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FABRICATION AND CHARACTERIZATION OF LEVOFLOXACIN LUNGS TARGETED DRUG DELIVERY BY THE WAY OF MICROSPHERES

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ABSTRACT

Objective: Pulmonary drug delivery systems offer many advantages over other conventional drug delivery system such as avoidance of first pass metabolism, large surface area suitable for drug absorption and quicker onset of pharmacological activity. One of the drug, Levofloxacin, if formulated into microspheres, could be trapped by filtration in the capillary bed of the lungs. The biodegradable and biocompatible polymer BSA was selected as matrix forming agent. **Method:** Biodegradable microspheres of levofloxacin has been prepared by emulsification heat stabilization technique using BSA as polymer and characterized by drug loading, size distribution, scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC), percentage entrapment efficiency, and in vitro release studies. The pharmacokinetics and targeting efficiency of microspheres was studied in vivo in albino rats. **Result and Discussion:** The microspheres showed drug loading (encapsulation) in the range of 11.00 to 21.10 % with an average size range of 12.33–18.73 µm, depending upon the drug–polymer ratio and emulsifier concentration. They were spherical in nature and free from surface drug as evidenced by the SEM photographs. FT-IR and DSC studies revealed the absence of drug–polymer interaction. In vitro drug release studies showed a biphasic release pattern for all formulations with an initial burst effect followed by slow release for almost 24 hours. The in vitro release profile of the microspheres matched the Korsmeyer's Peppas release pattern. Formulation F7 with good drug content and particle size less than 15 µm was selected for in vivo pharmacokinetic and drug targeting studies in comparison with conventional levofloxacin injection. The in vivo pharmacokinetic evaluation on albino rats demonstrated increased bioavailability with microsphere formulation in comparison to the traditional solution form. The significant increase in bioavailability shall enable one to reduce the frequency of levofloxacin administration and would effectively reduce the dose related side effects. The average targeting efficiency of drug loaded microspheres was found to be 53.23% of the injected dose in lungs, 37.96% in liver and 4.63% in spleen after 12 hours, whereas the concentration of drug from levofloxacin injection was 38.52% in lungs, 46.35% in liver, and 4.12% in spleen. These results revealed that the drug loaded microspheres showed preferential drug targeting to lungs followed by liver and spleen. Stability studies revealed that at 40±2ºC microspheres are stable after 90 days. Histopathological studies proved that the tissue compatibility of levofloxacin microspheres is safe. **Conclusion:** From the experimental data obtained from injectable microsphere was found to be an efficient tool in encapsulating levofloxacin with an excellent controlled release profile. This formulation could be an effective tool in targeting to lungs and helps in treating pneumonia.

KEYWORDS: Levofloxacin, Bovine serum albumin, injectable microspheres, pneumonia.

INTRODUCTION

Microspheres are characteristically free flowing powders consisting of proteins or synthetic polymers which are biodegradable in nature and ideally having a particle size less than 200 µm. A well designed controlled drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic efficacy of a given drug. There are various approaches in delivering a therapeutic substance to the target site in a controlled release fashion. One such approach is using microspheres as carriers for drugs. It is the reliable means to deliver the drug to the target site with specificity, if modified, and to maintain the desired concentration at the site of interest without untoward effects.

A well designed controlled drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic efficacy of a given drug. To obtain maximum therapeutic efficacy, it becomes

necessary to deliver the agent to the target tissue in the optimal amount in the right period of time there by causing little toxicity and minimal side effects.

The reason behind the development of controlled drug delivery systems is to make a therapeutic agents do its best when administered into the body. This means a high therapeutic efficacy with minimal toxicity successful application of many therapeutic agents are hampered by a multitude of problems. Drugs administered normally distribute throughout the body interacting not only with the target cells but also with the normal healthy cells which often results in toxic effects. Conventional therapy requires frequent administration of the therapeutic agent to the patient which reduces patient compliance. Systemic administration of the drug often requires high concentrations to maintain a therapeutic effect because of the dilution effect and the difficulty of drug placement in the target site. To obtain maximum therapeutic efficacy, it becomes necessary to deliver the agent to the target tissue in the optimal amount for the right period of time thereby causing little toxicity and minimal side effects. A well designed controlled drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic efficacy of a given drug.[1]

There are various approaches in delivering a therapeutic substance to the target site in a sustained or controlled release fashion. One such approach of using polymeric microspheres as carriers for drugs. Microspheres for biodegradable and non-biodegradable polymers have been investigated for sustained release depending on the final application. In the case of non-biodegradable drug carriers, when administered parentally, the carrier remaining in the body after the drug is completely released poses the possibility of carrier toxicity over a long period of time. Biodegradable carriers which degradable the body to non-toxic degradation products do not pose the problem of carrier toxicity and are more suited for parenteral application.[2]

MICROSPHERES AS DRUG CARRIERS

Microsphere based drug delivery have received considerable attention in recent years. It is the most important characteristic of microspheres of the microphase separation morphology which endows it with a controllable variability in degradation rate and also drug release.

Microspheres Based on Biodegradable Polymers

Biodegradable microspheres can be prepared from certain synthetic as well as natural polymers. An important requirement of such polymers is that the degradation products should be non-toxic because such products eventually enter circulation or result in tissue deposition. Long term toxicological evolution of the degradation products therefore is important in determining the clinical suitability of such carriers. Biodegradability carrier matrices can be designed to deliver the therapeutic agent for periods ranging from a few days to a few years.[3]

Natural polymer such as proteins and polysaccharides undergo enzymatic degradation in the body. Most synthetic biodegradable polymers contain hydrolysable linkages like amide, esters, ureas and urethanes. Polypeptides undergo enzymatic degradation while synthetic polyesters such as poly(lactic acid) and poly (glycolic acid) degrade by simple hydrolysis. Enzymes area also reported to exert influence on the degradation of synthetic polyesters.[4]

The preparation of microspheres from natural polymers involves three steps. First the solution of the polymer is disperse in a continuous medium such as vegetable oil or an organic solvent using a suitable agent. Dispersion is accomplished by stirring or by ultrasonication or by high speed homogenization depending on the particle size required. The second step involves the hardening of the polymer droplets either by heat denaturation (in the case of proteins) or by chemical cross-linking using suitable cross-linking agent. The third step involves separation of the solid microspheres formed, purification and drying.[5,6]

Chemical cross-linking of protein microspheres can be achieved using cross-linking agents such as formaldehyde, glutaraldehyde or by using diacid chlorides such a terephthalate chloride, the method is also limited to drugs that do not have any chemical interaction with the cross-linking agent. For example glutaraldehyde interacts with salbutamol and methotrexate and formaldehyde interacts with epinephrine.

Microspheres of synthetic polymers could be prepared either by the polymerization of the monomer using the techniques of suspension or dispersion polymerization or from the polymer using a suitable solvent evaporation technique.[7]

Methods used for preparation of microspheres

1. Solvent evaporation method, a) Single emulsion technique. b) Double emulsion technique.
2. Coacervation phase separation method.
3. Spray drying and spray congealing method.
4. Polymerization method.

General Methods of Preparation

Choice of the technique mainly depends on the nature of the polymer used, the drug, he intended use & the duration of therapy. These include in situ polymerization, solvent evaporation, Coacervation-phase separation, spray drying & spray congealing, etc.

1. Solvent Evaporation Method

a) Single Emulsion Technique

The microparticulate carriers of natural polymers, i.e. those of proteins & carbohydrates are prepared by single

emulsion technique. The natural polymers are dissolved/ dispersed in aqueous medium followed by dispersion in the non aqueous medium. Ex: oil. In the 2nd step, cross linking of the dispersed globule is carried out either by means of heat or by using chemical cross linkers. The chemical cross linking agents used –glutaraldehyde, formaldehyde, terephthalate chloride, diacidchloride, etc. Crosslinking by heat is effected by adding the dispersion to previously heated oil. Heat denaturation is not suitable for the thermolabile drugs while the chemical cross-linking suffers disadvantage of excessive exposure of active ingredient to chemicals if added at the time of preparation.[26]

Figure no. 1.1: Processing Scheme for Microspheres-preparation by Single Emulsion Technique.

b) Double Emulsion Technique

Involves the formation of the multiple emulsions or the double emulsion of type w/o/w & is best suited to the water soluble drugs, peptides, proteins & the vaccines. The aqueous protein solution is dispersed in a lipophilic organic continuous phase which is generally consisted of polymer solution that eventually encapsulates protein contained in dispersed aqueous phase. The primary emulsion is then subjected to the homogenization before addition to aqueous solution of PVA .this results in formation of double emulsion which is then subjected to solvent removal by solvent evaporation maintaining the emulsion at reduced pressure or by stirring so that organic phase evaporates out.[27] Examples: hydrophilic drugs like LHRH agonist, vaccines and proteins.

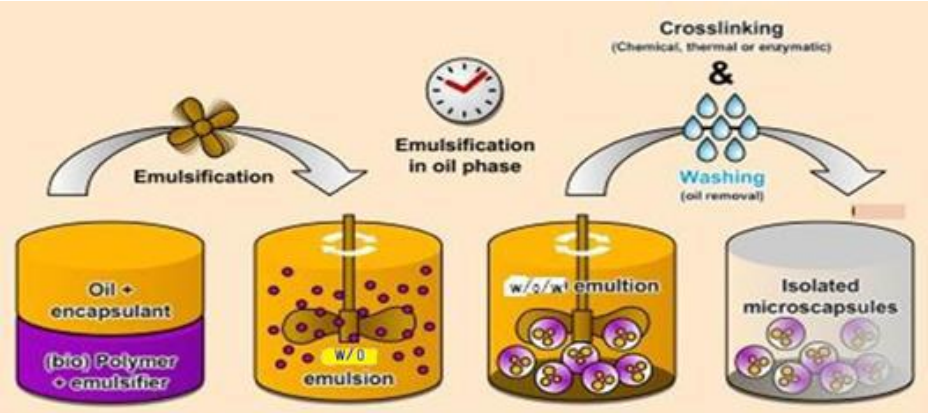


Figure 1.2: Processing Scheme for Microsphere-preparation by Double Emulsion Technique.

2. Coacervation phase separation method

Specially designed for preparing the reservoir type of the system, i.e., to encapsulate water soluble drugs e.g. peptides, proteins, matrix type particularly, when the drug is hydrophobic in nature e.g., steroids. In matrix type device, the drug or the protein is soluble in the polymer phase. The process is based on the principle of decreasing the solubility of the polymer in the organic phase to affect the formation of the polymer rich phase called the coacervates. The coacervation can be brought about by addition of the third component to the system which results in the formation of the two phases, one i.e. supernatant, depleted of the polymer. In this technique, the polymer is first dissolved in a suitable solvent & then drug is dispersed by making its aqueous solution, if hydrophilic or dissolved in the polymer solution itself, if hydrophobic. Phase separation is then accomplished by changing the solution conditions.[28]

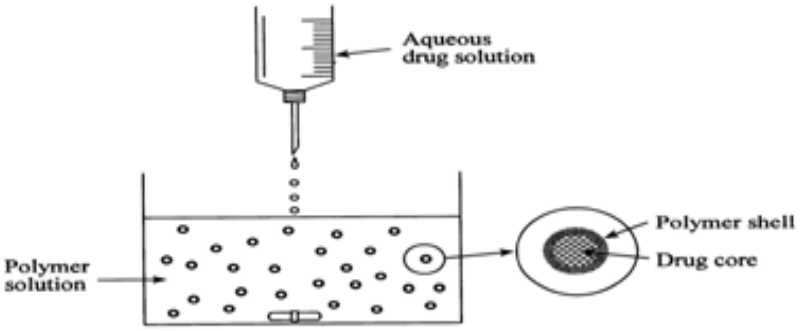


Figure 1.3: Coacervation Phase Separation Method.

3. Spray drying and spray congealing

These methods are based on the drying of the mist of the polymer and drug in the air. Depending upon the removal of the solvent or cooling of the solution, the two processes are named spray drying and spray congealing respectively. The polymer is first dissolved in a suitable volatile organic solvent such as dichloromethane, acetone, etc. The drug in the solid form is then dispersed in the polymer solution under high speed homogenization. This dispersion is then atomized in a stream of hot air. The atomization leads to the formation of the small droplets or the fine mist from which the solvent evaporates instantaneously leading the formation of the microspheres in a size range 1-100 µm. Microparticles are separated from the hot air by means of the cyclone separator while the traces of solvent are removed by vacuum drying. One of the major advantages of the process is feasibility of operation under aseptic conditions. The spray drying process is used to encapsulate various penicillins. Thiamine mononitrate and sulpha ethylthiadizole are encapsulated in a mixture of mono- and diglycerides of stearic acid and palmitic acid using spray congealing. Very rapid solvent evaporation, however leads to the formation of porous microparticles.[29]

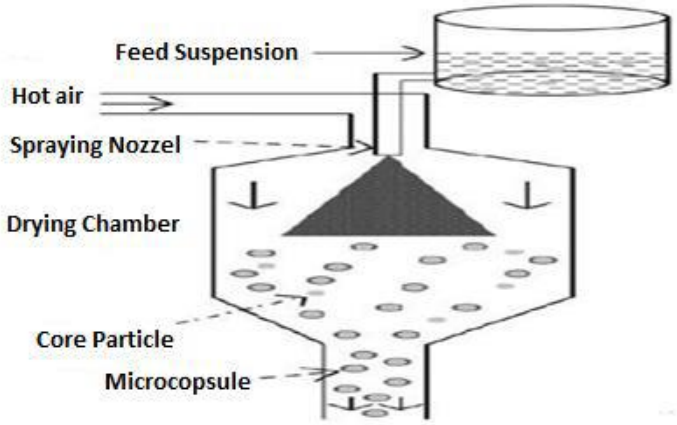


Figure 1.4: Spray Drying and Spray Congealing.

4. Polymerization method

I. Normal polymerization

II. Interfacial polymerization.

NORMAL POLYMERIZATION

Proceeds using techniques like bulk, suspension precipitation, emulsion & micellar polymerization processes. In bulk polymerization, a monomer along with initiator is heated to initiate polymerization. Initiator is added to accelerate the rate of reaction. Drug is added during process of polymerization. The polymer so obtained is fragmented to microspheres.

ADVANTAGE- Formation of the pure polymer,

DISADVANTAGE- Very difficult to dissipate the heat of reaction, which can adversely affect the thermolabile active ingredients.[30]

SUSPENSION POLYMERIZATION

Suspension polymerization is also called as bead/pearl polymerization. It is carried out by heating the monomer or mixture of monomers with active principles (drug) as droplets dispersion in a continuous phase. The droplets may also contain an initiator & other additives.

The emulsion polymerization differs from the suspension polymerization as due to presence of initiator in the aqueous phase, which later on diffuses to the surface of the micelles or the emulsion globules.

The suspension & emulsion polymerization can be carried out at lower temperature, since continuous external phase is normally water through which heat can easily dissipate. The 2 processes also lead to the formation of higher mol. wt polymer at relatively faster rate.

Major disadvantage of suspension & emulsion polymerization - association of polymer with the unreacted monomer & other additives.[30]

INTERFACIAL POLYMERIZATION

Involves reaction of various monomers at the interface between the 2 immiscible liquid phases to form a film of polymer that essentially envelopes the dispersed phase. In this 2 reacting monomers are employed one of which is dissolved in the continuous phase while the other being dispersed in the continuous phase. Monomer present in either phases diffuse rapidly 7 polymerize rapidly at the interface. If the polymer is soluble in the droplet it will lead to the formation of monolithic type of the carrier on the other hand if polymer is insoluble in the monomer droplet, the formed carrier is of capsular (reservoir) type. The degree of polymerization can be controlled by the reactivity of monomer chosen, their concentration, and the composition of the vehicle of either phases & by the temperature of the system. The particle size can be controlled by controlling the droplets or globule size of the disperse phase. The polymerization reaction can be controlled by maintaining the concentration of the monomers, which can be achieved by the addition of an excess of the continuous phase.[31]

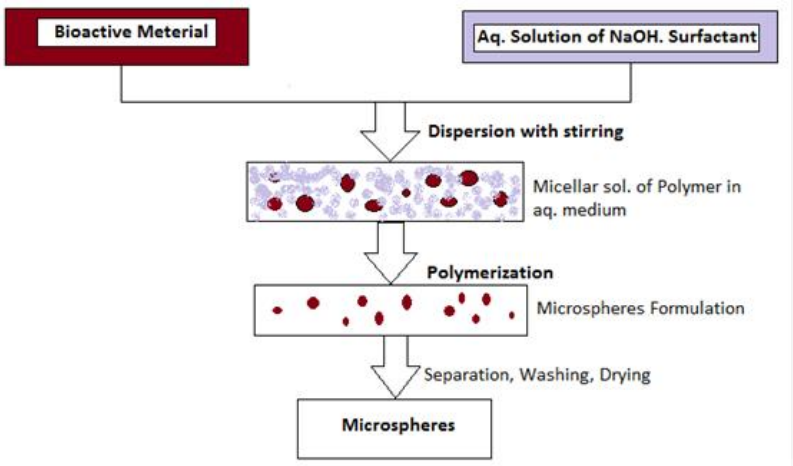


Figure 1.5: Polymerization Method.

Drug Loading and Drug Release Kinetics

The active components are loaded over the microspheres principally using two methods, i.e. during the preparation of the microspheres or after the formation of the microspheres by incubating them with the drug/protein. The active component can be loaded by means of the physical entrapment, chemical linkage and surface adsorption. The entrapment largely depends on the method of preparation and nature of the drug or polymer (monomer if used). Maximum loading can be achieved by incorporating the drug during the time of preparation but it may get affected by many other process variables such as method of preparation, presence of additives (e.g. cross linking agent, surfactant stabilizers, etc.) heat of polymerization, agitation intensity, etc. Release of the active constituent is an important consideration in case of microspheres. The release profile from the microspheres depends on the nature of the polymer used in the preparation as well as on the nature of the active drug.

The release of drug from both biodegradable as well as non-biodegradable microspheres is influenced by structure or micro-morphology of the carrier and the properties of the polymer itself. The drugs could be released through the microspheres by any of the three methods, first is the osmotically driven burst mechanism, second by pore diffusion mechanism, and third by erosion or the degradation of the polymer. In osmotically driven burst mechanism, water diffuse into the core through biodegradable or non-biodegradable coating, creating sufficient pressure that ruptures the membrane. The burst effect is mainly controlled by three factors the macromolecule/polymer ratio, particle size of the dispersed macromolecule and the particle size of the microspheres. The pore diffusion method is named so because as penetrating water front continue to diffuse towards the core. The polymer erosion, i.e. loss of polymer is accompanied by accumulation of the monomer in the release medium. The erosion of the polymer begins with the changes in the microstructure of the carrier as water penetrates within it leading to the plasticization of the matrix.

Drug release from the non-biodegradable type of polymers can be understood by considering the geometry of the carrier. The geometry of the carrier, i.e. whether it is reservoir type where the drug is present as core, or matrix type in which drug is dispersed throughout the carrier, governs overall release profile of the drug or active ingredients.[32]

Method of Drug Incorporation

Drugs are incorporated into the microspheres either during their synthesis or after the microspheres are formed. High loading can be achieved by *in situ* loading if the drug is insoluble in the dispersion medium employed for microsphere stabilization. Washing the microspheres after their preparation to remove surfactants, oils, other impurities etc., using solvents in which the drug solubility is high may result in poor loading efficiency. *In situ* loading cannot be employed for drugs which are affected by temperature of microsphere preparation, solvents employed, cross-linked agents etc. if the drug is heat sensitive, the resulting drug-loaded microspheres cannot be sterilized by heat. Loading into preformed microspheres incorporated. The microsphere is swollen in a suitable solvent containing high concentration of the drug. The drug molecules diffuse into the matrix which is then dried to obtain drug-loaded microspheres. The method is best suited for cross-linked microspheres which do not dissolve but only swell when equilibrated in a suitable solvent. A high payload of water soluble drugs can be obtained if the microspheres are hydrophilic and swells to a high degree in aqueous solution. The method allows the most native form of the drug to be incorporated into the matrix.[9]

Drug-polymer binding

The binding force that holds the drug to the microsphere matrix can be physical or chemical. In addition to this, hydrophobic and electrostatic interaction may also exist. Depending on the force of attachment, drug release from the matrix also varies. The drug release is expected to be faster if only physical entrapment is achieved. Drug release in such cases is modulated by a diffusion controlled mechanism.

Slow release can be achieved by chemically binding the drug to the microsphere matrix. The polymer matrix should have reactive functionalities to which the drug can be bound through functionality available on the drug.[10]

Drugs can be either attached onto the preformed microsphere or a polymer-drug conjugate prepared by attaching the drug to the polymer chain can be further into the microsphere form. The linkage should be susceptible to degradation in the physiological environment so that the drug is released from the microsphere matrix. High loading is possible if sufficient functionalities to which drug can be bound exist on the polymer matrix. The drug release in this case will depend on the rate of cleavage of the bond linking the drug to the matrix.[11]

Route of Administration

Microspheres can be used for the delivery of drugs via different routes. Route of administration is selected depending on the drug properties, disease state being treated and the age and condition of the patient. Desirable properties of the microspheres to be used for the delivery will also change depending on the route of administration.

(a) Oral delivery

Oral delivery is the simplest way of drug administration. In oral drug delivery, the microspheres have to pass through frequently changing environment in the GI tract. There is also patient to patient variation in GI content, stomach emptying time and peristaltic activity. Although constants of the oral route are numerous, on the whole, it offers less potential danger than the parenteral route. The relatively brief transit time of about 12 h through the GI tract limits the duration of action that can be expected via the oral route. Bioavailability of drugs with limited solubility in the stomach or intestine and small absorption rate constant can be increased by increasing the retention time in the stomach.[12]

(b) Parenteral Delivery

Most of the microsphere base controlled delivery systems are developed with the aim of using them for parenteral administration. Drug is completely absorbed in this case. Microspheres used for parenteral delivery should be sterile and should be dispersible in a suitable vehicle for injection. Hydrophilic microspheres have the potential advantage of aqueous dispersibility as opposed to hydrophobic microspheres for reconstituting them for injection. Surfactants in small concentrations are often necessary for reconstituting hydrophobic particles for injection in aqueous vehicle which are reported to cause adverse tissue reaction and affect the release of the incorporated drug.[13]

Fate of microspheres in the body

Knowledge of the fate of microspheres after parenteral administration is very important in designing a drug delivery system. The biological fate of the administered particles has been studied by radiolabelled techniques. 14C, 131I, 125I and 9Tc have been used for labeling. Another method for the estimation of microspheres administered into the body is by using magnetite. Magnetite eatimation has the advantage that it can be easily incorporated into the microsphere matrix by physical entrapment without altering the chemical nature of the matrix. The recovery of the microspheres from the target organs can be achieved with the aid of an atomic absorption spectrophotometer.

MECHANISM OF DRUG RELEASE

Theoretically, the release of drugs from biodegradable microspheres can be classified broadly into four different categories. But in actual practice, the mechanism is more complex and an interplay of different mechanisms may operate.[14]

Degradation Controlled Monolithic System

In degradation controlled monolithic microsphere systems, the drug is dissolved in the matrix and is distributed uniformly throughout. The drug is strongly to

the matrix and is released only on degradation of the matrix. The diffusion of the drug is slow compared with the degradation of the matrix. When degradation is by homogeneous bulk mechanism, drug release is slow initially and increases rapidly when rapid bulk degradation starts. Drug release from such type of devices is independent of the geometry of the device.

Release from a sphere is governed by the equation, where Mt is the amount of the agent released at time t, M∞ is the amount at time t∞ is the time for total erosion. Progesterone release from poly (glycolic-co-lactic acid) polymer films containing 10 weight % steroids is an example of this type of release. $Mt /M\infty = 1-[(1-t/ t\infty)]^3$

Diffusion Controlled Monolithic System

Here the active agent is released by diffusion prior to or concurrent with the degradation of the polymer matrix. Degeneration of the polymer matrix affects the rate of release and has to be taken into account. Rate of release also depends on whether the polymer degrades by homogeneous or heterogeneous mechanism.

Diffusion Controlled Reservoir Systems

Here the active agent is encapsulated by a rare controlling membrane through which the agent diffuses and the membrane erodes only after its delivery is completed. In this case, drug release is unaffected by the degradation of the matrix. Polymer that remains as such till the complete, release of drug and then degrades by homogenous mechanism so that the device is removed from the body is better for this type of delivery.

TARGETING OF MICROSPHERES

Targeting is achieved by exploiting the natural pattern of a drug carrier called passive targeting i.e. by changing the natural pattern of the carrier by some means thereby directing the drug to the specific organ or tissue. This is called active targeting.

Passive Targeting

Particles administered into the body intravenously will distribute itself in different organs depending on the size of the particles. The administered particles pass through the heart with little or no uptake to the lungs where particles > 7 µm get entrapped in the capillary beds. Particles < 7µm enter into the systemic circulation.[15]

Active targeting

Active targeting includes coating the microspheres with hydrophilic coating agents which suppresses opsonisation, with colloidal particles are administered into the blood streams, they may be coated with proteins such as albumin, globulin etc., depending on the nature of the material, surface charge and hydrophilicity of the particles. This is called opsonization. By coating the particles with certain polymers like poloxamer, opzonization and removal of particles by macrophages can be reduced. It is thus possible to direct particles within the body to sites such as the lung, the liver, the bone marrow or to retain them for longer periods within the systemic circulation.[16]

Targeting Using Magnetic Microspheres

Another approach in this area is by using magnetic microspheres. In this method magnetic loaded microspheres is infused into an artery supplying a given target site. A magnet is placed externally over the target area which restricts the microsphere to that area.[17]

Intracellular Targeting

Certain cytotoxic drugs are active intracellularly, but are normally discarded due to their poor intracellular influx. Intracellular pathogens are usually protected from the immune system and the chemotherapeutic agents. The poor efficacy of many therapeutic substances for intracellular bacterial and parasitic therapy is well know. Commonly phagocytic cells are the sites of intracellular infection. Intracellular delivery of drugs by suitable means can obviate these problems.[18] Albumin microspheres were avidly taken up by macrophages. They have also observed that biologically active streptomycin was released from albumin microspheres inside the phagocytic cells after ingestion and intracellular degradation of microspheres.

MICROSPHERES BASED ON NATURAL POLYMERS

Protein and polysaccharides have been extensively investigated for targeted drug delivery. Natural polymers have the advantage that they pose less toxicity problems of their own. Majority of the natural polymers are susceptible to biodegradation and are generally biocompatible. A major problem with biopolymers is the presence of antigenic determinants in them. Biopolymers also differ in their molecular weight and their physical and chemical properties to varying extents depending on the source and method of isolation and purification.

Albumin Microspheres

Proteins have attained considerable attention as drug carriers because of the selective uptake in tumor cells coupled with known liposomal active of many cells which is necessary for the breakdown of the polymer-drug complex. Moreover, the large m=number of functional groups present in proteins offer sites for attachment of the drug. Proteins can be combined with selection of drugs to generate derivatives whose properties are often significantly different from the free drug.

Albumin microspheres received considerable attention because of their specific organ targeting property, biocompatibility and non-antigenicity in the cross-linked or heat denatured form. The preparation procedure involves dispersion of the aqueous albumin solution in a suitable medium, solidification of the dispersed phase and separation of the micro-particles formed.

Drug release from albumin microspheres can be controlled by changing their cross-linking density and the drug albumin ratio. But high cross-linking retards the rate of degradation. Drug to albumin ratio has its limiting factors such as solubility of the drug in albumin solution and the burst effect that occurs at high drug payloads. This can be chemically attaching the drug to the protein. Since there are many functionalities in the protein to which the drug can be anchored, high payloads can he obtained by chemically binding the drug to the matrix.

Albumin binds many drugs strongly and this binding would drug retard drug release from an injection site until the microspheres are degraded by proteolytic enzymes. Albumin microspheres of suitable size have been found to be useful for localized drug delivery. Albumin microspheres have also been investigated for oral drug delivery. Retention of the microsperes in the stomach can be improved by mixing microspheres with a bioadhesive polymer.[19]

Casein Microspheres

Serum albumin microspheres are prepared from albumin separated from outdated human blood. With the prevalence of AIDS, the risk of viral contamination in blood used for the preparation of albumin is a possibility. Recently there has been considerable interest in the milk protein casein as a carrier for drugs. The biodegradation rate of albumin and casein microspheres by radiolabelled techniques and found that casein system degraded slowly compared to albumin.[20]

Gelatin Microspheres

It is nontoxic, biocompatible and biodegradable. First order release was obtained for gelatin microspheres while zero order release was obtained from ethyl cellulose coated microspheres. Gelatin nanoparticles are reported to be taken up by some tumour cells which do not take albumin nanoparticles.[21]

Polysaccharide Microspheres

Biocompatibility and biodegradability of many naturally occurring polysaccharides make them useful as drug carriers. Chitin, starch, dextran etc., are some polysaccharides used in drug delivery applications. Many investigations have been reported on the use of chitin, chitosan and their derivatives as drug carriers for targeted, controlled or sustained delivery of drugs. It has been demonstrated that partially deacetylated chitin is a substrate for lysozyne.

Partially deacetylated chitin and its glutaraldehyde cross-linked hydrogels are degraded by lysozyme but the degradation rate is reported to be rather slow. There are many reports on the use of chitosan as a drug carrier for oral and parenteral formulations. The release of drugs from the microsphere matrix was a function of the cross-linking density of the microspheres, drug payload, particle size and aqueous solubility of the drug. Chitosan microspheres prepared by the glutaraldehyde cross-linking of an aqueous acetic acid dispersion of chitosan have loaded with proteins such as bovine serum albumin (BSA) and the release was examined in vitro.

Implantation of the placebo chitosan microspheres in the gluteal muscle of rats has shown that the glutaraldehyde cross-linked microspheres were well tolerated by the tissue and were not degraded completely even after six months. The degradation appeared to be mostly due to surface erosion. These studies have demonstrated the potential of cross-linked chitosan microspheres as a potential drug carrier for the prolonged delivery of drugs and macromolecules such as polypeptides.

Starch microspheres have also have been investigated for drug delivery. Starch is one of the most abundant biopolymers consisting of glucopyranose units and hydrolyzes completely to yield D-glucose. Because of the presence of free OH groups in starch, it is susceptible to derivatization with a number of reagents. Due to their biocompatibility and biodegradability, starch microspheres of size 15-80 μm diameter were used for the temporary embolization to improve regional drug delivery.

Dextran also attracted attention as a drug carrier because of it biocompatibility and biodegradability. The polymer is also susceptible to chemical transformations using a variety of regents. Carboxylated or sulphonated polymer can be prepared by carboxymethylation or sulphopropylation. This acts as cation exchange drug carrier. Anionic group of carboxylated or sulphonated dextran interacts with basic group of drug such as adriamycin to form ionic salt complex. This salt exchange the free drug for other cations.[22]

Chitosan Microspheres

Chitosan capsules for colon-specific delivery to treat ulcerative colitis. A 5-amino salicylic acid was encapsulated into Chitosan capsules and delivered in vivo to Male Wistar rats after induction of colitis. It was observed that Chitosan capsules disintegrated specifically in the large intestines as compared to the control formulation (in absence of Chitosan), which demonstrated absorption of the drug in small intestines. This data is a representative example of utility of Chitosan for colon-specific delivery.[23]

MICROSPHERES OF SYNTHETIC BIODEGRADABLE POLYMERS

Synthetic polymers have the advantage that can be easily and reproducibly prepared. They can be copolymerized with one another to alter their physical, chemical and mechanical properties and can be prepared as low or high molecular weight materials by suitable reaction conditions. Chemical bonds which are susceptible to degradation include amides, esters, ortho esters, acetals, glycosides and related groups.

Biodegradability of the polymer depends on many factors such as polymer structure, molecular weight, the physical form of the implanted material and the environment in which the polymer is placed. Since many proteolytic enzymes specifically catalyse the hydrolysis of peptide linkage adjacent to substituents in proteins, substituted polymers containing benzyl, hydroxyl, carboxymethyl and phenyl groups have been prepared to improve biodegradability.

Polyester microspheres

Aliphatic polyesters have been extensively investigated as drug carriers. Degradation mechanism for all polyesters appears to e due to homogeneous erosion by random hydrolytic chain scission. Poly (lactic acid), poly(glycolic acid) and their copolymers have been extensively studied as drug carriers. The most important property of these polymers which makes them attractive as degradable drug carriers is that they form endogenous metabolites lactic and glycolic acids on biodegradation. Effect of enzymes on the biodegradation of these polymers is a subject of controversy. They possess excellent tissue compatibility. The polymers have prepared in microspherical form for use as drug carriers by the solvent evaporation techniques. Methylene chloride is the solvent usually employed for dissolving the polymer. If the drug incorporated is insoluble in the solvent used for dissolving the polymer, drug is distributed as discrete particles through the microsphere. If the drug is completely soluble, it could crystallize as the solvent evaporates to give discrete drug-rich domains scattered throughout the microsphere matrix or should remain molecularly dispersed in the matrix and not crystallize. Low temperature phase separation has been another method used for the preparation of polylactide microspheres. Drugs particles are dispersed in the polymer solution and a non-solvent is added to desolvate the polymer and the microspheres formed are separated. The lactide and glycolide polymer induce minimal inflammatory response when introduced into the body. The degradation products are removed by body constituents. The rate of degradation of copolymers depend on the ratio of poly(Lactic acid) to poly(glycolic acid) in the copolymer. The polymers in their microspherical form have been studied as carriers for the prolonged delivery of a number of drugs and as an embolization agent.[24]

Polyanhydride microspheres

Polyanhydrides have received increasing attention in recent years because they are biocompatible and non toxic. The easily hydrolysable linkage makes it possible to prepare anhydrides with wide range of degradation rates by changing the chemical structure of the back bone. Highly hydrophobic polyanhydrides degrade by surface erosion. Degradation rate was found to change with the length of the alkyl group in the polymer backbone degradation rate can also be varied by copolymerizing with sebacic acid. By changing the composition of the copolymer a wide range of degradation rate can be obtained. But the presence of hydrophilic matrix causes the release of drug by diffusion and a good correlation between drug release and polymer degradation is not obtained. Because the anhydride bond is highly sensitive to water, normal method of microsphere preparation like solvent evaporation cannot be used which requires an aqueous phase. Microspheres are made from polyanhydrides by interfacial condensation and by hot melt technique. Microspheres made by polycondensation method were found to e irregular in shape and had a rough surface. Release was found to be fast and polymer degradation always lagged behind the drug release.[25]

Applications[33]

- Assay - Coated microspheres provide measuring tool in biology and drug research.
- Buoyancy - Hollow microspheres are used to decrease material density in plastics (glass and polymer).
- Ceramics - Used to create porous ceramics used for filters (microspheres melt out during firing, Polyethylene Microspheres)
- Cosmetics - Opaque microspheres used to hide wrinkles and give color, Clear microspheres provide "smooth ball bearing" texture during application (Polyethylene Microspheres).
- Drug Delivery - Miniature time release drug capsule (polymer).
- Electronic paper - Dual Functional microspheres used in Gyricon electronic paper.
- Personal Care - Added to Scrubs as an exfoliating agent (Polyethylene Microspheres).
- Spacers - Used in LCD screens to provide a precision spacing between glass panels (glass).
- Standards – Monodisperse microspheres are used to calibrate particle sieves, and particle counting apparatus.
- Retroreflective - Added on top of paint used on roads and signs to increase night visibility of road stripes and signs (glass).
- Thickening Agent - Added to paints and epoxies to modify viscosity and buoyancy.

AIMS AND OBJECTIVES

Fabrication and characterization of Levofloxacin lungs targeted drug delivery by the way of microspheres

Since Conventional drug delivery of Levofloxacin in the treatment of Pneumonia is unsatisfactory, it is planned to modify the drug delivery of the same drug in same disease by preparing its microspare and accordingly study the efficacy of levofloxacin in the case of Pneumonia comparatively.

Levofloxacin, a fluoroquinolone anti-infective, optically active L-isomer of ofloxacin and two fold more potent than ofloxacin and reported to be more effective in the treatment of various bacterial infection.

Levofloxacin is available in the market as a conventional dosage forms such as tablets, modified release tablets and parenteral (i.v. infusion) having the dose of 250mg to 750mg / 250mg twice a day or 500mg once a day, and it is having half life of 6-8 hours.

Hence, passive targeting of drug to lungs by the way of microsphere by increasing the quantum of drug reaching the site and simultaneously decrease the amount being distributed to other parts of the body and protected from adverse reaction due to repeated dosing or high dosing.

In the present investigation, an attempt has been made to prepare microsphere of levofloxacin using natural, biodegradable and biocompatible polymer in order to release the drug at a controlled rate into target site.

The efficacy of a drug in a specific application requires the maintenance of appropriate drug blood level concentration during a prolonged period of time. However the conventional administration of drugs, give a poor control of the concentration of these substance in the plasma because of variation in the concentration. In many cases, conventional drug delivery provides sharp increase in drug concentration often achieving toxic level and following a relatively short period at the therapeutic level of the drug concentration eventually drops off until re-administration. These difficulties have been called for the development of new administration technique for bioactive compounds, directed towards attaining the steady state plasma concentration.

Microspheres are solid colloidal particle ranging in the size from 1- 1000µm, particles with size 7-15 µm when given through intravenous route are trapped in the capillary bed of the lung. Exploring alternative route of administration to develop as targeted drug delivery system to act locally on organ of infection will improve therapeutic efficacy, reduced side effects and there by provide patience compliance.

WORK PLAN AND METHODOLOGY

1. Review of literature related to drug profile, excipient profile and formulation development.

2. Procurement, characterization and identification of pure drug.

a) UV
b) IR spectroscopy
c) DSC
d) HPLC

3. Preparation of different formulation.

4. Optimization of formulation.

❖ Formulation shall be optimized on the basis of rotation speed, polymer concentration and surfactant concentration.

5. Preparation of different batches of optimized formulation.
6. Evaluation of formulation for their yield, particle size, particle surface morphology, entrapment efficiency, *in-vitro* release, release kinetics and stability studies.
7. To improve the hypothesis of targeted drug delivery of microspheres to lungs by studying the *in-vivo* tissue distribution in comparison to conventional dosage form of drug in an animal model.
8. Finally to improve the safety of the formulation by carrying out histopathological studies.

REVIEW OF LITERATURE

PAST WORK DONE ON LEVOFLOXACIN

➢ **Shen Hong-Liang., et al.**[34]

Prepared levofloxacin sustained-release microsphere with carboxymethyl chitosan by implementing emulsification and cross linking process. The prepared formulations were evaluated for the various parameters like particle size, surface characteristics, encapsulation efficiency and drug release pattern. The results of the drug release study showed that the appreciable sustained release of the drug.

➢ **Thakkar VT., et al.**[35]

Prepared levofloxacin hemihydrates floating tablets by direct compression method using Gelucire 43/01 (hydrophobic) and hydroxy propyl methyl cellulose (hydrophilic) polymer in different ratios. The floating tablets were evaluated for uniformity of weight, hardness, friability, drug content, *in vitro* buoyancy and *in vitro* release studies. Various models were used to estimate kinetics of drug release. *In vitro* release study reveals that the release rate of drug was decreased by increasing the proportion of Gelucire 43/01, 5 to 40%. The release rate of levofloxacin hemihydrates from matrices was mainly controlled by the hydrophilic and hydrophobic polymer ratio.

➢ **Shen Hongliang, et al.**[36]

Prepared microspheres of levofloxacin-chitosan for the treatment of the soft tissue wound healing. The prepared microspheres were evaluated for the sustained release drug effect for the treatment of the wound healing. The prepared microspheres were evaluated for the drug release pattern. The results of the study indicated that the microspheres of the levofloxacin prepared with chitosan gave the sustained and local effect at the specific site of action.[16]

➢ **Kavitha K, Rajas NJ.**[37]

Prepared sustained release *In situ* ocular gels of levofloxacin hemihydrates using gelrite as gel forming polymer, which is used in treatment of various bacterial infections. Formulations were evaluated for physical parameters like clarity, pH, drug content, gelation, rheological studies, sterility test, *in vitro* drug release and ocular irritancy studies. The formulated gels were transparent, uniform in consistency and had spreadability

with a pH range of 7.1 to 7.4. Six different formulations with increasing polymer concentrations were prepared which was found to have drug content of 72-86%. From the preliminary studies it was observed that as the concentration of polymer was increased, the rate of drug release decreased to produce sustained drug delivery for prolonged period of more than 8 hours. A maximum of 90.2% drug release was observed. Further *in vivo* results conclude that it is be possible to formulate *in situ* ocular gel containing levofloxacin hemihydrate.

- **Hemant Sahu., et al.**[38]

Levofloxacin effervescent sustained release tablets were developed in eight different formulations (F1 to F8) by employing different grades of polymers and effervescent agents such as sodium bicarbonate and citric acid. The formulations were evaluated for various physical parameters, dissolution parameters and drug released mechanisms. F8 formulation showed maximum floating time of 12 hours and gave slow and maximum drug release of levofloxacin spread over 12 hours and whereas levofloxacin released from marketed tablet was rapid and maximum within 8 hours.

- **Shreeraj H Shah., et al.**[39]

Developed a gastric floating drug delivery system (GFDDS) containing levofloxacin against the H.pylori infection using gas-forming agents, like sodium bicarbonate, citric acid and hydrocolloids, like hydroxypropyl ethylcellulose (HPMC) and carbopol 974P. The prepared tablets were evaluated in terms of their pre compression parameters, physical characteristics, *in vitro* release, buoyancy, floating lag time (FLT), total floating time (TFT) and swelling index. The formulations were optimized for the different viscosity grades of HPMC, carbopol 974P and its concentrations and combinations. Stability study was also performed after storage at 40°C / 75% RH for three months. All the formulations showed good floating lag time i.e. less than 3 mins. The batch containing combination of HPMC K4M, HPMC K100M and Carbopol 974P (i.e. L12) showed total floating lag time more than 24 hrs. The batch L12 showed the highest swelling index among all the prepared batches (i.e. 95%). The batch L12 was chosen as the optimized batch since, it was also stable for three months during stability study.

- **Arunachalam A., et al.**[40]

Prepared tablets by melt granulation method, using the polymer, hydroxy propyl methyl cellulose (HPMC K100M) with different amounts and other excipients and sodium bicarbonate as gas generating agent. The present study was to develop a floatable drug delivery system of levofloxacin hemihydrate for sustained drug delivery and gastric retentive property with special emphasis on optimization of formulations for floating matrix tablets. Thus the study aims to improve the oral bioavailability of the drug and to achieve extended retention in the stomach which may result in prolonged absorption. Tablets were evaluated by different parameters such as weight uniformity, content uniformity, thickness, hardness, IR spectral analysis, *in vitro* release studies, buoyancy determination and kinetic analysis of dissolution data, stability studies levofloxacin floating tablet drug delivery system showed improved *in vitro* bioavailability and extended drug release which may favour the reduced dose frequency and patient compliance**.**

- **Nagesh C., et al.**[41]

Developed new intra-gastric floating microspheres for controlled delivery of levofloxacin for the treatment of peptic ulcer caused by *Helicobacter pylori.* Floating microspheres of levofloxacin were prepared by emulsion solvent evaporation technique. The drug was encapsulated with HPMC and Eudragit S 100 in different polymers ratios. i.e. 1**:**1, 1**:**2, 1**:**3. Prepared microspheres were evaluated for % entrapment, particle size, buoyancy, dissolution study and drug release kinetics. The % yield of microspheres was high in HPMC batches over Eudragit S 100 batches. The particle sizes of microspheres were increased by increasing the polymer concentration. Percentage buoyancy of microspheres was found to be in the range of 63.38% - 75.58% indicated that most of the microspheres were still floatable after 12 hours because of their low density and internal voids. Microspheres of levofloxacin with HPMC showed enhanced release rate when compared to levofloxacin with Eudragit S 100. From the results it was concluded that, the prolonged GI residence time of the formulation & its controlled release in the gastric environment makes complete eradication of H.pylori from GIT more effectively than conventional tablets.

- **Abbaraju prasanna lakshmi., et al.**[42]

Formulated levofloxacin hemihydrate immediate release and Ambroxol HCl sustained release bilayer tablets in order to improve patient compliance. All the formulations were prepared by wet granulation method because of poor flow property exhibited by pure drugs. HPMC K4M and HPMC K100M were used for sustaining the release rate of Ambroxol from Ambroxol layer and superdisintegrant like sodium starch glycolate was used for immediate release of levofloxacin from immediate release layer. All the prepared formulations were evaluated for weight variation, hardness, thickness, friability, disintegration and dissolution tests. The release pattern of Ambroxol was fitted to different kinetic models and among all the formulations, F11 of Ambroxol has shown a release rate up to 12 hours. All the formulations could be good expressed by Higuchi equation as the plots shows good linearity, and the correlation coefficient (r^2) for the best formulation F_{11} was 0.9991 with slope n=0.530, which appears to showed a coupling of diffusion and erosion mechanism, so called anomalous diffusion. F_2 of levofloxacin disintegrated less than 12 min. So, F_{11} of Ambroxol and F_2 of levofloxacin were selected as best formulations. Stability studies were conducted at 25^0 C for long term

studies and 45^0 C for accelerated studies for the best formulations.

➢ **Nagabhushan BS., et al.[43]**

Orodispersible tablets of levofloxacin were prepared by direct compression method using different superdisintegrants. Taste masking was done for the selected formulation using Eudragit E-100. The tablets were evaluated for hardness, weight variation, friability, disintegration time, water absorption ratio, wetting time and *in vitro* dissolution. The disintegration time of all the formulations ranged between 20.0 to 76.16 seconds with rapid *in vitro* dissolution. Orodispersible tablets of levofloxacin can be prepared by direct compression method using superdisintegrants with enhanced dissolution and taste masking. Hence, the prepared formulations may improve patient compliance especially in the pediatric and geriatric patients and increase the efficacy of drug for treating infections.

➢ **Rajalakshmi R., et al.[44]**

Developed a novel gastro retentive oral floating *in situ* gelling system for controlled release of levofloxacin hemihydrate (LIG) for the eradication of *Helicobacter pylori* and other microorganisms causing local complications in stomach and proximal small intestine. Polymer based floating *in situ* gelling systems of levofloxacin hemihydrate were prepared by dissolving varying concentrations of gellan gum and sodium alginate in deionized water containing sodium citrate, to which varying concentrations of drug and calcium carbonate were added and dissolved by stirring. Calcium carbonate added to the formulation provides calcium ions and carbon dioxide. Calcium ions, due to ion interactions with the polymer, help in gelation. Carbon dioxide entrapped in the gel and facilitated buoyancy of the gel. The formulation variables, concentration of gellan gum and sodium alginate significantly affected the *in vitro* drug release of drug from the prepared LIG. Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) were used to check the presence of any interaction between the drug and the excipients. The drug release from the *in situ* gels follows the Hixson-crowell model, which indicated a drug release through the polymeric matrix. A stomach specific *in-situ* gel of levofloxacin hemihydrate could be prepared using floating mechanism to increase the residence time of the drug in stomach and thereby increase the absorption.

PAST WORK ON BOVIN SERUM ALBUMIN

➢ **Sayyed Abolghassem Sajadi Tabassi., et al.[45]**

Prepared Bovine Serum Albumin (BSA) based microspheres bearing propranolol hydrochloride by an emulsion-internal phase stabilization technique. The prepared microspheres were studied for particle size distribution, drug loading, release characteristics, bioadhesion and *in-vitro* controlled diffusion across the rat intestine. The microspheres had mean diameters 1-25 µm of which more than 50 percent were below 5 µm. The encapsulated drug was found to be about 9% w/w of that initially added to microspheres and the superficial drug was 25% of the total amount of the encapsulated drug. Also AMS were noted to possess good bioadhesion in such a way that about 70% of microspheres remained adherent on the surface mucosa of rat jejunum. The drug release from albumin microspheres was mainly controlled by diffusion and showed a biphasic pattern with a high initial release (burst effect), followed by a more gradual terminal release. The total amount of drug released from microspheres after 12h was 70%. *In vitro* experiments on the rat intestinal segments revealed that the microspheres could effectively pass their content through intestinal membrane.

➢ **Hetal Thakkar., et al.[46]**

Investigated the preparation of celecoxib loaded albumin microspheres and the biodistribution of technetium-99m (99mTc)-labeled celecoxib as well as its microspheres after intravenous administration. Microspheres were prepared using a natural polymer BSA using emulsification chemical cross-linking method. The prepared microspheres were characterized for entrapment efficiency, particle size, and *in vitro* drug release. Surface morphology was studied by scanning electron microscopy. Biodistribution studies were performed by radiolabeling celecoxib (CS) and its microspheres (CMS) using 99mTc and injecting arthritic rats intravenously. The geometric mean diameter of the microspheres was found to be 5.46 µm. *In vitro* release studies indicated that the microspheres sustained the release of the drug for 36 days. Radioactivity measured in different organs after intravenous administration of celecoxib solution showed a significant amount of radioactivity in the liver and spleen. In case of celecoxib-loaded microspheres, a significant amount of radioactivity accumulated in the lungs. No significant difference ($P > .1$) in the radioactivity was observed between the inflamed joint and the noninflamed joint following intravenous injection of 99mTc-CS. However, in case of the microspheres (CMS), the radioactivity present in the inflamed joint was 2.5-fold higher than in the noninflamed joint. The blood kinetic studies revealed that celecoxib loaded albumin microspheres exhibited prolonged circulation than the celecoxib solution.

➢ **Praveen B., et al.[47]**

Prepared Aceclofenac microsphere by suspension cross-linking method using albumin as polymer to prevent the gastric irritation and to achieve oral sustained / controlled release of the drug. Preformulation studies revealed that the drug aceclofenac and the polymer albumin were satisfactorily compatible without any change in the chemical nature of the drug. In present study two formulations were formulated by using bovine serum albumin. The formulations were subjected to various evaluation parameters like % practical yield, actual drug content, entrapment efficiencies, particle size distribution and *in vitro* release studies. The results of all parameters are tabulated and depicted graphically in the

results and discussion section. The advantages of such controlled release formulations containing non-steroidal anti-inflammatory drugs (NSAIDs) over the conventional dosage forms have been reported. Such formulations minimize the gastric irritant side effects of the conventional NSAID preparations.

- **Ashvini Urs., et al.**[48]

Prepared Ketoprofen loaded albumin microspheres for sustained delivery for long duration of time, by solvent evaporation technique. The microspheres were found to have incorporation efficiency of 48% to 79%. The effect of albumin concentration was evaluated with respect to entrapment efficiency, particle size, surface characteristics and *in vitro* release behaviours. The results of FTIR spectral showed that there was no significant interaction between the drug and polymer. These results indicated that Ketoprofen loaded albumin microspheres could be prepared providing a sustained release property. Albumin microspheres showed smooth surface were confirmed by Scanning Electron Microscopic (SEM) study. The mean particle size and entrapment efficiency were found to be varied by changing various formulation parameters. The *in vitro* release profile was altered significantly by changing various formulation parameters to gave a sustained drug release from the microspheres. The microspheres were in the suitable particle size range of 27.5-203µm. The release kinetics studies were found to fit into zero order and Non Fickian diffusion controlled mechanism was observed. The drug was released continuously for a period of 12 hours with a maximum release of 90.5%. From the preliminary trials it was concluded that it is possible to formulate sustained release Ketoprofen loaded albumin microspheres.

- **Vikas Parashar., et al.**[49]

The aim of present study was to develop biodegradable microspheres of Tinidazole. Bovine serum albumin was used for the preparation of microspheres. They were made in four batches. The emulsion cross-linking method was used for the preparation. The quantity of BSA varies for each formulation. Formulations were evaluated for particle size, melting point, TLC, entrapment efficiency and *in vitro* release studies. Depending upon the drug to polymer ratio, the entrapment, loading were found to range between 48, 55, 75 and 78 (in %) respectively. Particle size of prepared microspheres was measured using a compound microscope. The surface topography and internal textures of the microspheres was observed by scanning electron microscopy. The microspheres were spherical, discrete and compact and size distribution was between 33.28 to 36.25 µm. *In vitro* studies were carried out at different pH for a period of 18 h and compared with marketed formulation. From all the batches it was concluded that when concentration of polymer increased microspheres showed more controlled and prolonged release. The drug release was between 66, 51, 48, 42 % respectively. The drug release from 1:4 was most prolonged and constant. Both IR spectra of drug and formulation were almost same.

- **Kirti Rani.**[50]

Microspheres were prepared using a natural polymer Bovine serum albumin using emulsification chemical cross-linking method. Glutaraldehyde was used as cross linking agent and mustard oil was used as emulsifier which gave the newly formed structure by exposing the tryptophan residues as its diene adduct. The emulsification with mustard oil was increased the stability of encapsulated microspheres as well as responsible for sustained release of enzyme in the delivery system in the presence of proteolytic enzymes. Glucose oxidase was immobilized in bovine serum albumin for entrapment using cross linking reagent such as glutaraldehyde and mustard oil. The microspheres prepared under these conditions were spherical in shape and exhibit fluorescent at 495nm. The specific activity of glucose oxidase were studied at different time intervals as well as using different concentration of proteases such as trypsin, chymotrypsin and papin to understand the biodegradability of the cross linked entrapped glucose oxidase. The enzyme activity of glucose oxidase in emulsified encapsulated bovine serum albumin microspheres was estimated by the method as published in Worthington Manual. Among them the chymotrypsin was found to be the best proteolytic enzymes which release the maximum activity of glucose oxidase in solution which was 22U/ml of chymotrypsin at the fifth day, thereby confirming the proper immobilzation of enzyme in microsphere under specified conditions. In the absence of proteolytic enzymes, glucose oxidase was not detected. This immobilized glucose oxidase in emulsified encapsulated bovine serum albumin microspheres will convert glucose into gluconic acid and hydrogen peroxide. Hydrogen peroxide is completely soluble in solution and gluconic acid is metabolized easily in the body which makes the drink clear and free from opalescence. Hence, removal of excess glucose is essential before packing in the bottles. This immobilized glucose oxidase in emulsified encapsulated bovine serum albumin microspheres can be packed in columns and samples of beverages/ soft drinks/ in vitro drugs delivery system. Cross linked entrapped Glucose oxidase microsphere has become a useful tool in drug delivery system and in preparation of beverages industries as a preservative to remove the excess of glucose from soft drinks to make it opalescent.

- **Licy CD., et al.**[51]

Hepatitis B vaccine loaded microspheres were prepared by microencapsulation using albumin as polymer and sodium taurocholate as permeation enhancer. Bacitracin and aprotinin were incorporated as protease inhibitors. After *in vitro* studies, optimized drug-loaded microspheres were encapsulated in hard gelatin capsules which were further enteric coated with cellulose acetate phthalate. *In vivo* experiments were conducted in rabbits. Control group was administered with 20 µg of HBsAg

intramuscularly and the test groups were given different formulations of oral vaccine as per the current vaccination schedule of 0, 1 and 6 months.

Immunogenicity was compared with IM injected vaccine control group. Results showed HBsAg microspheres with albumin as polymer and bacitracin as protease inhibitor induced immune response with specific antibodies and cell mediated immunity better than the formulation with aprotinin as protease inhibitor ($p = 0.047$). Vaccine-related adverse events were absent in all immunized rabbits. Basic haematological, biochemical and physical parameters were within normal limits. The stability exhibited by this vaccine at room temperature can be extremely useful to overcome the incomplete vaccine coverage due to cold chain requirement. Impressive large logistical advantages and elimination of blood-borne infections were other major benefits, which may have a great impact on global HBsAg immunization acceptance and compliance and on hepatitis B disease burden.

- **Basavraj K Nanjwade., et al.**[52]

Albumin microspheres of antihypertensive drug hydralazine hydrochloride were prepared by emulsion cross-linking method by using glutaraldehyde as cross-linking agent. Drug and polymer compatibility was determined by Fourier-Transform Infrared spectroscopy. To determine the effect of polymer concentration and amount of glutaraldehyde, formulations were characterized for their entrapment efficiency, particle size, surface morphology and release behavior. *In vivo* study was carried out on hypertensive wistar rats. Maximum percentage entrapment efficiency (%EE) was found to be 68.20±1.03 %. Laser particle size analyzer confirmed mean particle size in the range of 31.7 to 39.6 µm. *In vitro* drug release studies showed a biphasic release pattern for all formulations with an initial burst effect followed by slow release for almost 24 hrs. *In vivo* study to determine antihypertensive effect of selected formulation strongly correlates with *in vitro* drug release behavior. The release behavior was significantly regulated by polymer concentration and volume of glutaraldehyde. The study revealed that hydralazine hydrochloride loaded albumin microspheres exhibited prolonged reduction of systolic and diastolic arterial pressure compared to hydralazine hydrochloride solution.

- **Tunçay M., et al.**[53]

Prepared microsphere formulations of Diclofenac sodium using a natural biodegradable polymer as a carrier for intra articular administration to extend the duration period of the dosage form in the knee joint. Microsphere formulations of DS which were prepared were evaluated *in vitro* for particle size, yield value, encapsulation efficiency, surface morphology, and *in vitro* drug release. Two appropriate formulations were selected for *in vivo* trials. For the *in vivo* studies, Technetium-99m labelled polyclonal human immunogammaglobulin (99mTc-HIG) was used as the radiopharmaceutical to demonstrate arthritic lesions by gamma scintigraphy. After the induction of arthritis in knee joints of rabbits, the radio-labelled microspheres loaded with DS were injected directly into the articular cavity and at specific time points gamma scintigrams were obtained to find the residence time of the microspheres in knee joints in order to determine the most suitable formulation.

- **Bozdag S., et al.**[54]

Prepared Naproxen Sodium (NS), (a NSAID) loaded microsphere formulation using natural Bovine serum albumin (BSA) and synthetic biodegradable polymers such as polylactide-co-glycolic acid) (PLGA) (50:50 MW 34000 and 88 000 Da) for intra-articular administration, and to study the retention of the drug at the site of injection in the knee joint. NS incorporated microspheres were evaluated *in vitro* for particle size (the mean particle size; for BSA microspheres, 10.0±0.3um, for PLGA microspheres, 9.0 ± 0.2 and 5.0±0.1um for MW 34000 and 88 000 Da, respectively), yield value, drug loading, surface morphology and drug release. For *in vivo* studies, monoarticular arthritis was induced in the left knee joints of rabbits by using ovalbumm and Freund's Complete Adjuvant as antigen and adjuvant. A certain time (4 days) is allowed for the formation of arthritis in the knee joints, then the NS loaded microspheres were injected directly into the articular cavity. At specific time points, gamma scintigrams were obtained to determine the residence time of the microspheres in knee joints, in order to determine the most suitable formulation. This study indicated that PLGA, a synthetic polymer, is more promising than the natural type BSA microspheres for an effective cure of mono-articular arthritis in rabbits.

- **Li FQ., et al.**[55]

Ciprofloxacin albumin microspheres were prepared by the spray drying technique, with bovine serum albumin as the natural biodegradable material. The spherical microspheres, flowed well, were organic solvent free and in the size range 1-5 microm. The drug release from the microspheres could be retarded by further thermal denaturation. The sustained-release microspheres were suitable for dry powder inhaled lung drug delivery systems.

- **Jayaprakash S., et al.**[56]

Prepared sustained-release methotrexate microspheres of bovine serum albumin in different ratios by the emulsion cross-linking method. The prepared microspheres were subjected to various physicochemical evaluation and *in vitro* release studies. The drug release from microspheres of 1:6 ratio was the most constant and prolonged drug release was diffusion followed by erosion. The characteristics of the prepared microspheres were conducive to the formulation of the sustained release drug delivery system.

➢ **Wang C., et al.**[57]

Bovine serum albumin microspheres (BSA-MSs) containing Pingyangmycin hydrochloride (PYM) were prepared for the interventional embolization by an emulsification-crosslinking method. The average diameter of the MSs was 83.6 microm with 80% ranging from 35 to 200 microm. The MSs showed a rather high entrapment efficiency (EE%) of 87.6+/-5.7% and the drug loading efficacy (DL%) was 20.2+/-1.3%. The drug release behaviors were evaluated both *in vitro* and *in vivo*, using UV-spectroscopy and HPLC, respectively. The *in vitro* results showed a significantly delayed release of drug for 10 days. The central auricular artery of rabbit was chosen as an embolization sites to study the *in vivo* drug release and the pharmacokinetics of the MSs compared with PYM injections. Experiments performed by artery perfusion and embolization in rabbits central auricular artery revealed that the PYM loaded BSA-MSs (PYM-BSA-MSs) could obviously prolong *in vivo* drug release, extend the mean residence time (MRT) and had equal bioavailability compared with plain PYM injections. These results demonstrated that by embolization of the central auricular artery with PYM-BSA-MSs, the local drug concentration could maintain at a relative high level for a longer time, thus achieved the aim of tumor targeting therapy. PYM-BSA-MSs are excellent potential alternatives of interventional embolization materials for the treatment of maxillofacial region tumors.

➢ **Haswani DK., et al.**[58]

Prepared and evaluated microsphere formulations of gentamicin using bovine serum albumin (BSA) as a polymer matrix and glutaraldehyde as a cross-linker. Microsphere formulations of gentamicin were prepared using a spray dryer and were evaluated for product yield, encapsulation efficiency, particle size and *in vitro* drug release. The anti-microbial testing was performed using a modified Kirby-Bauer technique which showed that encapsulated gentamicin had an equivalent anti-microbial activity against *E. coli* bacteria as compared to gentamicin solution. Since it was the goal to deliver a high drug load intra-cellularly, the formulation with the least burst release profile in PBS was evaluated for its pharmacokinetic performance in rats. The *in vivo* pharmacokinetic evaluation on rats demonstrated increased bioavailability with microsphere formulation in comparison to the traditional solution form. The significant increase in bioavailability shall enable one to reduce the frequency of gentamicin administration and would effectively reduce the dose related side effects of gentamicin such as ototoxicity and nephrotoxicity.

➢ **Sam T Mathew., et al.**[59]

Prepared and evaluated ketorolac tromethamine-loaded albumin microspheres using a factorial design. Albumin microspheres were prepared by emulsion cross-linking method. Selected formulations were characterized for their entrapment efficiency, particle size, surface morphology, and release behavior. Analysis of variance (ANOVA) for entrapment efficiency indicated that entrapment efficiency was best fitted to a response surface linear model. From the statistical analysis it was observed that as the drug: polymer (D:P) ratio and volume of glutaraldehyde increased, there was a significant increase in the encapsulation efficiency. Scanning electron microscopy of the microspheres revealed a spherical, nonporous and uniform appearance, with a smooth surface. Based on the entrapment efficiency and physical appearance, 9 formulations were selected for release study. The maximum particle size observed was below 40 µm. The release pattern was biphasic, characterized by an initial burst effect followed by a slow release. All selected microspheres, except those having less polymer proportion (D:P ratio is 1:1), exhibited a prolonged release for almost 24 hours. On comparing r^2 values for Higuchi and Peppas kinetic models, different batches of microspheres showed Fickian, non-Fickian, and diffusion kinetics. The release mechanism was regulated by D:P ratio and amount of cross-linking agent. From the experimental data obtained with respect to particle size and extent of drug relaase, it could be concluded that the prepared microspheres are useful for once-a-day intramuscular administration of ketorolac tromethamine.

PAST WORK ON INJECTABLE MICROSPHERES

➢ **Indranil Banerjee., et al**.[60]

Attempt has been made to characterize the effect of PLGA microsphere incorporation on the physical properties of freeze-dried gelatin scaffold and its influence on cyto compatibility. Scaffolds loaded with varying amount of PLGA microspheres (10%, 1%, 0.1% w/w) were subjected to microarchitecture analysis, swelling, porosity, mechanical properties, biodegradation, cell adhesion, and cell proliferation studies. Results revealed that an increase in percentage loading of microspheres reduced the pore size and uniformity of the pore structure. Moreover, loading of PLGA microspheres up to 1% w/w significantly increased porosity, swelling, and mechanical properties of the scaffold but variations were not proportional for 10% w/w loading. Results also showed that PLGA microspheres have no significant effect on cell adhesion but influenced the growth kinetics.

➢ **Suja C Jayan., et al.**[61]

In the present study, gelatin microspheres containing Salbutamol sulphate were prepared by coacervation phase separation method and characterized by optical microscopy and scanning electron microscopy. The microspheres were analyzed for drug entrapment, and *in vitro* release pattern. The different batches of microspheres were prepared by altering drug: polymer ratio and cross linking with glutaraldehyde. The size of microspheres was in the range of 5.6µm- 22.4µm and the average diameter was found to be 12.34 µm. They were spherical in shape as evidenced by scanning electron microscopic photographs. The percent drug entrapment

was up to 80% and they could sustain drug release over a period of 8 ½ hours.

➢ **Muniyandy Saravanan., et al.**[62]

Formulated Diclofenac sodium loaded gelatin microspheres for intra-articular administration. Drug-loaded microspheres were prepared by the emulsification/crosslinking method, characterized by drug loading, size distribution, scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC), X-ray diffraction (XRD), gas chromatography, and *in vitro* release studies. The targeting efficiency of microspheres was studied *in vivo* in rabbits. The microspheres showed drug loading of 9.8, 18.3, and 26.7% w/w with an average size range of 37–46 µm, depending upon the drug–polymer ratio. They were spherical in nature and free from surface drug as evidenced by the SEM photographs. FT-IR, DSC, and XRD revealed the absence of drug–polymer interaction and amorphous nature of entrapped drug. Gas chromatography confirms the absences of residual glutaraldehyde. The formulated microspheres prolonged the drug release up to 30 days *in vitro*. About 81.2 and 43.7% of administered drug in the microspheres were recovered from the target joint after 1 and 7 days of post intra-articular injection, respectively, revealing good targeting efficiency.

➢ **Hiremath G Jagadeesh., et al.**[63]

The main objective of this study was to develop a polymeric drug delivery system for tamoxifen, injectable microspheres for breast cancer. To achieve this goal tamoxifen loaded poly (ε-caprolactone) (PCL) microspheres were prepared by solvent evaporation method. Microparticles were characterized in terms of particle size, surface morphology, drug physical state by using master size analyzer (MSA), scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and Differential scanning calirometry (DSC). Tamoxifen loading over different concentration was analyzed by high performance liquid chromatography. *In vitro* drug release studies were performed in phosphate buffer saline (PBS, pH 7.4 at 37^0 C). Cytotoxicity study by MCF-7 breast cancer cells. The mean particle size of microspheres was 27-47µm and were spherical shape, with smooth surface. The DSC studies revealed that the entrapment tamoxifen in microspheres was in the form amorphous state. The encapsulation efficiency was found to 61-67%. The *in vitro* release of tamoxifen was relatively fast initially followed by a slower and sustained release. The cytotoxicity against the MCF-7 cells was affected significantly by the released amount of tamoxifen. FTIR and DSC studies demonstrated that did not indicate changes in either chemical stability of the polymer or thermal properties such as glass transition or melting temperature, indicating the absence of chemical degradation or polymer-drug interaction during long term stability studies.

➢ **Wahid Khan., et al.**[64]

Paromomycin (PM) loaded albumin microspheres (MS) (of size 5 µm) to target macrophages for treatment of visceral leishmaniasis. PM loaded MS were prepared by spray-drying method using albumin as a polymer matrix and stabilized using heat treatment. These MS were evaluated for product yield, encapsulation efficiency, particle size, size distribution, contact angle, drug–polymer interactions, and for *in vitro* drug release. Fluorescent labeling and *in vitro* uptake of these MS was assessed in RAW 264.7 cell line.PM loaded albumin MS were prepared with a mean particle size 3 µm. Free albumin content and contact angle study confirmed the stabilization of these MS. Release studies showed biphasic release pattern. Interaction studies ruled out any possibility of drug–polymer interaction. Uptake study in macrophage confirmed the suitability of prepared MS for macrophage targeting. The proposed drug-delivery system was found suitable for targeting macrophages *in vitro* and may serve as an optimum carrier to target macrophages where Leishmania parasite reside.

➢ **Raghuvir Singh., et al.**[65]

Pulmonary drug delivery systems offer many advantages over other conventional drug delivery system such as avoidance of first pass metabolism, large surface area suitable for drug absorption and quicker onset of pharmacological activity. One of the drug, ofloxacin, if formulated into microspheres, could by the mechanical interception of capillary bed, could effectively be targeted to the lungs. The biodegradable and biocompatible polymer chitosan was selected as matrix forming agent. The formulated nanoparticles were then evaluated for various parameters like for particle size, shape and entrapment efficiency etc. The microspheres were evaluated by DSC studies, IR spectroscopy and *in vitro* drug release study followed by *in vivo* lung targeting study. The microspheres could be successfully utilized for targeting ofloxacin to the lung tissue.

➢ **Sheth Zankhana., et al.**[66]

Biodegradable microparticulate delivery system of 5-fluorouracil has been developed by solvent evaporation technique by using polymethacrylate polymers like eudragit L100, eudragit S100, eudragit P4135F and methylcellulose. Four different formulations were prepared by using these polymers in drug to polymer ratio of 1:2. The formulations were evaluated with respect to particle size analysis, entrapment efficiency, in vitro drug release studies, in vivo drug targeting studies and stability studies. The formulated magnetic microspheres were found to be spherical with average particle size of 3-12 µm in diameter and incorporation efficiency up to 78.80%. In vitro drug release after 12 hr was 86.41 %, 92.84 %, 79.88 % and 82.38 % for formulation F1, F2, F3 and F4 respectively. Formulation F2 with highest drug content was selected for in-vivo drug targeting studies. The average targeting efficiency of drug loaded microspheres was found to be 26.16 % of the injected dose in liver, 11.40 % in lungs, and 15.08 %

in spleen, whereas the concentration of pure drug was 15.52 % in liver, 9.0 % in lungs, and 9.50 % in spleen. These results reveal that the drug loaded microspheres showed preferential drug targeting to liver followed by spleen and lungs. Stability studies revealed that 4ºC is the most suitable temperature for storage of 5 - fluorouracil loaded microspheres. Overall, this study showed that the 5 - fluorouracil can be formulated in a micro particulate drug delivery system by using various polymers and it showed significant prolonged drug release.

- **Sachiro Kakinoki., et al.,**[67]

TNP-470 (AGM-1470, 6-0-(N-chloroacetylcarbamoyl)-fumagillol), a derivative of fumagillin, is a promising angiogenesis inhibitor. However, as TNP-470 is very unstable in *in vitro* and *in vivo*, it has been difficult to verify its pharmacological efficacy in the clinical medicine. The preparation of a drug delivery system (DDS) in a microsphere form was studied for the stable inclusion and controlled release of TNP-470. Medium-chain triglyceride (MCTG) as an effective stabilizer and poly-lactic acid (PLA) as a biodegradable carrier were used for this purpose. The release of TNP-470 from the MCTG containing DDS continued for approximately 2 weeks, while the release of TNP- 470 from the one without MCTG stopped after only 5 days. It was proved that TNP-470 could be released much more stable for much longer period from the MCTG containing DDS compared to the one without DDS.

- **Kadriye Ciftci., et al.,**[68]

Poly (DL-lactide-co-glycolide) PLAGA (50/50) microspheres containing an antineoplastic drug, 5-fluorouracil (5-FU) were prepared by a solvent evaporation process in order to passively target liver carcinomas. The microspheres were spherical with diameters 2-5µm and encapsulated more than 70% (w/w) of the 5-FU. *In vitro* release patterns of 5-FU from microspheres were determined for various systems. It was found that drug release depended upon the amount of entrapped drug, the polymer molecular weight and pH of the dissolution medium. The *in vitro* release mechanism was diffusion controlled and followed a square-root of time relationship. *In vivo* distribution of 99mTc labeled microspheres after intravenous injection into mice was characterized by an initially high uptake by organs of the mononuclear phagocyte system (MPS). Following i.v. administration of fluorescein-labelled PLAGA microspheres, accumulation was into the MPS, mainly the Kupffer cells cytoplasm and near the liver sinusoids.

- **Sree Harsha., et al.,**[69]

Lung-targeting albumin loaded ofloxacin microspheres (ALOME) were prepared by water in oil emulsion method. The appearance and size distribution were examined by scanning electron microscopy, and the aspects such as *in vitro* release characteristics, stability, drug loading, loading efficiency, pharmacokinetics and tissue distribution in albino mice were studied. The experimental results showed that the microspheres have an average particle size of 11.32µm. The drug loading and loading efficiency were (66.95 and 94.8%) respectively. The *in vitro* release profile of the microspheres matched the Korsmeyer's Peppas release pattern, and the release after 1 h was 42%, while for the original drug, ofloxacin, under the same conditions, 90.02% released in the first half an hour. After intravenous administration (15 min), the drug concentration of microspheres group in lung of albino mice was 432 gg^{-1} while that of controlled group was 1.32 gg^{-1} ALOME found to release the drug to a maximum extent in the target tissue, lung. Histopathological studies proved the tissue compatibility of ALOME to be safe.

- **Dandagi PM., et al.,**[70]

In the present study, bovine serum albumin (BSA) based microparticles bearing captopril were prepared by an emulsification–heat stabilization technique. Four batches of microparticles with varying ratio of drug and polymer were prepared. The prepared microparticles were studied for drug loading, particle size distribution, *in vitro* release characteristics, *in vivo* tissue distribution study and stability studies. The microparticles had mean diameter between 2 and 11 µm of which more than 70% were below 5µm and incorporation efficiency of 41–63% was obtained. *In vitro* release profile for formulations containing captopril-loaded albumin microparticles with heat stabilizing technique shows slow controlled release up to 24 h. The *in vivo* result of drug-loaded microparticles showed preferential drug targeting to liver followed by lungs, kidneys and spleen. Stability studies showed that maximum drug content and closest *in vitro* release to initial data were found in the formulation stored at 4^0 C. In the present study, captopril-loaded BSA microparticles were prepared and targeted to various organs to a satisfactory level and were found to be stable at 4^0 C.

- **Yalcin Ozakan., et al.,**[71]

prepared clarithromycin microspheres and evaluated by an emulsion polymeration technique. Two matrix materials have been consider as the basis in preparing the selected model active substance. Natural human serum albumin (HAS) and bovin serum albumin (BSA), Albumin microspheres containing clarithromycin were prepared by heat stabilization at different stirring rate. In the first part of our study, drug content, payload, particle size, surface morphology and release characteristic from microspheres prepared. The release of clarithromycin from both types of prepared microspheres was measured for 24 hr. The release profile of clarithromycin from HAS and BSA microspheres were extended for 24 hrs.

MATERIAL AND METHODS

The following materials that were either AR/LR grade or the best possible Pharma grade available were used as supplied by the manufacture.

Table no. 4.1: List of chemicals with grade and suppliers.

Sl. No.	Materials	Manufacturer
1.	Levofloxacin	Ronak Enterprises, Gujarat
2.	Bovine serum albumin	S.D. Fine Chem. Ltd., Mumbai.
3.	Petroleum ether	S.D. Fine Chem. Ltd., Mumbai.
4.	Tween 80 and span 80	S.D. Fine. Chem. Ltd., Mumbai.
5.	Potassium dihydrogen phosphate	S.D. Fine Chem. Ltd., Mumbai.
6.	Sodium hydroxide	S.D. Fine Chem. Ltd., Mumbai
7.	Anhydrous petroleum ether	S.D. Fine Chem. Ltd., Mumbai
8.	Cotton seed oil	N.K Proteins Ltd. Gujarat

Table no. 4.2: List of instruments used with manufacturers.

Sl. No.	Equipments	Manufacturers
1.	Electronic Weighing Balance	Acculab ALC-210.4
2.	UV-Vis Spectrophotometer (UV-1700)	Shimadzu, Japan.
3.	FTIR Spectrophotometer (8400S)	Shimadzu, Japan.
4.	Scanning electron Microscope	JSM 6380 A JOEL, Japan
5.	Dissolution test apparatus TDT-08T	Electrolab.
7.	Digital pH meter 7007	Hanna Electronics Mumbai.
8.	Hot Air Oven	M.C. Dallal, Chennai.
9.	Stability Chamber	Lab Control Equipment Co. Mumbai.
10.	Centrifuge	Remi electrotechnik Ltd. Vasai
11	Mechanical stirrer	Remi electrotechnik Ltd. Vasai
12	Magnetic stirrer	Remi electrotechnik Ltd. Vasai

METHODS

4.1 PREFORMULATION STUDIES

It is one of the important prerequisite in development of any drug delivery system. Preformulation studies were performed on the drug, which included melting point determination, compatibility studies.

4.1.1 Determination of Melting Point[78]

Melting point of Levofloxacin was determined by taking small quantity of drug in a capillary tube (previously sealed at one end) and was placed in digital melting point apparatus and temperature range at which the drug melted was noted.

4.1.2 Drug excipient compatibility studies

Excipients are integral components of almost all pharmaceutical dosage form. The successful formulation of stable and effective dosage form depends on careful selection of excipients, which are added to facilitate the administration, promote the consistence release, bioavailability of drug and protect it from degradation. The drug excipient compatibility studies are carried out by FTIR and DSC studies.

4.1.2.1 Fourier Transmittance Infra-Red spectroscopy[79]

Infra Red spectroscopy is one of the most powerful analytical technique, when it comes to the determination of presence of various functional groups involved in making up the molecule. It provides very well acceptable spectral data regarding any change in functional group characterization of a drug molecule occurring while in the processing of a formulation.

Procedure

Integrity of the pure drug and physical mixture of pure drug with excipient was checked by taking an IR spectrum. The spectra were obtained using shimadzu FTIR 8700 spectrophotometer.

In this study, pelletization of potassium bromide (KBr pellets) was employed. Before forming the pellet of potassium bromide, it was completely dried at 100^{o}C for one hour and after drying it was thoroughly mixed with the sample in the ratio 1 part of drug and 100 part of KBr. The mixture was compressed to form a disc using the dies. This disc was placed in the sample chamber and a spectrum was obtained which was further subjected to interpretation.

4.1.2.2 Differential Scanning Calorimetry[80]

Differential scanning calorimetry or DSC is a thermo analytical technique in which difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature. Both the reference and the sample are maintained at the same temperature throughout the experiment.

DSC (Perkin-Elmer thermal analysis) studies were carried out in order to characterize the physical state of drugs. Sample of pure drug and physical mixture were placed in the aluminium pans and thermatically sealed. The heating rate was 10^{o}C per min using nitrogen as pure gas. The DSC instrument was calibrated for temperature using indium. In addition, the enthalpy calibration indium was sealed in aluminium pan with sealed empty pans as reference.

4.2 ANALYTICAL METHODS[81]

4.2.1 Preparation of standard solution

100 mg of levofloxacin was accurately weighed and transferred into 100 ml volumetric flask and dissolved in small quantity of pH 7.4 phosphate buffer. The required volume was made with the pH 7.4 phosphate buffer to get a concentration of 1000µg/ml. From the above prepared stock solution, 1 ml was withdrawn and diluted to 100ml to get a concentration of 10µg/ml.

4.2.2 Determination of analytical wavelength of levofloxacin

Most of the drugs absorb light in the ultraviolet region (200nm - 400nm), since they are generally aromatic or contain double bonds. The solution containing 10 µg/ml of levofloxacin in pH 7.4 phosphate buffer was prepared and scanned over the range of 200nm – 400nm against the pH 7.4 phosphate buffer as blank using double beam UV spectrophotometer. The maximum peak obtained in the graph was considered as λ max.

4.2.3 Standard calibration curve of Levofloxacin

A precise sensitive and accurate method for estimating levofloxacin was developed using UV spectrophotometer. For the preparation of standard calibration curve, stock solution was prepared by dissolving 100mg of accurately weighed levofloxacin in 100 ml of pH 7.4 phosphate buffer to get 1000 µg/ml solution. Further 1 ml of this solution was pipetted out into 100ml volumetric flask and diluted to 100ml with pH 7.4 phosphate buffer to get 10µg/ml solution. From this, pipetted out 2,4,6,8 and 10 ml into a series of 10ml of volumetric flask and volume was made upto 10 ml with pH 7.4 phosphate buffer to get a concentration of 2,4,6,8 and 10 µg/ml respectively.

The absorbance of each concentration was measured using UV-Visible spectrophotometry. The observation were recorded in table no 5.2 and the calibration curve was drawn by plotting absorbance versus concentration of levofloxacin(µg/ml) as shown in figure no 5.8.

4.2 FORMULATION DEVELOPMENT

4.3.1 Selection of polymer

Natural biodegradable polymer are classified as proteins and polysaccharides. Proteins include gelatin and albumin and polysaccharides include alginate, dextran, chitosan and agarose. Polysaccharides are non-biocompatible material and may cause allergic reaction, dermatitis, nausea and dizziness. Hence, protein biodegradable polymer are preferred.[82]

Natural biodegradable polymers remain attractive primarily because they are natural products of living organism, readily available, relatively inexpensive and capable of multiple of chemical modification hence, albumin was found suitable polymer for the preparation of microsphere.

4.3.2 Selection of method of preparation of microspheres

Many different techniques have been proposed for the production of microspheres and it was suggested that more than 200 methods are identified from the patent literature.[83] It has been further reported that many scientists prepared microsphere by w/o technique for lung targeting.[84,85,86]

4.3.3 Preparation of levofloxacin albumin microspheres[70]

Bovine serum albumin microspheres containing levofloxacin were prepared by emulsification heat stabilization technique. Weighed quantity of BSA was dissolved in 2 ml of deionized water containing tween 80 to which levofloxacin was added and used as aqueous phase. The oil phase comprised of 3ml of cotton seed oil and 1 ml of petroleum ether with span 80 as emulsifier, which were mixed together and stirred for 10 min at 900 rpm on a magnetic stirrer. The aqueous phase was added dropwise to the oily phase and stirred at the same rpm for 30 min to form primary emulsion. This emulsion was then added to 40 ml of cotton seed oil, preheated to 110-120^{o} C using 21 no. needle and stirred at 1500 to 2500 rpm for 15 min to allow the formation and solidification of microsphere using mechanical stirrer. The suspension was then allowed to cool to room temperature with continuous stirring using a magnetic stirrer, on cooling, 100 ml of anhydrous ether was added. The suspension containing the microspheres was centrifuged at 3500 rpm for 30 min and the settled microspheres were washed for 3 times with petroleum ether to remove traces of oil on microsphere surface. The obtained microspheres were vaccum dried and stored in desiccators over night.

The preparation of microspheres was carried out as shown in table no 4.3, the process parameters such as stirring rate, concentration of albumin and concentration of emulsifying agents were varied according to the process.

Table no. 4.3: Composition of injectable microspheres of Levofloxacin.

Formulation	Drug (mg)	BSA (mg)	RPM	Span 80(%)	Tween 80(%)
F_1	50	100	1500	0.1	0.1
F_2	50	100	2500	0.1	0.1
F_3	50	200	1500	0.1	0.1
F_4	50	200	2500	0.1	0.1
F_5	50	100	1500	0.5	0.5
F_6	50	100	2500	0.5	0.5
F_7	50	200	1500	0.5	0.5
F_8	50	200	2500	0.5	0.5

4.4 EVALUATION PARAMETERS OF FORMULATED MICROSPHERES

4.4.1 Percentage practical yield[52]

Microspheres after the preparation were collected and weighed to determine practical yield from the following equation.

Percentage practical yield

$$= \text{X } 100 \frac{\text{Practical mass (microsphere)}}{\text{Theoretical mass (polymer + drug)}}$$

4.4.2 Entrapment efficiency[52]

To determine amount of drug encapsulated in microsphere 50 mg of microspheres were suspended into screw capped vial with pH 7.4 phosphate buffer and digested for 24 hours on magnetic stirrer in order to extract the entrapped drug completely. The amount of drug was determined using a double beam UV-Visible spectrophotometer after diluting suitably with pH 7.4 phosphate buffer. The percentage encapsulation efficiency of microsphere was calculated by using following formula.

$$\% \text{ EE} = \text{X } 100 \frac{\text{ED}}{\text{AD}}$$

Where, % EE is the percentage encapsulation efficiency
ED is the amount of encapsulated drug
AD is the amount of drug added.

4.4.3 Surface morphology

Examining the surface of polymeric drug delivery system can provide a vital information on porosity and microstructure of these system. The distribution and morphology of the surface and encapsulated matrix can be directly observed.

Most common technique used for characterizing the surface morphology of the system of drug delivery is scanning electron microscopy (SEM). This method offers several advantages by its versatility of the method, simplicity of the sample preparation and ease of operation. The sample sizes which can be analyzed using this method range from nanometer to micrometer to centimeter. This method also used unlimited amount of samples offering innumerable view points by means of its translational, tilting and rotary moments and having various resolving power of the instrument.

Procedure

Scanning electron microscopy of the albumin microsphere was performed to examine the surface morphology. The microsphere were mounted on a metal stubs and the stubs was then coated with conductive gold with sputter coater attached to the instrument the photographs were taken using SEM photography.[87]

4.4.4 Particle size

Particle size characterization of microsphere is an important study to ensure that particle size of the formulation lies in the optimal range. This is crucial factor since the target activity has intended in this study exclusively depends on particle size of microsphere produced.[88]

A wide variety of methods have been developed to measure particle size depending upon the size itself. These methods use different physical principles for the determination of size. For the determination of microsphere size Malver zeta sizer analyzer was used.

The microsphere were added to the sample dispersion unit containing stirrer and stirred to reduce the aggregation between microspheres. The average volume mean particle size was measured after performing the experiment in triplicates.

4.4.5 In vitro dissolution studies[69]

The *in-vitro* release studies were performed using dialysis method. In this method a weighed quantity of microsphere was placed in dialysis bag, which was immersed in a beaker containing continuous acceptor fluid. The compartment was stirred and the drug which diffuses out of microsphere into the continuous phase was periodically sampled and assayed.

In this method 50 mg of albumin microsphere were placed in dialysis bag and dialyzed against 200 ml of pH 7.4 phosphate buffer at 37^0C ± 2^0C. Sink condition was maintained throughout course study and stirred continuously using a magnetic stirrer. Aliquots were withdrawn at specific time interval and same volume of dissolution medium was added to the beaker to maintain sink condition. The withdrawn aliquots were estimated using UV-Visible spectrophotometer at 287 nm and the data were used to calculate a % cumulative drug release profile from microsphere.

4.5 RELEASE KINETICS

The results of *in-vitro* release profiles obtained for all the formulations were fitted into five models of data treatment as follows

1. Cumulative percent drug released versus time (zero-order kinetic model).
2. Log cumulative percent drug remaining versus time. (First-order kinetic model).
3. Cumulative percent drug released versus square root of time (Higuchi's model).
4. Log cumulative percent drug released versus log time (Korsmeyer-Peppas equation).
5. Cumulative percentage drug released versus cube root of time (Hixson and crowell's cube root law of dissolution).

- **Zero order kinetics[89]**

A zero-order release would be predicted by the following equation.

$$A_t = A_0 - K_0 t \quad (7)$$

Where
A_t = Drug release at time't'
A_0 = Initial drug concentration
K_0 = Zero-order rate constant (hr^{-1}).

When the data is plotted as cumulative percent drug release versus time, if the plot is linear then the data obeys zero-order release kinetics, with a slope equal to K_0.

➢ **First order kinetics[89]**
A first-order release would be predicted by the following equation
$\text{Log } C = \text{Log } C_0 - 303.2Kt$ (8)

Where
C = Amount of drug remained at time't'
C_0 = Initial amount of drug
K = First-order rate constant (hr^{-1}).

When the data is plotted as log cumulative percentage drug remaining versus time yields a straight line, indicating that the release follows First-order kinetics. The constant 'K' can be obtained by multiplying 2.303 with slope values.

➢ **Higuchi model[90]**
Drug released from the matrix devices by diffusion has been described by following Higuchi's classical diffusion equation.

$$Q = \left(\frac{D\varepsilon}{\tau}(2A - \varepsilon C_s)C_s t\right)^{1/2} \quad \ldots\ldots\ldots\ldots\ldots(9)$$

Where,
Q = Amount of drug released at time't'
D = Diffusion coefficient of the drug in the matrix
A = Total amount of drug in unit volume of matrix
C_S = The solubility of the drug in the diffusion medium
ε = Porosity of the matrix
τ = Tortuosity
t = Time (hrs) at which 'Q' amount of drug is released.

Equation (9) may be simplified if one assumes that D, C_S and A are constant. Then equation (9) becomes
$Q = Kt^{½}$ ……(10)

When the data is plotted as cumulative drug released versus square root of time, yields a straight line, indicating that the drug was released by diffusion mechanism. The slope is equal to 'K'.

➢ **Korsmeyer and peppas release model[91]**
The release rates from controlled release polymeric matrices can be described by the equation (10) proposed by korsmeyer et al.

$$Q = K_1 t^n \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(11)$$

Q is the percentage of drug released at time 't', K is a kinetic constant incorporating structural and geometric characteristics of the tablets and 'n' is the diffusional exponent indicative of the release mechanism.

For Fickian release, n=0.45 while for anomalous (Non-Fickian) transport, n ranges between 0.45 and 0.89 and for zero order release, n = 0.89.

➢ **Hixson and crowell's cubic root law of dissolution[92]**
The Noyes-Whitney's equation assumes that surface area of the dissolving solid remains constant during dissolution, which is practically not possible for dissolving particles. Hence, dissolution methods that involve use of constant surface area discs are employed to determine the rate of dissolution.

To account for the particle size decrease and change in surface area accompanying dissolution, Hixson and Crowell's cubic root law of dissolution is used:

$$W_0^{1/3} - W^{1/3} = Kt \quad \ldots\ldots\ldots\ldots\ldots\ldots(12)$$

4.6 IN-VIVO STUDIES

4.6.1 Pharmacokinetic and tissue distribution studies

4.6.1.1 Pharmacokinetic studies[69]

The pharmacokinetics of the optimized microsphere (F_7) in comparison with levofloxacin injection was carried out in albino rats. The study protocol was approved by institutional animal ethical committee S.C.S College of Pharmacy, Harapanahalli (approval no. SCSCP/583/10/2011-12. Dated-26-11-2011).

36 albino rats were used. Prior to administration the animals were divided into 12 groups with 3 animals in each group. i.e. standard group (levofloxacin injection) and test group (Levofloxacin microspheres, F_7). Standard group was administered (60 µg/kg body weight) with levofloxacin injection via tail vein, while the other group (test group) receive equivalent amount of drug in microsphere in suspension form.

Then blood samples were collected at predetermined time periods of 10 mins, 30 mins, 1hour, 3hour, 6hour and 12hour from retro orbital sinus. The plasma was separated immediately by using cold centrifugation at 5000 rpm for 10 minutes and the plasma was stored at -40^{0}C until further analysis.

4.6.1.2 *In-vivo* tissue distribution studies[68,69,70]

After collecting blood from orbital vein at various interval of time, rats were lightly anaesthetized and perfused with normal saline through heart. The tissue of interest such as lungs, liver and spleen were isolated and weighed accurately.

1) Sample preparation

The extracted organs were homogenized by adding saline at the ratio 0.1 g/ml (250 mg/2.5 ml) and the protein in the mixture was precipitated to prevent interference during quantification process by adding perchloric acid (100µl). The mixture was then vortexed for 1 min and centrifuged at 5000 rpm. The prepared samples were used to determine the amount of levofloxacin present in the tissue sample quantitatively using the HPLC method.

2) Analysis of drug in plasma and tissue[93]

A sensitive high performance liquid chromatographic (HPLC) method was used to analyze the amount of levofloxacin in plasma and tissue. The HPLC system with a variable wavelength programmable UV-visible detector and a reverse phase C-18 column was used.

- **Preparation of stock and working standard solution**

Stock solution of 1 mg/ml of levofloxacin along with propyl paraben as an internal standard was prepared separately using acetonitrile: water: phosphoric acid: tri ethyl amine in 4:6:0.5:0.3 as mobile phase. From this stock solution, working standard solution were prepared to contain 0.5, 1, 1.5, 2, 2.5, and 3 µg/ml of levofloxacin was prepared using the mobile phase.

Table no. 4.4: Chromatographic condition.

1.	Mobile phase	Acetonitrile: water: Phosphoric acid: triethyl amine
2.	Column	Phenomenex luna 5u C-18 (2) 100 A, 250 X 4.60mm 5µ
3.	Flow rate	1 ml/ min
4.	Injection volume	20 µl
5.	Temperature	25^{o} C
6.	Run time	8 min
7.	Detection wavelength	275 nm
8.	Internal standard	Propyl paraben

- **Method**

The standard and sample solutions were injected with the above chromatographic conditions and chromatograms were recorded. From the response factor (peak area) of the standard solution, the concentration of levofloxacin present in the plasma and tissue sample was calculated.

- **Data analysis**

The pharmacokinetic parameters were calculated by using PK 2.0 solution pharmacokinetic data analysis software.

4.6.2 HISTOPATHOLOGICAL STUDIES[69]

After 12 hours of administration of the formulation, animals (including control group) were sacrificed by excess anesthesia and the lungs were dissected and washed with cold saline. The organ was pressed between filter pads and weighed. Lung tissues was fixed in 10% neutral formalin using standard techniques and stained with hematoxylin and eosin for histopathological examination. All tissue samples were examined and graded under light microscopy with 400X magnification.

4.7 STABILITY STUDY[94,95]

Stability of a dosage form has been defined as the ability of a particular formulation in a specific container to remain within its physical, chemical, therapeutic and toxicological specification. Accelerated stability study was carried out as per the ICH guidelines.

➢ **Procedure**

As per ICH guidelines, optimized microsphere (F_7) was subjected to accelerated stability studies. Microsphere were kept in zip lock pouch and exposed to controlled temperature (40±2ºC) and RH (75±5%) for a period of 3 months in humidity control oven. After 30, 60 and 90 days, the sample were taken out and analyzed for physical appearance and drug content.

RESULT AND DISCUSSION

Targeting of drug to any organ is mainly depending on particle size and drug loading in any particular formulation are the key parameter for its successes. It is also pertinent to mention that scale up of the formulation is another key factor to take formulation from bench to market. Taking all factor under consideration in the present study microsphere of desired size less than 20µm were prepared by emulsification heat stabilization method.

5.1 MELTING POINT OF LEVOFLOXACIN

The melting point of levofloxacin was found to be **228^{0}C** which is within reported range of 224 - 229^{0}C. It complies within official standard. Thus indicating the purity of sample.

5.2 DRUG EXCIPIENTS COMPATIBILITY STUDIES

5.2.1 Fourier Transform Infrared Spectroscopy

IR spectra analysis for pure drug and in combination with polymer was carried out. In absence of any interaction, the IR spectra of mixture show patterns corresponding to those of the individual components. In the event that interaction occurs, this indicated in the IR spectrum of a mixture by the appearance of one or more new peaks or disappearance of one or more peaks corresponding to those of the components.

The principle IR peaks of pure levofloxacin and physical mixture are shown in the Table no 5.1 and Figure no.5.1-5.3.

There were no considerable changes in the IR peaks of mixture of drug and polymer when compared to pure levofloxacin. These observations indicated the absence of interaction between levofloxacin and Bovine serum albumin.

Table no. 5.1: Major Peaks of Levofloxacin in FTIR spectra.

Formulation code	C-H Stretching	COOH Stretching	C=C Stretching	C-X stretching	C-H_2 Bending
Pure drug	2937.83	2359.75	1604.51	1030.81	1467.82
Drug and BSA	2926.29	2361.37	1657.56	1091.64	1417.25

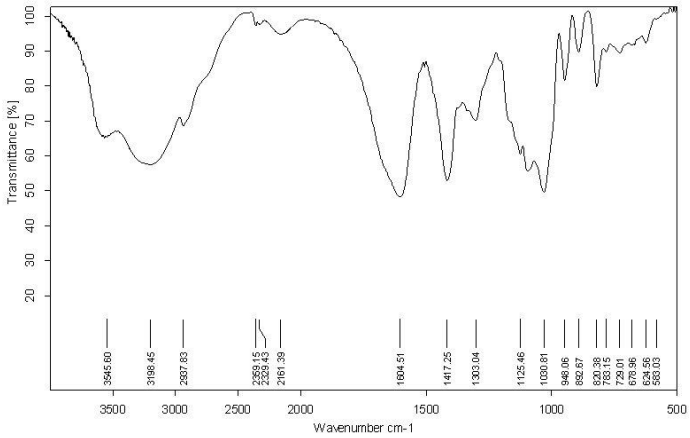


Figure 5.1: FT-IR Spectra of Levofloxacin.

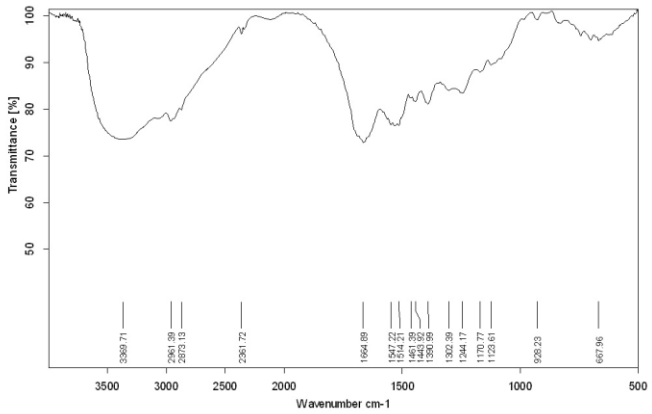


Figure 5.2: FT-IR Spectra of BSA.

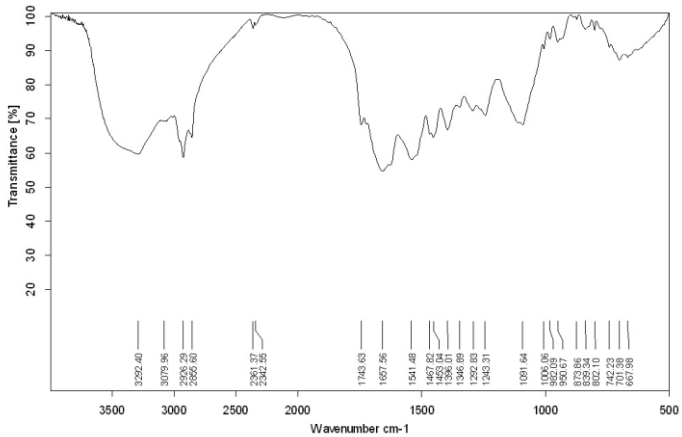


Figure 5.3: FT-IR Spectra of Drug + BSA.

5.2.2 Differential Scanning Calorimetry (DSC) Analysis

DSC is useful in the investigation of solid state interactions. Thermograms are generated for pure drug and physical mixture of drug along with other excipients. If there is no change in peaks of mixture when compared to pure drug, it indicates the absence of interaction. In the event if interaction occurs this indicated in the thermogram of mixture by the shift in the value of melting endotherm of the pure drug.

The DSC thermogram of pure Levofloxacin and its physical mixture are shown in Figure no. 5.4–5.6. Pure Levofloxacin showed a sharp endotherm at 230.79^{0}C corresponding to its melting point temperature.

However, there was a small change in the melting point of drug in physical mixture (203.78^{0}C) compared to that of pure levofloxacin this may be due to drug has been engulfed in the polymer matrix.

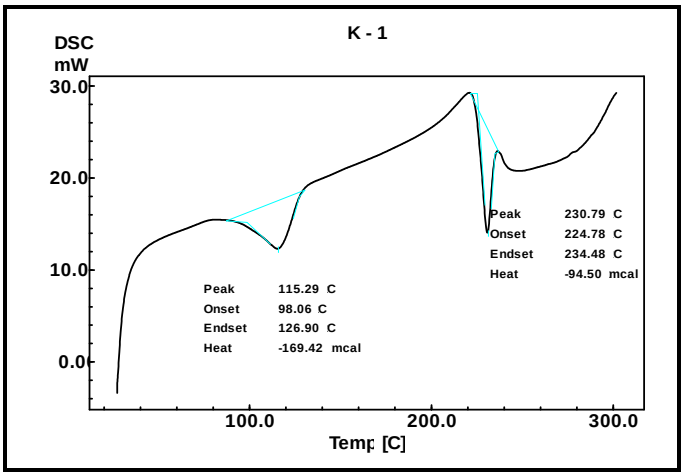


Figure no. 5.4: Differential Scanning Calorimetry of Levofloxacin.

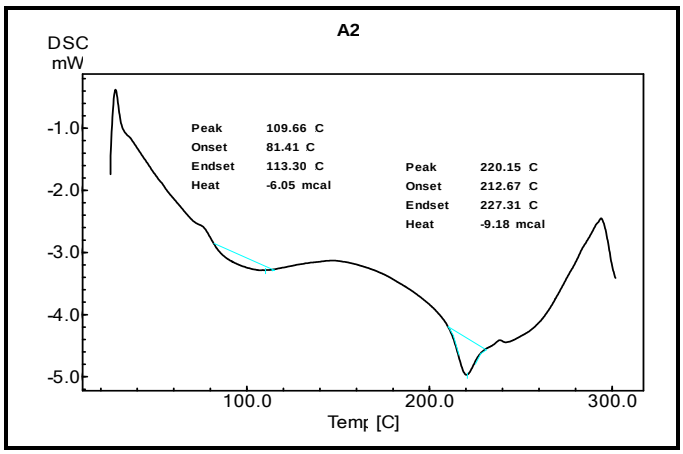


Figure no. 5.5: Differential Scanning Calorimetry of Bovine serum albumin .

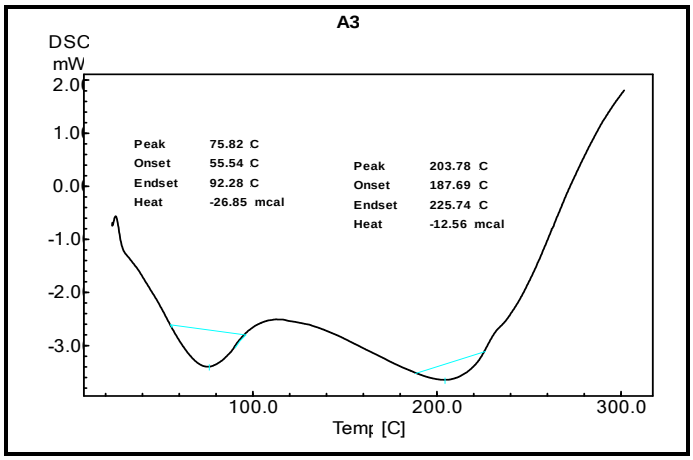


Figure no. 5.6: Differential Scanning Calorimetry of physical mixture.

5.3 ANALYTICAL METHOD

5.3.1 Scanning of levofloxacin in pH 7.4 phosphate buffer

The solution of 10 µg/ml of levofloxacin in pH 7.4 phosphate buffer was scanned over the range between 200-400 nm against pH 7.4 phosphate buffer as blank.

The absorption spectra of levofloxacin showed only one strong absorption peak at 287 nm, which represent the maximum absorption (λ max) of the drug in the pH 7.4 phosphate buffer as shown in figure no.5.7. This was similar to the reported values.

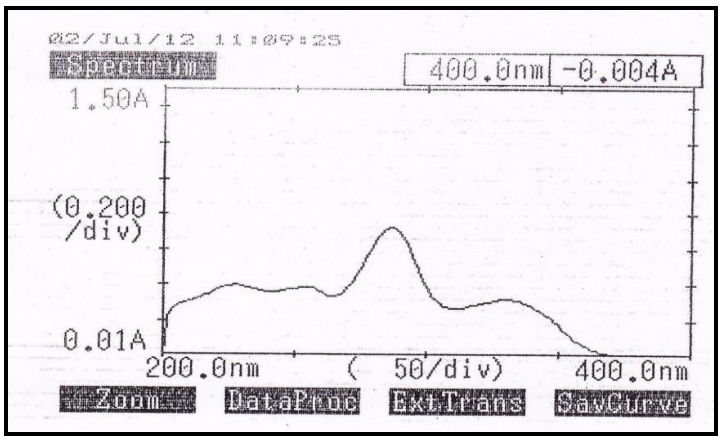


Figure no. 5.7: Analytical wavelength of Levofloxacin.

5.3.2 STANDARD CALIBRATION CURVE OF LEVOFLOXACIN

Table no.5.2, shows the absorbance value of standard solution of levofloxacin in pH 7.4 phosphate buffer at 287 nm. Figure no.5.8, shows standard calibration curve for levofloxacin in pH 7.4 phosphate buffer. The curve was found to be linear in the range of 2 -10 µg/ml with a regression value of 0.9991.

The calculation of the drug content and *in vitro* drug release rate studies are based on this standard calibration curve.

Table no. 5.2: Calibration data of Levofloxacin in pH 7.4 phosphate buffer.

Sl.no.	Concentration (µg/ml)	Absorbance at 287nm
1	0	0 ± 0
2	2	0.163±0.030
3	4	0.299± 0.005
4	6	0.436 ± 0.008
5	8	0.587 ±0.008
6	10	0.748± 0.007

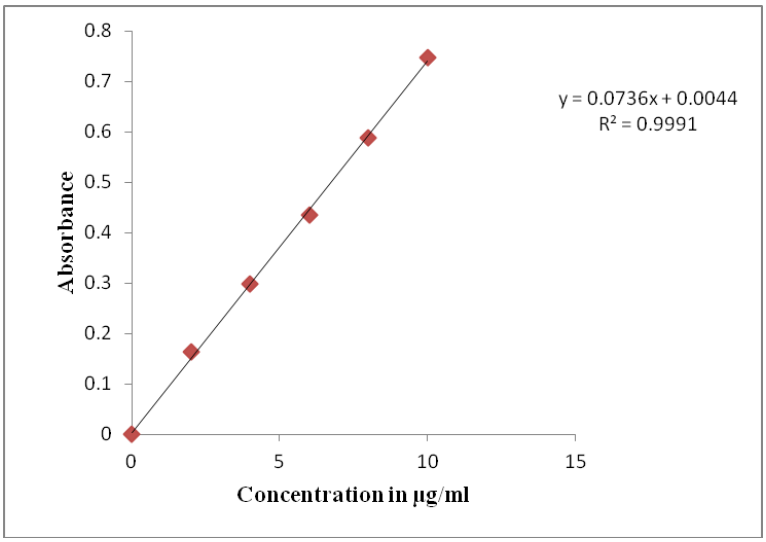


Figure no. 5.8: Standard calibration curve of Levofloxacin in pH 7.4 phosphate buffer.

5.4 FORMULATION OF LEVOFLOXACIN MICROSPHERES

In the present study an attempt was made to formulate levofloxacin microparticulate drug delivery system in order to localise the drug at absorption site, enhances its bioavailability, reduce dose thereby improving the patient compliance.

Levofloxacin microspheres were formulated using Bovine serum albumin (natural polymer) as carrier by emulsification heat stabilisation method. Table no 4.3 gives the experimental design and various independent variables like drug to polymer ratio, stirring speed and emulsifier concentration which have been changed at different levels. Each of these independent variables significantly influenced the various physico-chemical parameters of the microspheres.

5.5 CHARACTERISATION OF MICROSPHERES

5.5.1 Entrapment efficiency

Incorporation of therapeutic agent into albumin microsphere can be influenced by number of factors such as the method of preparation, drug and albumin concentration, drug and protein binding, physico chemical characteristics of drug and size of the microsphere. In the present study encapsulation of levofloxacin in albumin microsphere was investigated at drug to polymer ratio of 1:2 and 1:4 and encapsulation efficiency was found less i.e. in the range of 11.88 to 21.10 %. This may be due to fact that particles prepared by emulsification heat stabilisation method and particles have a room for loss of drug during manufacturing process.

The values of entrapment efficiency are shown in table no.5.3. The drug entrapment efficiency was found to be maximum in formulations F_7 and F_8. It was observed that drug entrapment efficiency was increased with increase in concentration of polymer added in consecutive formulation.

Table no. 5.3: Evaluation parameters of levofloxacin albumin microspheres.

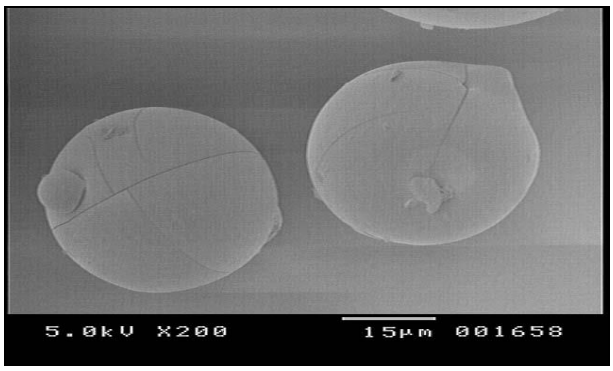
Formulation Code	Drug: Polymer	Theoretical loading (mg)	Actual Drug Loading(mg)	Encapsulation Efficiency (%)	Yield (%)
F_1	1:2	10	1.36 ± 0.215	13.63	78.00
F_2	1:2	10	1.34 ±0.035	13.43	81.66
F_3	1:4	10	1.56± 0.345	15.63	92.64
F_4	1:4	10	1.95± 0.185	19.53	87.40
F_5	1:2	10	1.25±0.285	12.52	84.60
F_6	1:2	10	1.10±0.150	11.00	81.10
F_7	1:4	10	1.81± 0.070	18.10	81.20
F_8	1:4	10	2.11±0.365	21.10	98.60

5.5.2 Surface morphology

Surface topography, particle size, morphology and internal cross sectional structure of microspheres were investigated with a scanning electricon microscopy. SEM is one of the commonly used method owing to the simplicity of sample preparation and easy operation.

SEM of the formulation F_7 is shown in figure no 5.9. The microspheres with smooth, spherical without porous/pores, discrete particles were obtained. The smoothness of the surface may be because of the drug concentration in the mixed solvent.

Very less particulate matter were seen on the surface of microsphere indicating uniform distribution of drug in the polymeric network.



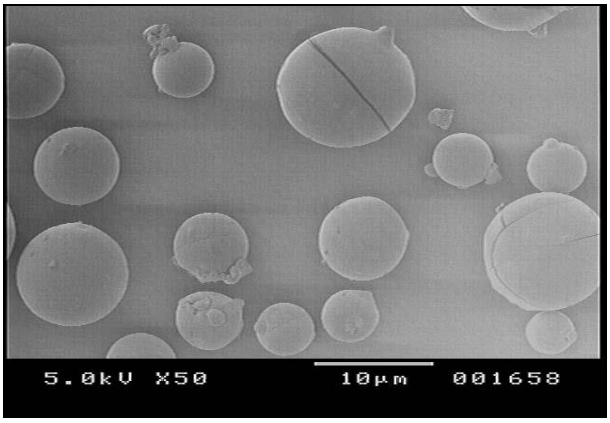


Figure no. 5.9: SEM photography of levofloxacin albumin microspheres.

5.5.3 Particle size

In vivo uptake of levofloxacin microsphere formulation largely depends on their particle size and route of administration. Following intra venous administration, larger particles (>7 µm) are reported to be trapped by mechanical filtration in the capillary bed in the lungs. Therefore, particle size is the key parameter that dictates the *in vivo* fate of a particulate drug delivery system.

Table no. 5.4 and figure no. 5.10 shows the average particle size of levofloxacin microsphere prepared by emulsification heat stabilization method. Results shows that no significant difference was observed in mean particle size in all batches.

The particle size of levofloxacin albumin microsphere ranged from 12.33 to 18.73 µm, it was observed that the size of microspheres was increased with increase in concentration of polymer, the size of microsphere was decreased with increase in stirring rate and increase in concentration of emulsifying agent.

All these results indicated that the particle size of microspheres could be modified by varying factors such as concentration of polymer, concentration of emulsifying agents and stirring speed.

Table no. 5.4: Average particle size of levofloxacin microspheres ($F_1 – F_8$).

Formulation Code	Average particle size(µm)
F_1	16.66 ±4.50
F_2	14.23 ±1.72
F_3	18.73 ±2.41
F_4	16.66 ±5.50
F_5	16.00 ±3.60
F_6	13.00 ±2.00
F_7	12.33 ±2.51
F_8	15.66 ±3.05

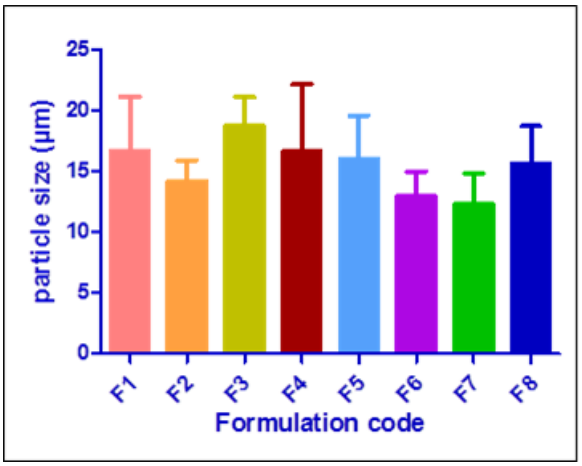


Figure no. 5.10: Average particle size of levofloxacin microspheres ($F_1 – F_8$).

5.6 In vitro drug release

In vitro drug release pattern were studied using a conventional dialysis technique, levofloxacin albumin microspheres were placed in dialysis bag and dialyzed against 200 ml of pH 7.4 phosphate buffer.

In vitro drug release profile of levofloxacin albumin loaded microsphere for different time periods are shown in table no 5.5 and figure no.5.11. Levofloxacin microsphere exhibited a biphasic release pattern, where an initial burst release of around 25-35% of drug was observed during initial 1 hour, this burst release was followed by a controlled release. Cumulative % of drug release was found to be between 90-100% after 24 hours. These results indicated that water soluble nature of levofloxacin might be the reason for initial burst release.

In clinical practice this would lead to burst effect which enables the preparation to show fast effect to the patient. However the levofloxacin release from albumin microspheres was extended upto 24 hours. During this period the released amount was more than 95%. This result indicated that albumin containing levofloxacin had a well controlled release efficacy**.**

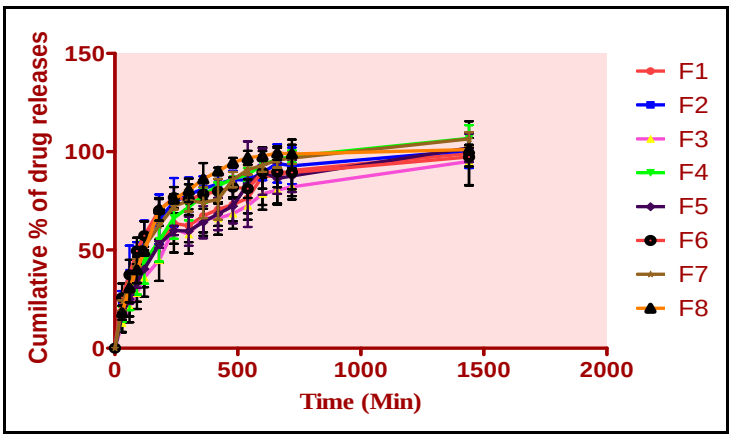


Figure no. 5.11: *In Vitro* Dissolution profile of Levofloxacin microspheres (F_1-F_8).

5.7 Release kinetics

The data obtained from *in vitro* release studies fitted to various kinetics equation (i.e. First order, Higuchi, Korsmeyer-Peppas and Hixson Crowell equation) to determine the mechanism of drug release and the correlation coefficient values R^2 is showed in the table no 5.6, taken into account to decide upon the relevance of the model curve fit which will best describe the extent of fit. According to the R^2 values given by different data fits form albumin microspheres, first order model to be an ideal fit having R^2 value 0.923 to 0.983.

According to Peppas fit, the release of the drug was decided diffusion of the drug from polymeric matrix and release is governed by Fick's law of diffusion.

The release pattern was classian Fickian diffusion aided by initial swelling of microspheres and releasing of drug particles was controlled and sustained for longer period of time due to diffusion.

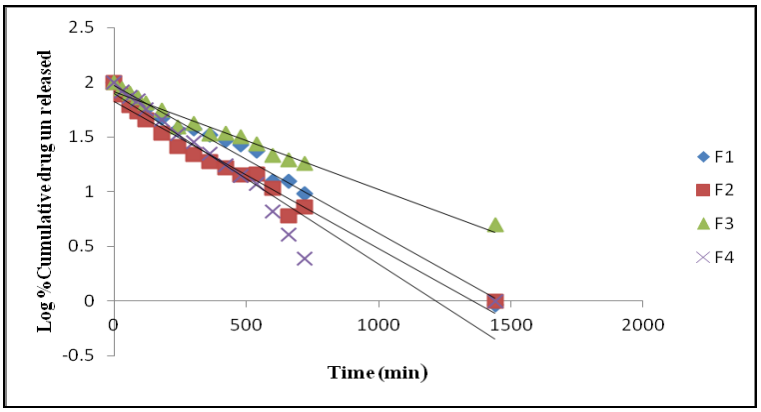


Figure no. 5.12: First order release kinetics of Formulation F_1-F_4.

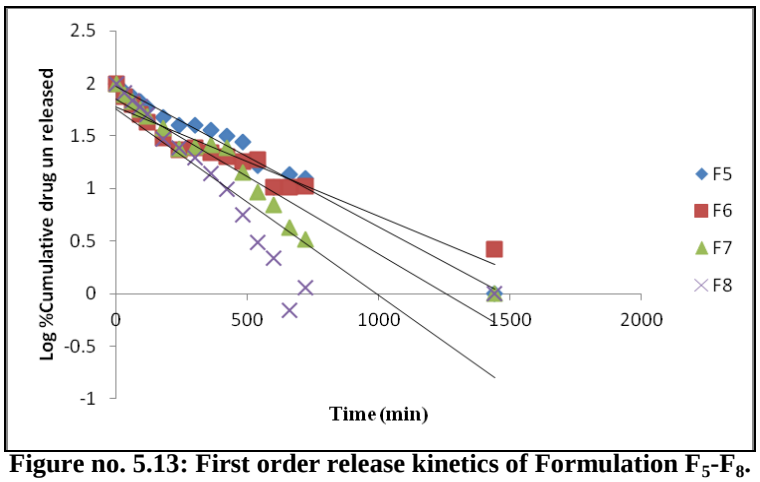


Figure no. 5.13: First order release kinetics of Formulation F_5-F_8.

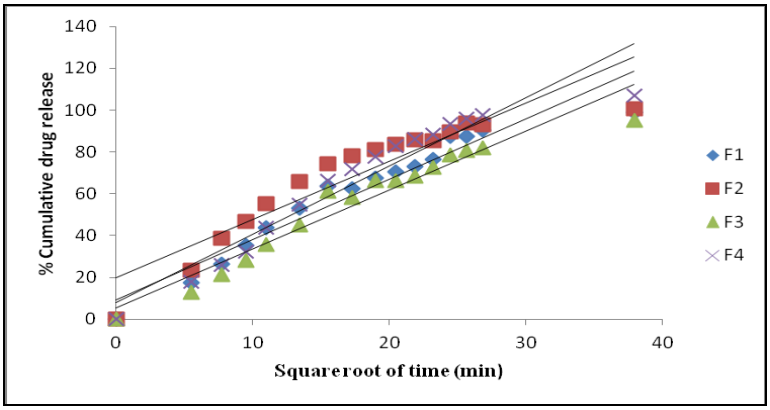


Figure no. 5.14: Higuchi release kinetics of Formulation F1-F4.

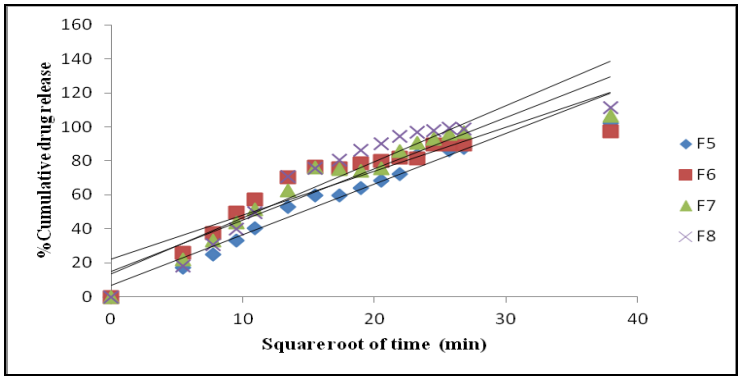


Figure no. 5.15: Higuchi release kinetics of Formulation F5-F8.

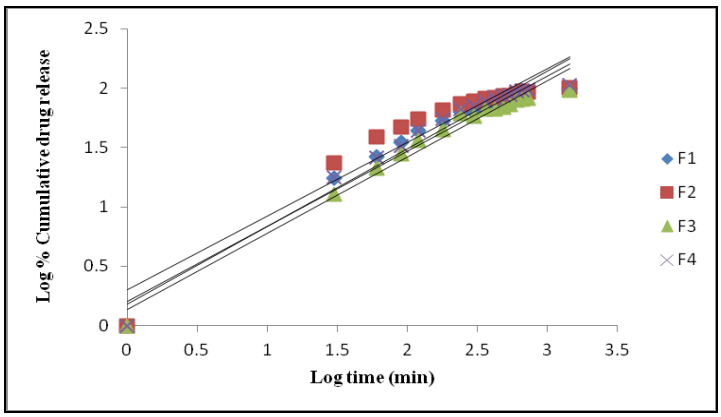


Figure no. 5.16: Korsmeyer-Peppas release kinetics of Formulation F1-F4.

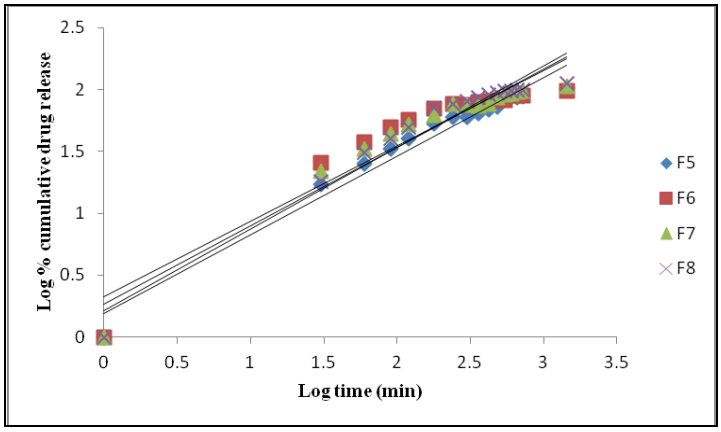


Figure no. 5.17: Korsmeyer-Peppas release kinetics of Formulation F4-F8.

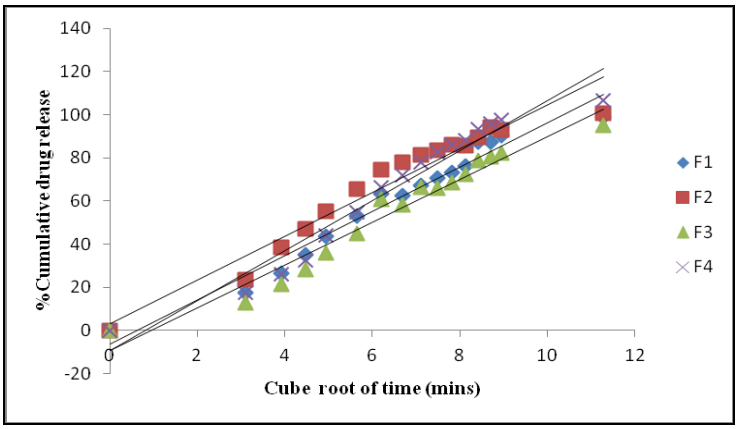


Figure no. 5.18: Hixson-Crowell release kinetics of Formulation F1-F4

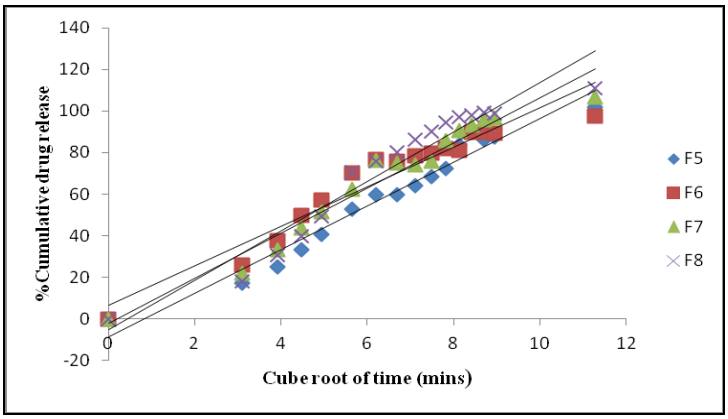


Figure no. 5.19: Hixson-Crowell release kinetics of Formulation F5-F8.

Table no. 5.6: Correlation coefficient data of Release Kinetics study.

Batch Code	Zero Order	First Order	Huguchi Matrix	Korsemeyer Peppas		Hixson- Crowel
	R^2	R^2	R^2	R^2	N	R^2
F_1	0.688	0.983	0.933	0.956	0.632	0.964
F_2	0.588	0.970	0.864	0.912	0.621	0.944
F_3	0.726	0.977	0.939	0.973	0.643	0.956
F_4	0.709	0.924	0.922	0.964	0.656	0.950
F_5	0.738	0.980	0.946	0.963	0.637	0.963
F_6	0.556	0.925	0.836	0.890	0.609	0.928
F_7	0.657	0.931	0.905	0.933	0.633	0.956
F_8	0.626	0.773	0.884	0.948	0.658	0.936

5.8 *IN-VIVO* STUDIES

5.8.1 Pharmacokinetic and tissue distribution studies

5.8.1.1 Pharmacokinetic studies

The reverse phase HPLC method was selected for estimation of levofloxacin in plasma. The commonly available UV detection with protein precipitation extraction was followed in the present study because of its simplicity and selectivity. The blank plasma was analyzed prior to the analysis of levofloxacin standard preparation. There was no interference from blank plasma. Propyl paraben was selected as internal standard, the peak were well resolved and the retention time of levofloxacin and Propyl Paraben were 2.3 and 8.6 min respectively, as shown in figure no 5.20. Table no 5.7 shows the standard levofloxacin concentration with peak area and figure no 5.21 show standard calibration curve with an linear regression value of 0.9732.

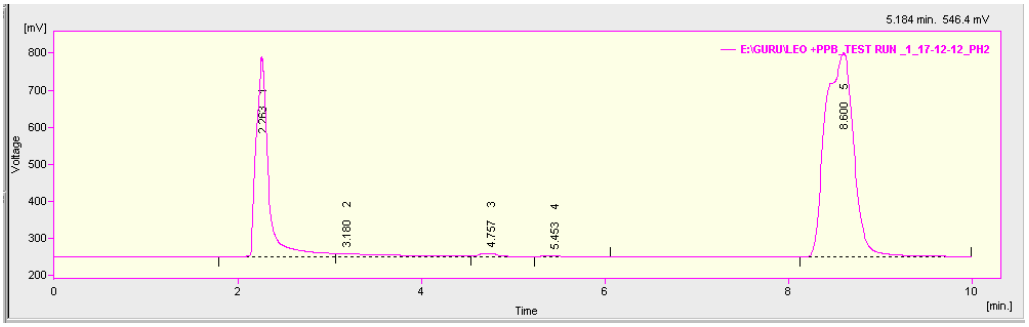


Figure no. 5.20: Typical chromatogram of reference standard with internal standard.

Table no. 5.7: Linear plot of levofloxacin by HPLC.

Sl.no	Concentration (µg/ml)	Average Peak area	Statistical parameter
1	0.5	28.00	y=23.186x + 12.397 R^2= 0.9732
2	1.0	32.70	
3	1.5	44.99	
4	2.0	55.94	
5	2.5	75.55	
6	3.0	81.15	

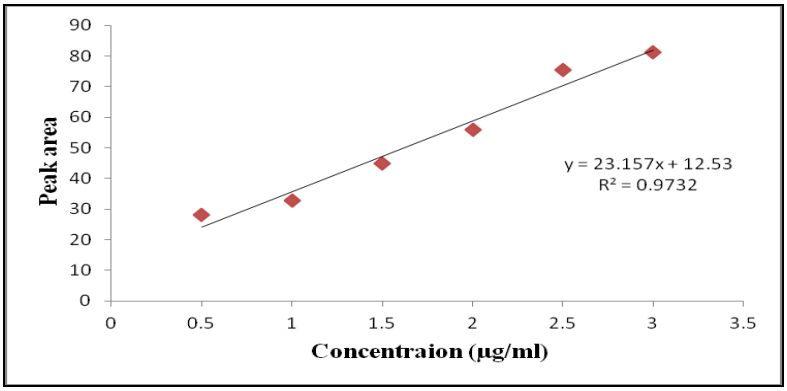


Figure no. 5.21: HPLC calibration curve of Levofloxacin.

Optimised formulation (F_7) with optimal particle size and satisfaction *in vitro* release was selected for *in vivo* pharmacokinetic studies. Plasma drug concentration of levofloxacin microsphere (F_7) and levofloxacin injection at different time intervals were showed in the table no 5.8 and figure no 5.22 respectively. The pharmacokinetic paremeter were calcultated from peak area. Levofloxacin absorption after intravenous administration was rapid with levofloxacin injection as indicated by high T_{max} value of 1 hour. However the C_{max} value was high with injectable microspheres (F_7) indicated minimum amount of drug in plasma. The elimination half life of levofloxacin with injection was less indicating that the drug rapidly excreated from body. It was further supported by high elimination rate constant value (K_e) of injection in comparision with prepared microsphere. The half life and Ke values indicated that prepared micropsheres remain in the body when compared to injection. Microspheres showed high AUC_{0-t} value in comparision with injection which may give greater bioavalibility of the drug from microsphere.

Hence the pharmacokinetic study indicates microspheres released the drug slowly with higher bioavailability in comparision with injection. This could be due to

controlled release of drug from microspheres (F_7). Pharmacokinetic parameters are shown in table no 5.9.

Table no. 5.8: Plasma concentration of Levofloxacin microsphere (F_7) against Levofloxacin injection.

Time in hours	Concentration in µg/ml	
	Levofloxacin injection	Levofloxacin microsphere (F_7)
0.17	23.07±0.99	27.41±1.21
0.5	26.29±0.56	23.54±0.87
1	79.29±0.44	26.66±0.79
3	20.85±0.98	30.24±0.45
6	20.85±1.12	25.49±0.66
12	20.95±0.34	21.06±0.91

All these values are expressed as mean ± Sd n=3.

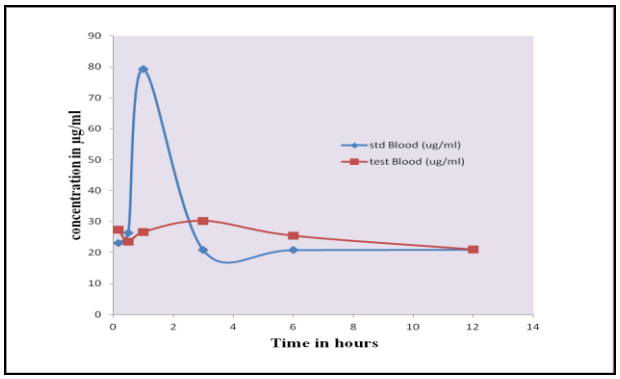


Figure no. 5.22 Plasma drug concentration of levofloxacin microspheres (F_7) and levofloxacin injection at different time intervals.

Table no. 5.9: Pharmacokinetic parameters from the plasma concentration time curve.

Parameters	Levofloxacin injection	Levofloxacin microsphere(F_7)
C_{max}	33.04 µg/ml	12.74 µg/ml
T_{max}	1.00 hrs	3.00 hrs
AUC_{o-t}	144.84 µg h/ml	89.34 µg h/ml
$t_{1/2}$	8.22 hrs	18.15 hrs
K_e	0.084 h^{-1}	0.038 h^{-1}

All these values are expressed as mean ± Sd n=3

Where,
C_{max} = Maximum concentration that a drug attains after dosing.
T_{max} = Time at which maximum concentration of drug present in the serum.
AUC_{o-t} = Area under curve
$t_{1/2}$ = Half life
K_e = Elimination rate constant.

5.8.1.2 *In-vivo* tissue distribution studies

Same optimized formulation (F_7) was selected for *in vivo* drug targeting studies. The comparison between the amount of drug targeted from microspheres and injection in various organs was analyzed using non-compartmental method using PK 2.0 solver version. The drug content (µg/ml) with time was determined in blood, lungs, liver and spleen as shown in table no 5.10, figure no.5.25 and 5.26 respectively.

Table no. 5.10: Distribution of drug in various organ.

Sl.no	Time (Hours)	Lungs		Liver		Spleen		Plasma	
		LI	LM	LI	LM	LI	LM	LI	LM
1	0.17	24.10	22.15	32.01	19.23	10.27	6.04	45.48	11.57
2	0.5	25.27	22.17	37.66	22.44	9.86	7.45	37.88	9.96
3	1.0	29.85	32.27	39.12	24.96	9.97	4.92	33.04	11.26
4	3.0	53.77	38.96	36.31	37.42	8.00	5.50	8.85	12.74
5	6.0	50.12	50.12	39.98	35.19	8.00	4.86	10.40	10.77
6	12.0	38.52	53.23	46.35	37.96	4.12	4.63	8.89	8.94

Where, LI= Levofloxacin injection and LM= Levofloxacin microsphere (F_7)

The organ targetability of levofloxacin albumin microsphere showed more amount of drug present primarily in the lungs, secondarily in liver and very small amount of drug present in spleen and plasma when compared to i.v administration of levofloxacin injection. Hence, the present study proved the hypothesis of passive targeting of microsphere formulation to lungs when compared with conventional levofloxacin injection.

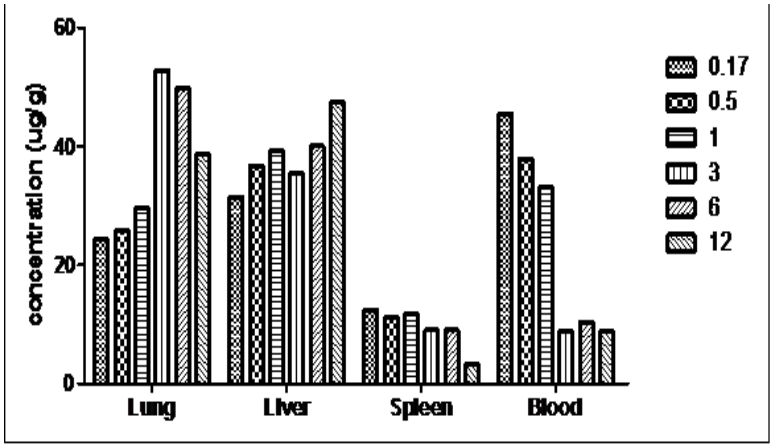


Figure no. 5.23: Distribution of drug in albino rats after levofloxacin injection in tissue and blood.

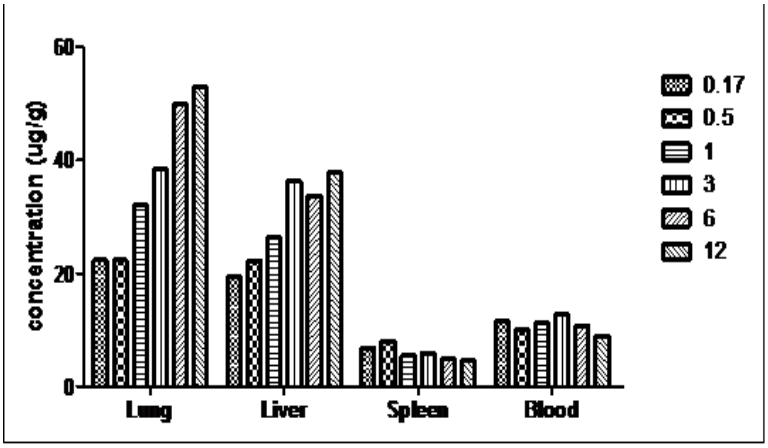


Figure no 5.24 Distribution of drug in albino rats after injection of levofloxacin microspheres in tissue and blood

Compartment model

***In vivo* pharmacokinetics of microspheres**

The *in vivo* data of levofloxacin microsphere was subjected to compartment modeling using PK 2.0 solver version pharmacokinetic software, and the data was fitted to one compartment model and two compartment model respectively, based on the analysis of models and parameters, the actual plot exactly superimposed on predicted line. From the graph it was concluded that the *in vivo* pharmacokinetic of microsphere in blood could be best described by two compartment model with i.v. injection.

One can see that in comparison with levofloxacin injection, levofloxacin microspheres altered the distribution of drug *in vivo* and also half life after i.v. injection of microspheres ($t_{1/2}$ of levofloxacin injection is 8.22 hrs, levofloxacin microspheres is 18.15 hrs) were remarkably higher, this results showed that prepared microspheres had sustained release efficacy.

The drug concentration in tissue indicated that the microspheres targeted the levofloxacin mainly to lungs after i.v. injection to albino rats and the concentration of levofloxacin in lungs was significantly more than that in any other organ and blood.

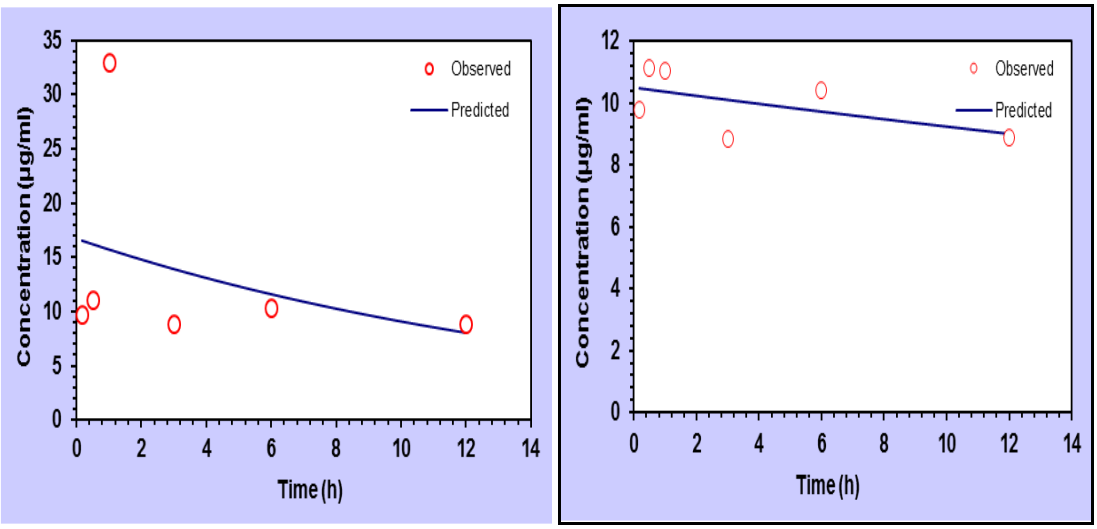


Figure no. 5.25: One-compartment model following i.v. administration of a) Levofloxacin injection b) Levofloxacin albumin microspheres.

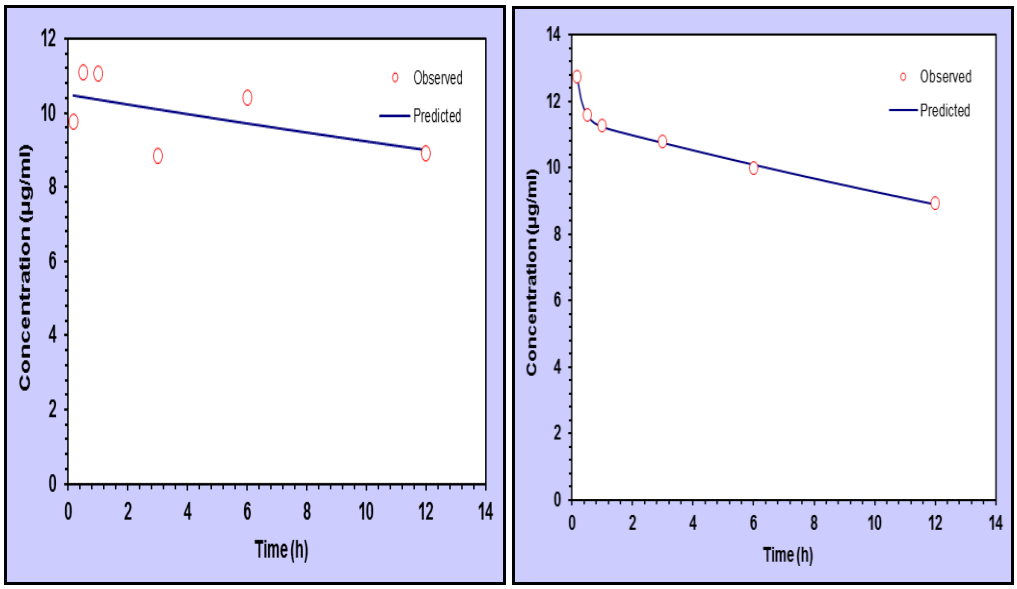


Figure no. 5.26: Two-compartment model following i.v. administration of a) Levofloxacin injection b) Levofloxacin albumin microspheres.

5.8.2 HISTOPATHOLOGICAL STUDIES

In case of targeted drug delivery system such as microspheres, major portion of drug and excipients were accumulated in specific tissue (lung) and therefore determining the compatibility between these tissues and the microsphere formulation become a necessity in order to rectify the safety of the formulation.

On comparison of microspheres formulation with control group, the cyto architecture of the tissue (lungs) did not show any major difference i.e. degenerative changes as evidenced in figure no 5.27 to 5.29, based on these observation, the constituents of the microsphere formulation were declared safe for parenteral use as a passive targeted drug delivery system to lungs.

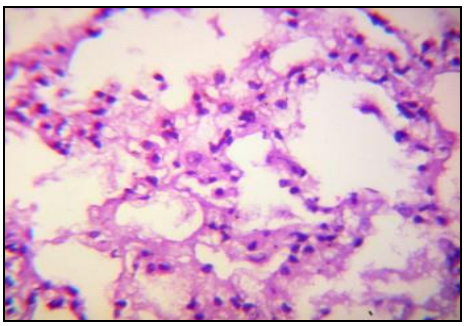


Figure no. 5.27: Cytoarchitecture of lung (control).

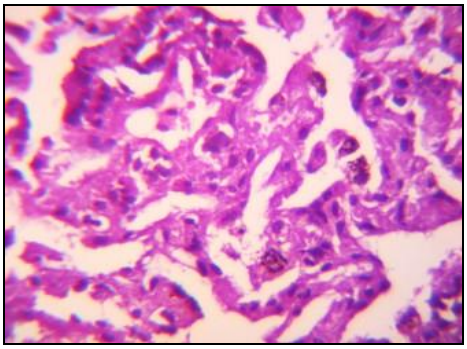


Figure no. 5.28: Cytoarchitecture of lungs after administration of levofloxacin injection.

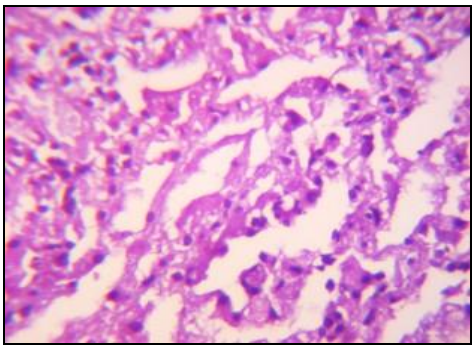


Figure no. 5.29: Cytoarchitecture of lungs after administration of levofloxacin albumin microspheres.

5.9 STABILITY STUDIES

All the regulatory bodies accept only real time stability data for any drug or pharmaceutical for the purpose of accessing shelf life and accelerated stability studies may only serve as tool for formulation screening and stability issue related to shipping or storage at room temperature. On storage, the optimized microsphere (F_7) did not show any change in physical properties and as well as no remarkable change in the drug content profile as indicated in the table no 5.11 which were in the acceptable range. At the end of 90 days indicating that the optimized formulation (F_7) was fairly stable at accelerated storage condition.

Table no. 5.11: **Stability study of formulation F_7.**

Time (month)	Drug content (%)
Zero	18.10±0.08
First	17.92±0.16
Second	17.81±0.14
Third	17.75±0.21

CONCLUSION

The major conclusion of this work was that microsphere constitutes a useful mean passive targeting of drug to the target organ and can be employed for effective antibacterial therapy.

Controlled release injectable microspheres of levofloxacin were made from biodegradable polymer such as bovine serum albumin (BSA). BSA has an excellent record of biocompatibility, biodegradability and non-immunogenicity.

- Levofloxacin loaded BSA microspheres were prepared by emulsification heat stabilisation method due to its ease of preparation and efficacy.
- Interaction studies from FT-IR and DSC ruled out any possibility of drug polymer interaction.
- The prepared microspheres were found to be possess suitable physico-chemical properties and particle size range ensured by optimisation and all the formulation showed ideal surface morphology.
- The *in vitro* release studies showed biphasic release pattern for all formulation, with an initial burst effect which may be attributed due to the more water soluble nature of the drug.
- Overall the curve fitting into various mathematical models and *in vitro* release of formulations best fitted into the First order with Korsmeyer peppas model, this indicated that the microspheres follow Fickian controlled release mechanism.
- On the basis of drug content, particle size, surface morphology and *in vitro* release characteristics formulation F_7 was selected as an optimum formulation for *in vivo* pharmacokinetic tissue distribution and stability studies.
- *In vivo* drug targeting studies indicated that microsphere could be targeted preferentially to lungs than other organs. The order of targeting was found to be lungs > liver> spleen.
- The present study showed that the targeting efficiency of drug loaded microspheres over injection was higher, which may provide increased therapeutic efficacy, moreover higher concentration of drug targeted to lungs may help in reduction of dose required for the treatment of pneumonia and thereby dose related side effects could be minimised.
- Stability studies of selected formulation F_7 showed that closest in drug content to initial data which focused at 40 ± 2^{o}C storage.

Future scope

Present work was a satisfactory in targeting of levofloxacin to lungs using BSA microspheres. Further detailed investigation and elaborated *in vivo* targeting studies need to be carried out. *In vitro in vivo* correlation need to be established to guarantee the efficacy and bioavailability of the formulation.