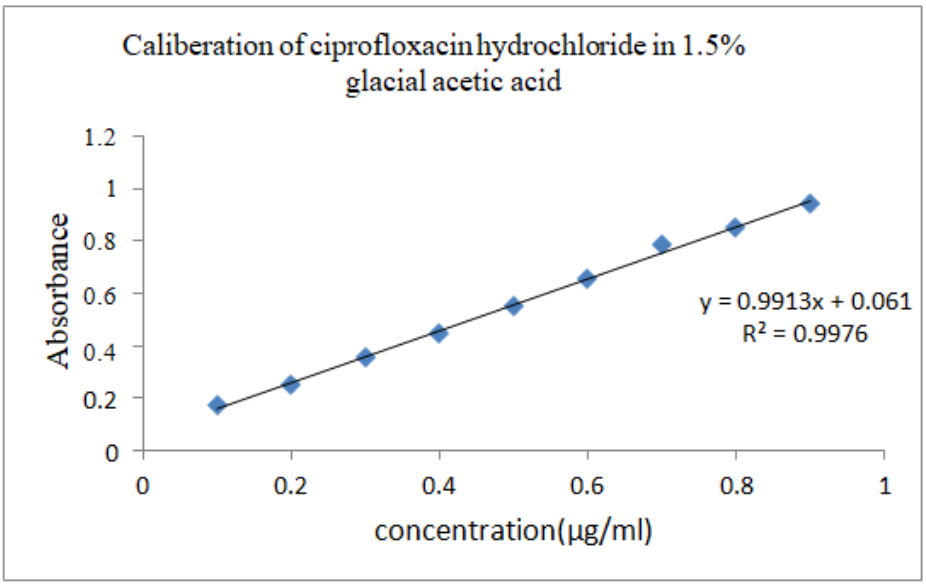


Figure 5.2: Calibration Curve of Ciprofloxacin hydrochloride in phosphate buffer pH 6.8.

Table 5.1: Calibration curve of ciprofloxacin hydrochloride in phosphate buffer pH6.8.

SR.NO	Concentration	Absorbance
1.	0.1	0.137
2.	0.2	0.255
3.	0.3	0.355
4.	0.4	0.441
5.	0.5	0.502
6.	0.6	0.623
7.	0.7	0.782
8.	0.8	0.809
9.	0.9	0.960

5.1.2 Caliberation of Ciprofloacin hydrochloride in 1.5% Glacial acetic acid:



6.1 Characterization of Nanoparticles:

6.1.1 Determination of %Drug loading:

The % drug loading in batches B1 to B9 was found to be:

Table 6.1: %drug loading in nanoparticle batch B1 to B9.

Batch	% Drug loading
B1	7.2±0.023
B2	4.49±0.0112
B3	4.52±0.03
B4	7.1±0.007
B5	4.34±0.102
B6	5.3±0.056
B7	4.5±1
B8	6.3±0.333
B9	6.9±0.023

6.1.2 Determination of %Entrapment efficiency

The % Drug entrapment efficiency of batches B1 to B9 is given in table below:

Table 6.2: % drug entrapment efficiency in nanoparticle batch B1 to B9.

Batch	% Drug entrapment efficiency
B1	68.5±0.03081
B2	62.9±0.01627
B3	70.9± 0.001574
B4	72.17± 0.01
B5	80.63± 0.02071
B6	71.48 ±0.023
B7	87.49 ±0.052203
B8	89.4± 0.03405
B9	94.9 ±0.025265

6.1.3 Determination of Particle size and Zeta potential:

The prepared nanoparticles were transferred to a disposable polystyrene cuvette with help of plastic syringe and Nano size of nanoparticles was determined via laser Doppler velocimetry and phase analysis light scattering at an angle of 90°. Before putting the fresh sample, cuvettes were washed with the methanol and rinsed using the sample to be measured before each experiment.

Necessary measurement condition:

- ✓ Temperature: 25°C
- ✓ Viscosity: 0.01 poise
- ✓ Scattering angle: 90°
- ✓ Run time: 30 sec

- **Particle size:** The particle size of Batch 9 was found to be lowest among the batches which is shown in figure below:

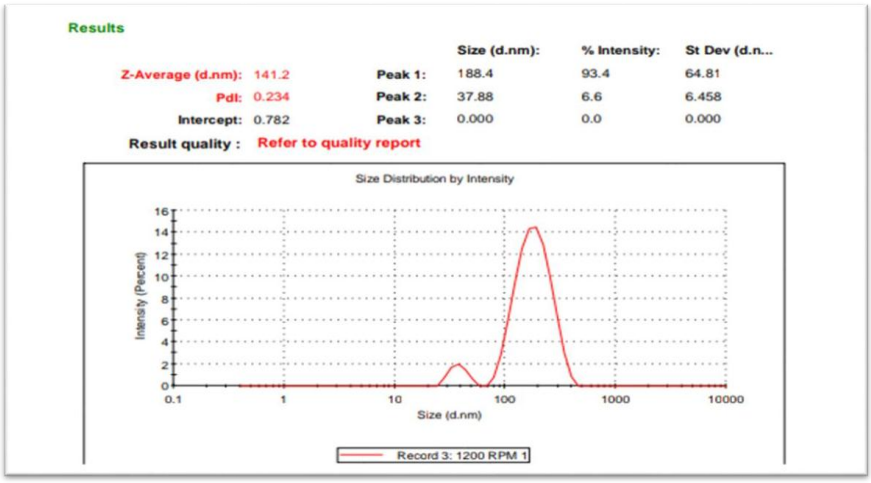


Figure 6.1: Particle size of Batch 9.

- **Zeta Potential:** The zeta potential of Batch 9 was also obtained which is shown in figure below:

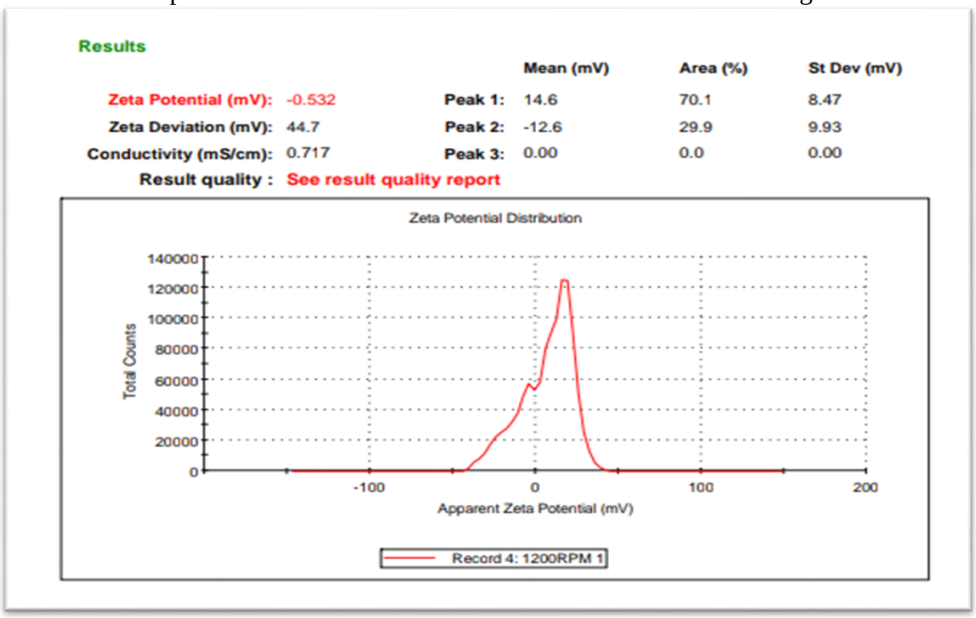


Figure 6.2: Zeta Potential of batch 9.

7.1 Scanning Electron Microscopy (SEM) study

Scanning electron micrographs of C_8 film of nanoparticle surface shows morphology and surface topography of prepared nanoparticle batch.

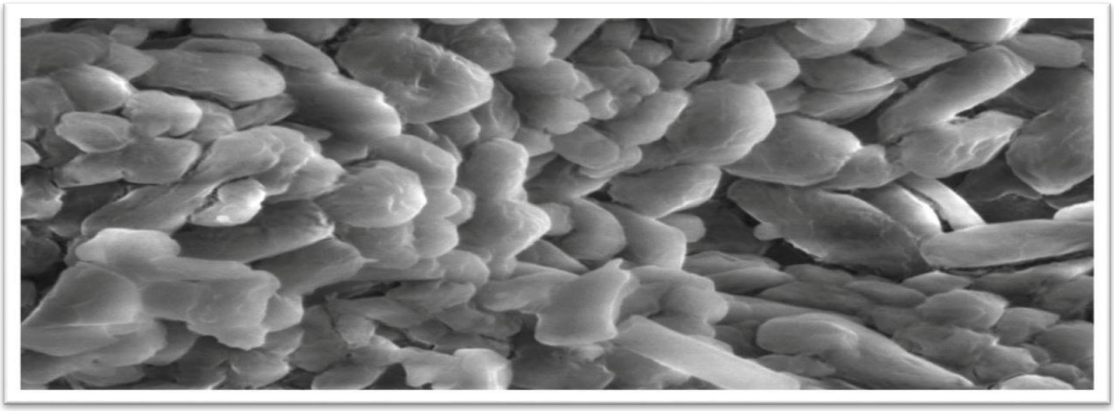


Figure 7.1: Scanning Electron Microscopy of nanoparticles.

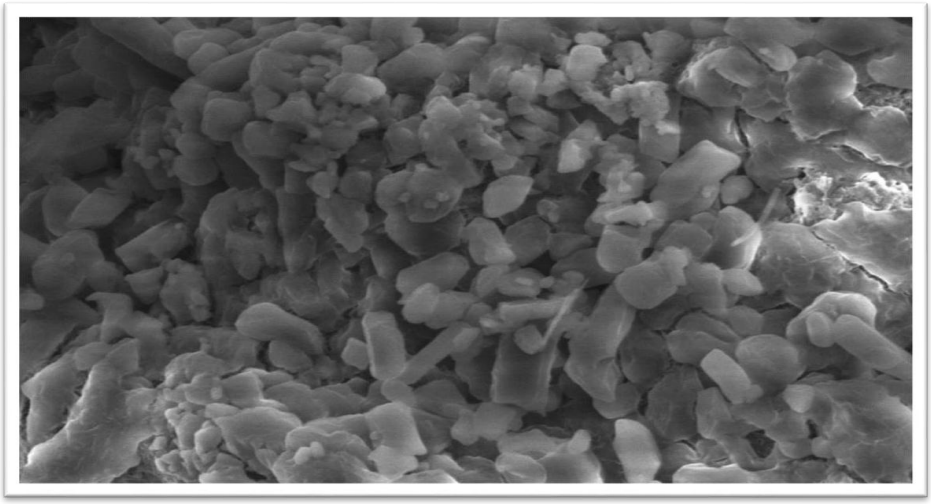


Figure 7.2: Scanning Electron Microscopy of nanoparticles.

8.1 Determination of In-vitro drug release study

8.8.1 Diffusion study of batch F1 to F9 in phosphate buffer pH 6.8

Medium: Phosphate buffer pH 6.8 Volume: 22ml in receptor compartment Temperature: 37± 0.5°C
RPM: 50

Table 8.1: Diffusion study of Batch B1 to B5 in phosphate buffer pH6.8 (%CDR).

Time (hours)	B1	B2	B3	B4	B5
0	0	0	0	0	0
24	16.49 ±0.181901	15.08 ±0.00688	12.36 ±0.02266	16.36 ±0.02266	17.36 ±0.03364
48	40.82± 2	46.16 ±0.0226	24.42 ±0.6774	30.70 ±0.4631	30.70 ±0.4633
72	78.05 ±0.004663	78.99 ±0.0126	78.97 ±0.00688	40.55± 0.022	49.55 ±0.03365
96	81.99 ±0.007874	83.53 ±0.00688	83.78± 0.493	60.96 ±0.00788	60.96 ±0.006785
120	84.38 ±0.023183	89.60 ±0.39977	87.66 ±0.0067	65.52 ±0.00788	65.55 ±0.006886
144	85.13 ±0.0217321	89.63 ±0.00688	89.66 ±0.00775	70.03± 0.033658	70.03 ±0.033658
168	86.32± 2	89.65 ±0.00589	89.77 ±0.006	78.79± 0.03997	78.79 ±0.03997

Table 8.2: Diffusion study of batch B6 to B9 in phosphate buffer pH6.8 (%CDR).

Time (hours)	B6	B7	B8	B9
0	0	0	0	0
24	13.64± 0.0294	14.79± 0.022457	14.43± 0.03305	14.51± 0.006885
48	21.50± 0.39499	31.55± 0.00775	43.55± 0.00688	35.66 ±0.005885
72	33.08 ±0.034095	44.36± 0.033085	66.51± 0.56786	76.89± 0.031148
96	38.21 ±0.036211	45.08± 0.84797	75.55± 0.78846	77.99± 0.04457
120	55.85 ±0.022785	52.70± 0.02365	80.72± 0.03365	83.42± 0.006784
144	72.89 ±0.036211	60.80± 0.03358	83.12± 0.0774	83.43± 0.0773
168	75.89 ±0.018421	73.88± 0.028411	84.32± 0.031146	85.77± 0.00781

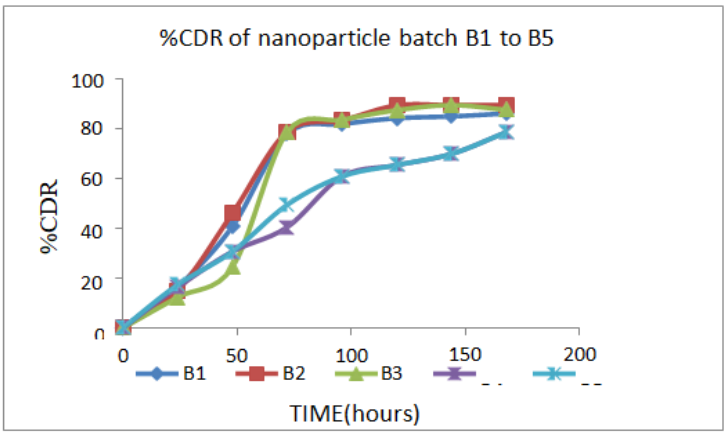


Figure 8.1: %CDR vs. Time in phosphate buffer pH 6.8 batches B1-B5.

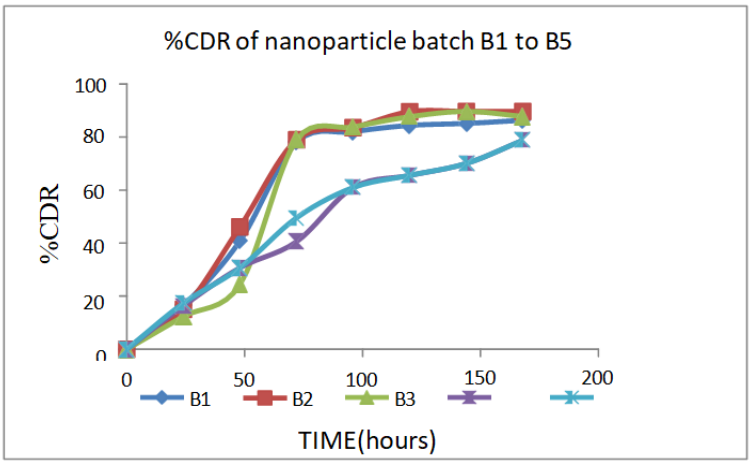


Figure 8.2: % CDR vs. Time in phosphate buffer pH 6.8 batches B6-B9.

9.1 Determination of In-vitro antimicrobial activity of nanoparticles

Table 9.1 MIC (µg/ml) of ciprofloxacin hydrochloride and nanoparticles in phosphate buffer pH6.8

Bacteria	MIC(µg/ml)	
	Ciprofloxacin hydrochloride solution	Nanoparticles
E.coli	1	0.12
S.aureus	0.25	0.2

Table 9.2: MIC (µg/ml) of ciprofloxacin hydrochloride and nanoparticles in 1.5%glacial acetic acid.

Bacteria	MIC(µg/ml)	
	Ciprofloxacin hydrochloride solution	Nanoparticles
E.coli	1	0.06
S.aureus	0.2	0.12

DISCUSSION

The solubility of Ciprofloxacin hydrochloride was performed by using different solvents. It was found that Ciprofloxacin hydrochloride showed good solubility in water and ethanol and poor solubility in isopropanol and acetone as per literature. The melting point of drug was found to be in the range 276-277°C. Figure 4.2 exhibits chemical interaction between drug and poymer were studied by FT-IR. No significant changes were observed in the IR spectra of the pure drug and drug- loaded nanoparticles. The peaks of the drug-Ciprofloxacin hydrochloride when compared with the formulation peak, showed that the drug and the excipients were compatible with each other due to almost same as –COOH stretch, -C==O vibration, C-H stretch, Aromatic C=C and C-F stretch were found to be near to each other and hence the formulation showed good compatibility between drug and excipient. The Figure 4.3 represents the endothermic and exothermic peak and enthalpies associated with each peak of pure drug- ciprofloxacin hydrochloride, physical mixture of drug and polymer and nanoparticles obtained by complexation of polyelectrolytes. Thermogram of pure drug was obtained as exothermic peak at 84.39°C and enothermic peak at 323.53°C. Endothermic peaks are correlated with loss of water associated to hydrophilic groups of polymers while exothermic peaks resulted from degrading polyelectrolytes due to degradation and depolymerisation reactions most probably to the partial decarboxylation of the protonated carboxylic group and oxidation reactions of polyelectrolytes. Thermogram of physical mixture show broad exothermic peak at 130.03°C. Peak of nanoparticles registered at 149.72°C, an intermediate and broader peak value compared with isolated polyelectrolyte which was interpreted as an interaction between both components. The λ_{max} of ciprofloxacin hydrochloride was obtained at 270 nm when scanned in ultraviolet spectrophotometer with phosphate buffer pH6.8 as reference standard. Regression co-efficient for the drug in phosphate buffer pH 6.8 were found to be near to one and in the linearity range, this standard concentration method obeys Beer's law and found to suitable for the determination of drug release study. Regression coefficient of drug was found to be in linearity range and the equation obtained as $y = m\,x + c$, is utilized for determining the percentage encapsulation efficiency and percentage drug loading of the optimized batches. % drug loading of nanoparticles is the first main indice for the characterization of nanoparticles. Batch B1 showed highest %drug loading among the 9 batches of formulation as 7.2±0.023%. Nanoparticles of ciprofloxacin hydrochloride were formulated by ionic gelation method followed by sonication. From the data in Table The %drug entrapment efficiency of nanoparticles is the second main indice in characterization of nanoparticles. Batch B9 had the % drug entrapment efficiency as 94.9 ±0.025265%. Batch B9 showed highest % Drug entrapment efficiency. The particle size of all 9 batches was found to be between 178.4 ± 119.36 to 141.2 ± 108.36. Batch 9 showed lowest particle size as 141.2 ± 108.36. The ability of nanoparticles to alter the bio-distribution and pharmacokinetics of drugs has importance in in-vivo therapeutic applications. The size of nanoparticles is of prime importance i.e. nanoparticles of particle size 141.2± 108.36 are easily captured by phagocytic cells that restrict their bio-distribution. Nanoparticles of this size have hydrophilic surfaces which have longer circulation in blood. Such systems prolong the duration of drug activity and also increases the targeting efficiencies to specific site. Batch 9 showed lowest particle size distribution. From above Figure6.2, Zeta potential of Batch 9 was found to be 44.7mV. It was also known that the zeta potential of nanoparticles was higher than the value of 30mV, implying the nanoparticles had good stability due to the nature of the potential value in the electrostatic repulsion between nanoparticles. So, this zeta potential value confirms the good stability of Batch 9**.** The SEM micrographs of Ciprofloxacin hydrochloride nanoparticles from above Figure 7.1 and 7.2, exhibited smooth surface of nanoparticles with spherical appearance and uniform particle size. The particle morphology of SEM image showed that each particle unit exhibited a nanostructure, less aggregation with uniform particle structure. The in-vitro drug diffusion study was carried out by Franz Diffusion Cell. The drug release profile from nanoparticle batch B1-B9 is shown in Figure 8.1 and Figure 8.2 and Table 8.1 and Table 8.2. The formulations showed good drug release from the polymer and the percentage of drug released after 168 hours i.e. 7days with Batch B3 with highest drug release. The in-vitro drug release profiles of all the formulations showed an initial burst effect followed by slow drug release and the remaining amount of drug was released in a

sustained manner over a period of 7 days. This release of drug was associated with those drug molecules dispersing close to the nanoparticle surface, which easily diffuse in initial incubation time. The chitosan nanoparticles were well distributed in bacterial suspension which showed a nice dispersion. Bacteria can adhere to the surface chitosan nanoparticles significantly in 30minutes and thus they exhibit antimicrobial activity. As chitosan possess antimicrobial activity against a number of gram negative and gram positive bacteria, the antimicrobial activity of nanoparticles were tested in phosphate buffer pH6.8 and 1.5% glacial acetic acid. According to this data, the antimicrobial activity of nanoparticles are higher than that of ciprofloxacin hydrochloride. Moreover, The MIC value of drug alone against bacteria is lower than those of drug loaded nanoparticles, which indicate higher antimicrobial activity.

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