POLYMER MEDIATED DELIVERY OF ANTIDIABETIC DRUG

A THESIS

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By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **POLYMER MEDIATED DELIVERY OF ANTIDIABETIC DRUG** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee, is an authentic record of my own work carried out during a period from July 2003 to February 2008 under the supervision of Dr. V. Pruthi, Asstt. Professor, Department of Biotechnology, IIT Roorkee and Dr. V.R. Sinha, Professor, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

With the aid of engineering and biotechnology, biomedical and pharmaceutical engineering has emerged as a fascinating field with applications in diverse areas such as drug delivery, medical imaging and nanotechnology. In the pharmaceutical industry, novel drug delivery technologies represent a strategic tool for expanding drug markets. These new technologies can address issues associated with current pharmaceuticals, by extending product life or by adding to their performance and acceptability, either by increasing efficacy or improving safety and patient compliance. Oral delivery of drugs, especially proteins, is difficult since bioavailability is limited by the epithelial barriers of the gastrointestinal tract and gastrointestinal degradation by digestive enzymes. Carrier technology with biodegradable polymers offers an intelligent approach for drug delivery by entrapping the drug in a carrier particle such as microspheres or nanoparticles which modulate the release and absorption characteristics. Protein delivery through polymeric microspheres and nanospheres is expected to create innovations and play a critical role in drug delivery. Biodegradable polymer microspheres are preferred because surgical removal of spent device, as in the case of implants, is not required. Further, there is minimal possibility of toxicological problems. Their release rates can be tailored and they degrade in biological fluids to produce biocompatible or non-toxic products in the body, which are removed by normal physiological pathways. Both natural and synthetic biodegradable polymers have been studied for drug delivery purposes. Among the biodegradable polymers, poly-ε-caprolactone (PCL) and its derivatives, with a high permeability to many therapeutic drugs and lack of toxicity, are well suited for oral and controlled drug delivery. Considerable research effort has been devoted to developing suitable, painless delivery systems to replace injectable

insulin dosage forms. An oral insulin dosage form would be preferred by diabetic patients over the currently available parenteral formulations.

Microspheres of PCL were prepared by using by w/o/w double emulsion solvent evaporation technique. The results showed the formation of smooth spherical PCL microspheres. The effect of different solvents (chloroform, dichloromethane and ethyl acetate) in combination with stabilizers (polyvinyl alcohol and hydroxypropyl methylcellulose) on the percent yield and surface morphology of PCL microspheres was studied. It was found that microspheres formed with dichloromethane as the solvent and HPMC as the stabilizer gave the best microspheres in terms of smoothness and were devoid of pits. Other formulational parameters like the stabilizers (PVA or HPMC) and their concentrations in the range of 0.5% to1.5%, polymer concentration in the range of 0.5% to 2.0% and the drug concentration, either 40 IHU/ml or 100 IHU/ml were varied. The responses were evaluated in terms of percent yield, percent entrapment efficiency, particle size and surface morphology. On the basis of these experiments, it was found that PCL microspheres prepared with 1% polymer concentration and 1% solution of HPMC as the stabilizer gave the optimum results of 92.2 ± 0.26% yield and an entrapment efficiency of 65.72 ± 0.14%. Micro BCA Protein Estimation Kit (PIERCE®) was used for the estimation of the amount of drug present in the microspheres. Surface morphology of the microspheres was studied by scanning electron microscopy. The morphology of the microspheres prepared with 1% PCL and 1% HPMC concentration was smooth and spherical, with the average mean particle diameter being 2.33 μm. The changes in morphology of the microspheres, when kept in contact with the release medium, with time were determined by scanning electron microscopy.

Characterization of biodegradable polymeric microspheres used for drug delivery is essential to ensure reproducible results of *in-vitro* and *in-vivo* drug release profiles. Different advanced techniques were used to further characterize the microspheres prepared. Confocal

laser scanning microscopy was used to determine the drug distribution within the microspheres. This was achieved by fluorescently labeling insulin with FITC and PCL with nile red and viewing the microspheres formed under laser adjusted in the green/red fluorescence mode resulting in two excitation wavelengths at 488 and 514 nm. Drug polymer interaction was studied by differential scanning calorimetry (DSC). The DSC curves of unloaded PCL microspheres and insulin-loaded PCL microspheres were compared. The T_m of PCL polymer was found to be 66°C, and T_m, for the unloaded and insulin-loaded PCL microspheres was found to be 59.7°C and 62°C respectively confirming no major change in the T_m of the polymer, since polymers usually exhibit a melting range instead of a sharp melting point. Residual solvent in the microspheres was determined by gas chromatography. Residual content of dichloromethane was found to be well within the pharmacopoeial limits of 600 ppm and indicated safe use of microspheres for oral administration.

The *in-vitro* insulin release profile from the PCL microspheres was evaluated. An initial burst release, followed by a slower release of insulin from the microspheres was observed. Since PCL is a slow degrading polymer, the predominant mechanism of insulin release from these microspheres is diffusion. The initial burst release may be explained due to the drug desorption from the particle surface. The effect of polymer, stabilizer and drug concentration on the *in-vitro* release profiles was also studied. It was found that microspheres prepared with increased polymer concentration showed the least drug release. When microspheres were formulated with a low stabilizer concentration in the external water phase, they showed a more rapid insulin release. A correlation was also established between the encapsulation efficiency and the release rate. Results showed that higher the insulin concentration used, higher was the insulin encapsulation within the microsphere which further led to a more rapid release rate.

The biological efficacy of the insulin-loaded microspheres was determined after oral administration in diabetic rats and rabbits. In order to determine hypoglycemic effect of the

insulin-loaded PCL microspheres, the microspheres were administered orally to overnight fasted diabetic rats and rabbits. Blood glucose concentrations were measured by AccuChek® blood glucometer (Roche, Germany) and the changes in blood glucose level versus time profiles were observed. The results showed that the encapsulation of insulin into microspheres prepared with PCL allowed the preservation of its biological activity alongwith its prolongation of action, following its oral administration in fasted and fed diabetic animal models.

To further evidence our *in-vivo* findings, pharmacoscintigraphic evaluation was carried out in the animal models to ascertain the absorption and distribution characteristics of the radiolabeled drug. Insulin was radiolabeled with radionuclide, ^{99m}Tc, and encapsulated into the polymeric microspheres. The radiolabeling efficiency of the ^{99m}Tc-insulin radiocomplex was evaluated by instant thin layer chromatography (ITLC) and found to be 99.2%. Stability studies of ^{99m}Tc-insulin complex revealed that the radiocaomplex retained high stability (97%) both in saline and serum. In order to determine the absorption and distribution characteristics of the radiolabeled insulin, gamma camera imaging of diabetic Male New Zealand rabbits and *Sprague-Dawley* rats administered with ^{99m}Tc-insulin loaded microspheres, free ^{99m}Tc-insulin and ^{99m}Tc-insulin loaded microspheres administered alongwith ethanol as enhancer was carried out. The gamma camera images showed showed that after 4 hours of oral administration, animals fed with ^{99m}Tc-insulin loaded PCL microspheres showed significantly higher diffusion into the stomach and intestine besides some diffusion in other tissues like paws and visceral organs as compared to those fed with free ^{99m}Tc-insulin without loading into PCL microspheres. Biodistribution of free ^{99m}Tc-insulin and ^{99m}Tc-insulin loaded PCL microspheres in heart, blood, liver, stomach and intestine in *Sprague-Dawley* rats was studied at various time intervals after its oral administration. It was observed that among all the organs studied, stomach exhibited highest radioactivity per gram organ for both ^{99m}Tc-insulin loaded PCL microspheres and free ^{99m}Tc-

insulin. On the contrary, intestine and stomach showed highest radioactivity per whole organ for ^{99m}Tc-insulin loaded microspheres and free ^{99m}Tc-insulin respectively.

To determine any toxic effects of the insulin-loaded PCL microspheres, toxicity studies were carried out in *Sprague-Dawley* rats. Histopathological studies of liver, stomach, small intestine and large intestine were carried out. Results showed that two month of repetitive dose of PCL microspheres to experimental animals showed no adverse effect on histopathology of the tissues studied.

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Anindita Mukerjee

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CHAPTER 1

INTRODUCTION

1.1 PREAMBLE

Major advances in the field of pharmaceutical biotechnology together with the explosion of genomic and proteomic information have increased the interest in newer proteins enormously. The understanding of protein functions in the etiopathology of currently incurable diseases has led to the discovery of a large number of bioactive molecules and vaccines based on peptides, proteins and oligonucleotides (Suriano et. al., 2006; Suriano et. al., 2005). This, in turn, could result in new therapeutic approaches and drug delivery systems to deliver such complex molecules.

Research in various fields of medicine and drug delivery technologies has increased steadily over the past few years (Glod et. al., 2006; Kamen and Glod, 2004; Foo et. al., 2007; Glod et. al., 2002). The cost involved in developing a new molecule, considering all the post-launch expenses which include marketing, advertising, education and training, is about $ 800 million to $1 billion. Thus, there is major emphasis on developing novel drug delivery technologies that are designed to target the therapeutic molecule to the desired site. Such delivery systems can be developed much faster (3-4 years) and at a relatively lower cost ($20 to $50 million). It has been seen that with the development of various drug delivery technologies, a rebirthing of existing molecules has taken place which have resulted in increasing their market values along with patent lives of such molecules. Drug delivery technologies also enable us to deliver difficult-to-deliver compounds, offer improved efficacy, safety and patent compliance to existing drugs. As targeting of the molecule at the desired site of action is possible with some technologies, smaller amounts of drugs are required to achieve the appropriate concentration at that specific site. This further results in reducing the side effects associated with higher doses of the drug. These advanced drug

delivery technologies are required to deliver complex molecules like proteins and other genctically engineered pharmaceuticals to their site of action so that their delivery is accurate, modulated and effective.

In the pharmaceutical industry, novel drug delivery technologies represent a strategic tool for expanding drug markets. The last two decades have witnessed an avant-grade interaction among fields of polymer and material science, resulting in development of novel drug delivery systems. These new technologies can address issues associated with current pharmaceuticals, by extending product life or by adding to their performance and acceptability, either by increasing efficacy or improving safety and patient compliance (Sahoo and Labhasetwar, 2003; Mishra et. al., 2006; Kumar and Mishra, 2006; Mishra et. al., 2005; Singh et. al., 2004; Ahuja et. al., 2003).

Despite the rapid progress in novel drug delivery systems, oral delivery of drugs, especially proteins, is difficult since bioavailability is limited by the epithelial barriers of the gastrointestinal tract and the gastrointestinal degradation by digestive enzymes. Indeed, their large molecular size, their short plasma half-life, their biodegradation by enzymes, and the importance of maintaining a correct conformation all limit their biological activity.

The development of suitable, convenient and effective oral delivery systems for proteins and peptides remains a major challenge for pharmaceutical scientists. Carrier technology offers an intelligent approach for drug delivery by entrapping the drug in a carrier particle such as microspheres and nanoparticles, thus protecting them against any enzymatic degradation and can also modulate the release and absorption characteristics. This concept of controlled drug delivery in order to obtain specific release rates or spatial targeting of active ingredients has led to an enormous impact on the health care system.

Polymeric micro and nanoparticle carriers have important potential applications for the administration of therapeutic molecules (Ravi Kumar, 2000). Incorporating the protein drug inside the microparticles has more advantages as compared to adsorbing the protein on the

particle surface (Armstrong et. al., 1997) as the later led to limited loading capacity and uncontrolled desorption of the drug, leading to erratic drug profiles where release rates cannot be tailored. Protein delivery through polymeric microspheres is expected to create innovations and play a critical role not only in drug delivery but also in target-specific drug therapy.

Biodegradable polymer microspheres are preferred because surgical removal of spent device, as in the case of implants, is not required. Further, there is minimal possibility of toxicological problems. Their release rates can be tailored and they degrade in biological fluids to produce biocompatible or non-toxic products in the body, which are removed by normal physiological pathways. Natural biodegradable polymers which have been studied for the delivery of various drugs include proteins like albumin, globulin, gelatin, collagen and casein and polysaccharides like starch, cellulose, chitosan, dextran and alginic acid. However, residual monomers could interact with the protein leading to its denaturation. In addition, residues of the polymerization medium can be more or less toxic. Therefore, encapsulation techniques were researched and developed by using new preformed polymers. Synthetic biodegradable polymers like polyorthoesters, polyanhydrides, polyamides, polyalkylcyanoacrylates, polyesters like PLA, PLGA, polyphosphazenes have been extensively studied for drug delivery purposes (Sinha and Trehan, 2003). Among the biodegradable polymers, poly-ε-caprolactone (PCL) has been receiving increasing attention as matrix materials for controlled release drug formulations. PCL and its derivatives, with a high permeability to many therapeutic drugs and lack of toxicity (Kim et. al., 1985), are well suited for oral and controlled drug delivery.

There are several reported techniques for the preparation of microspheres entrapping drugs like spray drying, coacervation technique, solvent emulsion-evaporation technique, ionotropic gelation etc. The double emulsion technique, among other incorporation methods, has been reported to show better protein stability (Tabata et. al., 1993) alongwith

higher microencapsulation of hydrophilic drugs and proteins within microparticles (Alex and Bodmeier, 1989; Cohen et. al., 1991).

1.2 RATIONALE OF STUDY

Throughout the world, considerable research effort has been devoted to developing appropriate, painless delivery systems to replace injectable dosage forms (Peppas, 2004; Fossa et. al., 2004; Kim and Peppas, 2003; Nakamura et. al., 2004; Morishita et. al., 2004). Since the parenteral administration is the only route of insulin delivery, alternative routes of administration (oral, nasal, rectal, pulmonary and ocular) have been extensively investigated (Owens et. al., 2003). Among them, the oral route seems to be the most convenient and being painless, would be preferred by diabetic patients over the currently available parenteral formulations. Also, insulin from an oral dose undergoes a first hepatic bypass, thus warranting a primary effect by inhibiting hepatic glucose output (Damgé et. al., 2007). Insulin, at the same time, is strongly degraded by proteolytic enzymes in the gastrointestinal tract and is poorly absorbed from the intestine. Therefore, to protect it from biodegradation and to improve its intestinal absorption, insulin has been associated to antiproteases (Liu et. al., 2003), hydrogels (Morishita et. al., 2004), combined with absorption enhancers such as cyclodextrins (Shao et. al., 1994), bile salts (Mesiha et. al., 2002) and surfactants (Eaimtrakarn et. al., 2002). Insulin was also encapsulated in polymeric biodegradable nanocapsules, nanospheres or microparticles associated or not to sufactants or antiproteases (Damgé et. al., 1988; Damgé et. al., 1997; Radwant et. al., 2002). Thiolated or mucoadhesive polymers of polyacrylic nature (Krauland et. al., 2004; Krauland and Bernkop-Schnurch, 2004), chitosan-coated liposomes (Takeuchi et. al., 1996) or liposomes encapsulated in alginate-chitosan gel capsules (Ramadas et. al., 2000) also increased the residence time of insulin in the vicinity of intestinal absorptive cells allowing its absorption (Damgé et. al., 2007).

1.3 SCOPE OF PRESENT STUDY

Based on these findings, the scope of our present work was defined as the development of a formulation technique for the efficient encapsulation of the protein drug, insulin, in microspheres of a biodegradable polymer, PCL, using a water-in-oil-in-water (w/o/w) double emulsion solvent evaporation method. The optimization of this formulation process would be carried out by varying some of the influencing process parameters during the preparation steps. The insulin loaded PCL microspheres would be characterized and studies on drug-polymer interaction, drug distribution and residual solvent analysis would also be done. These microspheres would be evaluated for their *in-vitro* release profiles. The biological efficacy of insulin-loaded micropheres would be determined after oral administration in diabetic rats and rabbits. Further to ascertain the absorption and distribution mechanisms of the insulin loaded PCL microspheres, pharmacoscintigraphic studies would be carried out in the animal models. Finally, the toxicity of these polymeric microspheres would be studied in healthy rats to determine any harmful effects of the formulation with histopathological analysis.

1.4 SPECIFIC OBJECTIVES OF PROPOSED RESEARCH

To design and develop a suitable dosage form of insulin loaded PCL microspheres using double emulsion solvent evaporation technique for oral drug delivery in order to improve the patient compliance.

Studies undertaken in this proposed research work include:

a) **Pre-formulation Establishment of Drug Estimation Procedures**

b) **Formulation of the Biodegradable Microspheres**

c) **Characterization of the Microspheres**

- *Determination of Percent Yield*
- *Determination of Percent Encapsulation Efficiency*

- *Surface Morphology Studies*
- *Particle Size Analysis*

d) Advanced Characterization Studies of the Microspheres

- *Drug Distribution Studies*
- *Drug Polymer Interaction Studies*
- *Atomic Force Microscopic Studies*
- *Determination of Residual Solvent*

e) Evaluation of the Microspheres

- *In-vitro Drug Release Studies*
- *In-vivo Studies*

f) Pharmacoscintigraphic Studies

- *Determination of Radiolabeling Efficiency*
- *Determination of Radiolabeling Stability*
- *Determination of Pharmacokinetics of Radiolabeled Insulin Loaded Microspheres*
- *Biodistribution Studies*
- *Gamma Imaging Studies*

g) Toxicity Studies

- *Conventional Acute Toxicity Studies*
- *Repetitive Dose Toxicity Studies*
- *Histopathological Studies*

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CHAPTER 2

LITERATURE REVIEW

2.1 DIABETES AND ITS PREVALENCE

The prevalence of diabetes has been rising at an alarming rate throughout the world. The International Diabetes Federation estimated that approximately 194 million people around the world suffered from diabetes in 2003. By 2025, this figure is expected to rise to about 333 million, amounting to almost 6.3% of the world population, living with diabetes. This steady growth in diabetes may be attributed to increase in life expectancy, sedentary lifestyle and obesity. Among the countries of the world, India has the largest diabetic population with an estimated 35 million people, amounting to almost 8% of the adult population (Wild et. al., 2004). It is further estimated that by the year 2010, India alone will have nearly 20% of world's diabetic population. The geographical distribution of diabetes in the world is depicted in the Figure 2.1 (WHO report, 2000). According to a survey conducted by the World Health Organization (WHO), developing country host more number of diabetic cases, in all the different age groups studied, than the developed countries as shown in Figure 2.2. In the developed countries, around 6 million people were reported to be diabetic in the year 2000 and this number is expected to almost double to 10 million by the year 2030. The number of diabetic people, in case of the developing countries, is expected to increase and reach 58 million by the year 2030 (WHO report, 2000).

2.2 DIABETES MELLITUS

The term diabetes mellitus describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (Wild et. al., 2004). An individual is diagnosed as suffering from diabetes mellitus when the fasting

plasma glucose level is in excess of 126mg/dl, the normal levels being less than 100mg/dl or when the plasma glucose levels is in excess of 200mg/dl at two time points during an oral glucose tolerance test (OGTT), one of which must be within 2 hours of ingestion of glucose (diabetesnet.com-diabetes_information; Pradeepa et. al., 2002).

2.2.1 Types of diabetes and its complications

Diabetes mellitus has been subdivided into a number of classes based on differences in etiology and pathogenesis. However, the two most common classifications are the Type-1and the Type-2 diabetes. These two account for 99.9% of the total diabetic population in the world.

2.2.1.1 Type-1 or insulin dependent diabetes mellitus (IDDM)

Insulin dependent diabetes mellitus (IDDM) results from the chronic autoimmune destruction of the β-cells, resulting in an absolute insulin deficiency. It is more prevalent in children and young adults and therefore also called as Juvenile Diabetes. By the time it is usually diagnosed in patients, 75% of them already develop antibodies against the β-cells and lose their capability to produce insulin. The symptoms of the (IDDM) include frequent urination, excessive thirst, extreme hunger, irritability and increased fatigue (Fish, S. medical encylopedia). IDDM accounts for only 5 to 10 % of the disease.

2.2.1.2 Type-2 or non insulin dependent diabetes mellitus (NIDDM)

Non insulin dependent diabetes mellitus (NIDDM) is caused when insulin is produced in the body but at reduced rate and there is progressive loss of β-cells in many patients, but to a much lower extent than IDDM. The onset of NIDDM occurs usually after forty years of age and generally occurs in overweight teenagers. The degenerative long term complications of

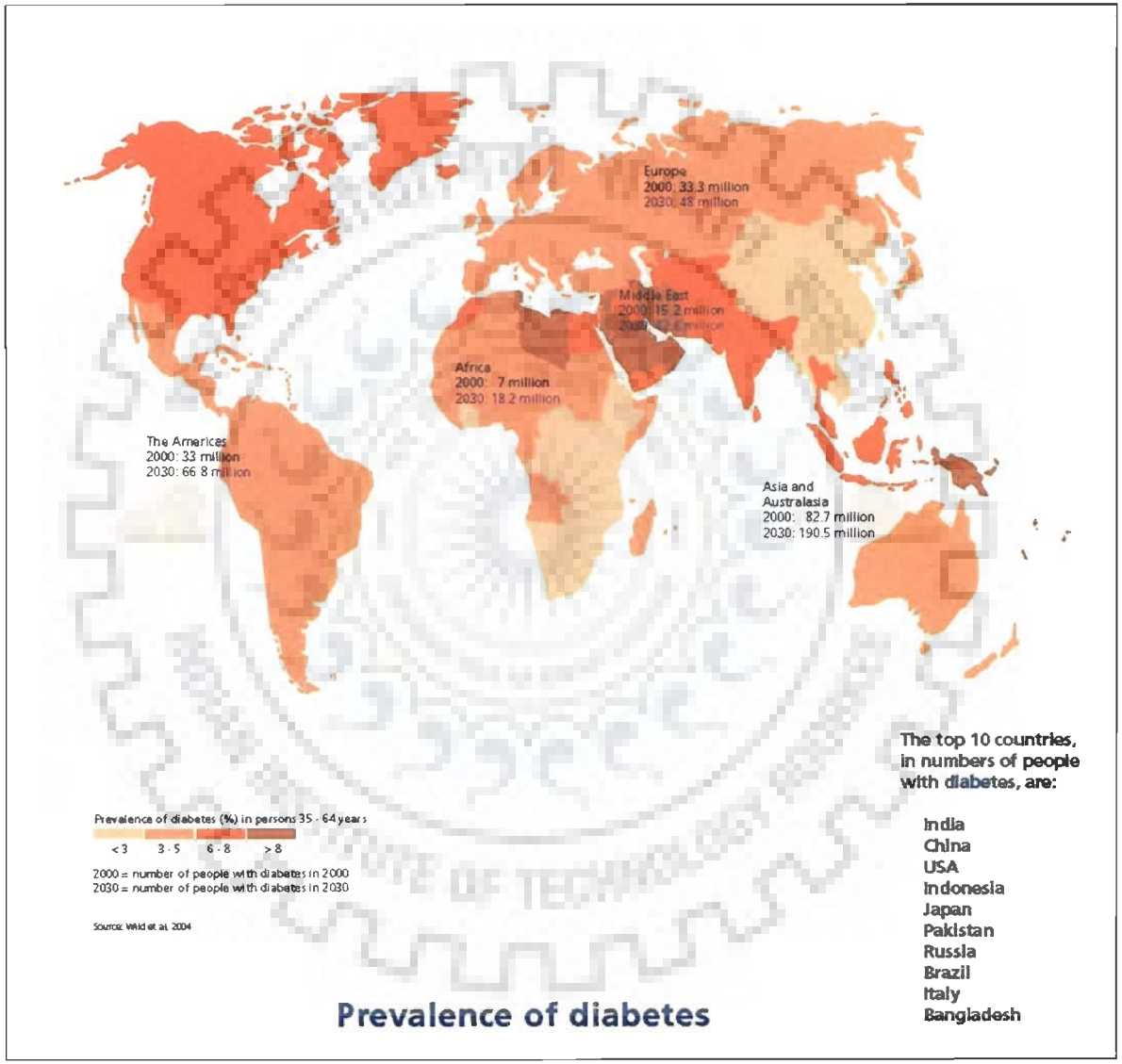

Figure 2.1: Prevalence of diabetes in the world (http://www.diabetesnet.com /diabetes_information)

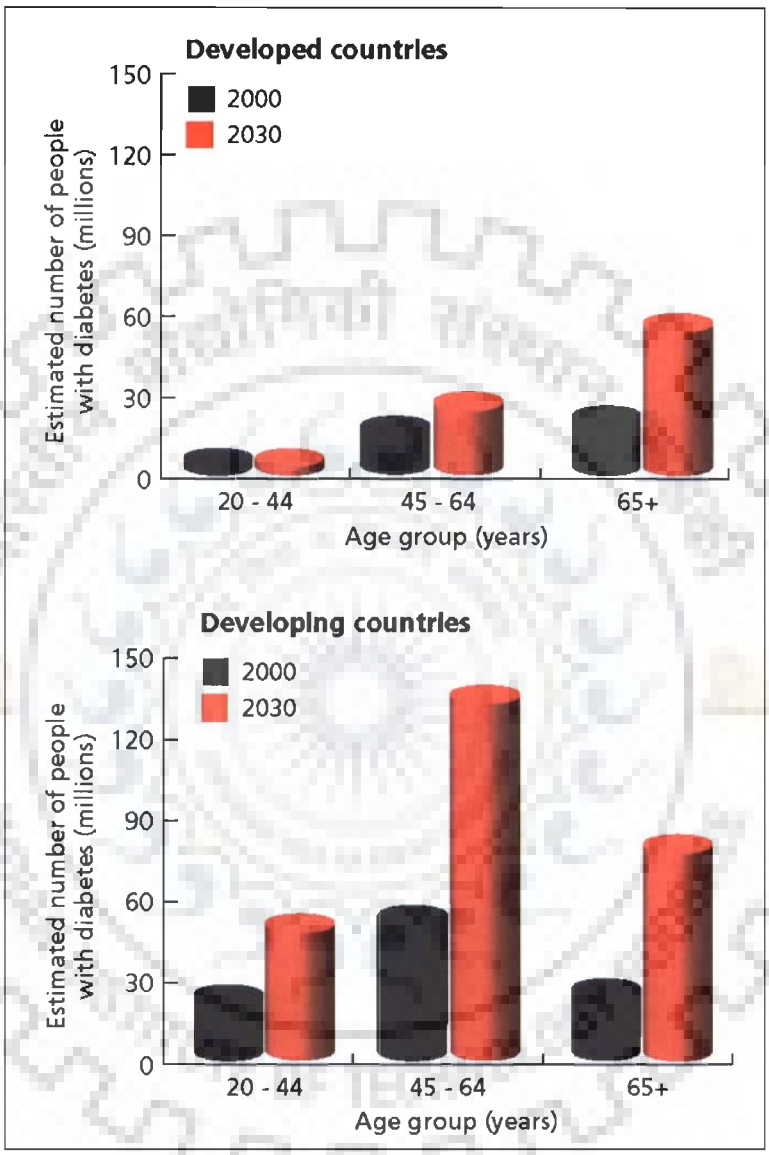

Figure 2.2: Comparison of the number of people with diabetes in the different age groups in developed and developing countries

Type-2 diabetes are same as that of Type1 diabetes. In addition, Type-2 diabetes predisposes the patient to a higher risk of cardiovascular disorders. The actual cause of Type-2 diabetes is still not very apparent. NIDDM is much more prevalent accounting for 90 to 95% of the diabetic population (King et. al., 1995). It has been observed that its prevalence is more in population with affluent lifestyles (Brown, medical encylopedia; Mahler et. al., 1999).

Both forms of diabetes ultimately lead to high blood sugar levels, a condition called hyperglycemia. Over a long period of time, hyperglycemia may leads to the following complications.

- Diabetic retinopathy: Damage to the retina leading to blindness.
- Diabetic nephropathy: Damage to the kidneys leading to kidney failure.
- Diabetic neuropathy: Damage to the nerves leading to foot wounds and ulcers, which frequently lead to foot and leg amputations.
- Damage to the nerves in the autonomic nervous system leading to paralysis of the stomach, chronic diarrhea, and an inability to control heart rate and blood pressure with posture changes.
- Atherosclerosis: Diabetes accelerates the formation of fatty plaques inside the arteries, which can lead to blockages or thrombus, which can then lead to decreased circulation in the arms and legs, a cardiac arrest or stroke.

(cdc.gov-diabetes-national diabetes fact sheet; diabetes.ca-NFP_Executive_Summary; Bate et. al., 2003; diabetes.org-federal commitment to diabetes prevention).

2.3 TREATMENT REGIMENS AND DRUGS

Conventionally, patients suffering with diabetes are required to take multiple injections of insulin daily. Repeated injections are painful and are not patient compliant. Moreover they donot provide adequate metabolic control (Pickup et. al., 1991; Brange et. al., 1990). There are three categories of treatments for diabetes mellitus:

- Permanent cure of diabetes
- Chemical agents
- Insulin

Successful pancreas transplantation as the permanent cure of diabetes is still a dream till date and research is under progress in the area of transplantation of pancreas but it is very costly and the chances of rejection are very high (Roller, 1999). People with type-2 diabetes are often prescribed tablets to control their blood glucose levels at the initial stages. Over a time period, tablets may no longer work and insulin injections may be needed. Sometimes multiple regimens where insulin injections are used in combination with tablets are prescribed.

2.3.1 Chemical agents for treatment of diabetes

There are several oral hypoglycemic agents that lower blood glucose in Type-2 diabetes. Some of the important widely used oral medicine and their drawbacks are listed in Table 2.1. They fall into one of these three groups:

1. Oral hypoglycemic agents which increase insulin production by the pancreas. Examples include Amaryl, Glucotrol, and Glucotrol XL, Micronase, Diabeta, Glynase, Prandin, and Starlix (Barry, 2001; Carino et. al., 1999; Björk et. al., 1990; emedicinehealth.com-medicine consumer health).

2. Oral hypoglycemic agents which increase sensitivity to insulin. Examples include Glucophage, Avandia, and Actos (Burke et. al., 2003; Chopra et. al., 1956; Al-Awadi et. al., 1987).
3. Oral hypoglycemic agents which delay absorption of glucose from the gut. Examples include Precose and Glyset (Ivorra et. al., 1989; Alarcon-Aguilara et. al., 1998).

2.3.2 Insulin

Insulin is the most important regulatory hormone in the control of glucose homeostasis. Following the analysis of data on multitude of factors affecting the supply of and demand for insulin, a WHO report estimated that out of a total of 11.9-16.5 million diabetic population in 2000, 3.8 (±0.6) million were insulin dependent diabetics and require daily parenteral injections of insulin to stay healthy and live normally (Figure 2.3) and the demand for Insulin was 55.9 (±8.6) billion units (Figure 2.4) in USA alone.

2.4 PHYSIOLOGICAL BASIS OF INSULIN

The connection between disturbance in pancreas functions and occurrence of diabetes mellitus was noted as early as 1788. Then, in 1889, diabetes mellitus was induced experimentally by disruption of pancreas. The pancreatic substance responsible for the lowering of blood glucose level was discovered to be produced in the islet of Langerhans cells. This unknown substance was later named as 'insulin' by de Meyer.

Table 2.1 Mechanism of action, advantages and disadvantages of oral chemical agents used in treatment of diabetes mellitus (Sadegholvad, 2002)

Agent	Activity & advantages	Disadvantages
Rosiglitazone	Decreases glucose production and plasma levels and increases glucose clearance; significantly increases insulin sensitivity improves lipid profile	Not indicated in patients with hepatic impairment, heart failure, increased bodyweight and fluid retention, particularly with insulin; relatively expensive
Metformin	Decreases hepatic production of glucose, slightly increases muscle insulin sensitivity; low risk of hypoglycemia; beneficial effects on some cardiovascular parameters; doesn't cause gain in bodyweight	Liver disease, respiratory insufficiency, hypoxaemia, severe infection, alcohol abuse or cardiac failure; associated with gastrointestinal adverse effects
Sulphonylureas	Increase endogenous insulin secretion; generally well tolerated; inexpensive	Adverse effects include hypoglycaemia (particularly in the elderly) and gain in bodyweight
Repaglinide	Increases endogenous insulin secretion in the presence of glucose; well tolerated; can be used by patients with renal impairment	Similar incidence of hypoglycaemia and similar efficacy to sulphonylureas; drug interactions with enzyme inhibitors or inducers; relatively expensive; gain in bodyweight
Acarbose, Miglitol	Inhibit carbohydrate absorption from the gastrointestinal tract; serious adverse effects are rare; hypoglycaemia is not a risk; no effects on body weight	Gastrointestinal adverse effects; hepatic toxicity (acarbose); less effective than sulphonylureas or metformin impairment.

2.4.1 Biosynthesis

The human pancreas consists of islets of Langerhans, each unit of which is composed of three different type of cells namely alpha, beta, gamma cells, which secrete various hormones vital for human body as depicted in Figure 2.5 (Banting and Best, 1922). In a healthy nondiabetic human, insulin is synthesized in the β-cells of the Langerhans islets. Investigations in the biosynthesis of insulin with labeled amino acids were made easier using isolated islet tissue of fish, whose exocrine pancreas is physically separated from the endocrine pancreas. The biosynthesis of insulin was noted to follow the general pattern of protein biosynthesis. It occurs in the endoplasmic reticulum (by ribosomes), with the synthesis of the B chain beginning at the N-terminus. The separate biosynthesis of the insulin's A and B chains was demonstrated in doubly labeled insulin from fish islets. The A and the B chains of insulin are linked via disulphide bridges. A glutathione insulin transhydrogenase, which is capable of splitting insulin molecule into its A and B chains by reducing its disulphide bridges, has been isolated from liver. The enzymes capable of carrying out a reductive cleavage of insulin appear to exist, not only in the liver but in the pancreas as well.

Physiologically, it requires around 12 days to synthesize the amount of insulin normally present in the pancreas, which is about 250 IU (10mg) in humans. This amount of insulin is 5-10 times the daily consumption of insulin for glucose homeostasis.

2.4.2 Secretion

After its biosynthesis in the endoplasmic reticulum, insulin is stored in the secretory granules by forming a complex with other proteins. This complex constitutes about 10% of the proteins in the granules. The transport of insulin from the synthesis site (in the ribosomes) to the secretory granules and the process of secretion (via the fusion of the granule membrane with the cell membrane, with the simultaneous liberation of insulin into the capillaries)

appear to be similar to the secretion process in the exocrine pancreas. Insulin secretion is regulated mainly by the levels of both glucose and insulin in the circulation. A direct dependence of secretion rate on the glucose level has been observed.

2.4.3 Mode of action

Insulin is an anabolic hormone, that is, it promotes the synthesis of glycogen, fat and protein. There are a number of theories in which most actions of insulin have been attributed to one primary effect. The best known to these is the theory of glucose permeability, which postulates that the primary effect of insulin is to promote the entry of glucose into the cell. The increased oxidation of glucose, the facilitated synthesis of glycogen and fat, the decreased degradation of protein and fat, and the prevention of ketone formulation can be easily explained on the basis of this primary effect.

Insulin has also been observed to promote the transport of some amino acids and of K^+ ion, even in the absence of glucose. Similarly, insulin also inhibits the liberation of free fatty acids and of glycerol from adipose tissue. It has been shown that all the effects of insulin effects on the adipose tissue can be initiated by phospholipase C, and the action of insulin on the plasma membrane is thus postulated to result from the modification of membrane lipids. This would expand the original glucose permeability theory into a more generalized theory of membrane permeability. However, some of insulin's effect, such as the stimulation of protein synthesis and of glycogen synthetase, cannot be explained even with this expanded theory.

2.4.4 Metabolism

From the pancreatic vein, insulin molecules are first transported into liver, where approximately half of the molecules are degraded and removed from hepatic circulation. The half-life of insulin is about 30 min in man. The liver and kidneys are the two principal organs

responsible for metabolism of insulin. Insulinase, which is probably identical to glutathione insulin transhydrogenase has also been reported to catalyze the reductive cleavage of interchain disulphide bridges in the insulin molecule; the reduced chains are further hydrolyzed to some smaller fragments.

In addition to the liver, the pancreas muscle tissue and the adipose tissue have also been reported to have the ability to cleave the insulin molecule by reduction or proteolysis.

2.4.5 Physiochemical properties of insulin

The insulin crystals are often externally similar, but they may differ if obtained from different animals. When insulin is crystallized isoelectrically from a buffered solution at pH 5.5-5.6, zinc is often detected in these crystals. Furthermore, it is reported that bivalent metal ions, such as Zn^{2+}, Ni^{2+}, Co^{2+} or Cd^{2+} are essential to the crystallization of insulin; Cu^{2+}, Mn^{2+}, Fe^{2+} etc. ions could do the same. In addition to chloride, other anions (such as acetate, citrate, phosphate, or carbonate) may also be present for the crystallization of insulin. Insulin crystals normally contain 30-51% of crystallization water, which shrinks in air to a water content of 10%. Part of water (30 moles per mole of insulin) cannot be released even by drastic drying.

2.4.6 Structural composition

Insulin was recognized as a protein molecule as early as 1926, but the sequence of its 51 amino acids was not resolved until 1955 by pioneering work of Ryle and his researchers Insulin contains an unusually high content of sulphur (3.3%), all of which is present in the form of cystine. It has been concluded that active insulin must consist of at least two parallel peptide chains held together by disulphide bridges, which was confirmed by Sanger. Sanger and his coworker established the overall structural formula of bovine insulin. They are the first research group to have determined the sequence of the 21 and 30 amino acids in the A

and B chains, respectively, and then the positions of amide groups and of three disulphide bridges. The molecular weight was found to be 5734 daltons.

Proinsulin (~ 9kD), which is about 1.5 times the size of insulin (~ 6 kD), has a molecular structure consisting of the A and B chains of insulin alongwith a C-peptide of 34 amino acids. It has biologic and immunologic activities that are approximately 10-20% that of insulin. The C-peptide is cleaved from proinsulin molecule by carboxykinase like enzyme or trypsin, with the removal of two basic amino acids from each end (Arg ^{31} and Arg ^{32}, and Lys ^{64} and Arg ^{65}). The A and B chains alongwith the C- peptide and their amino acid sequences are shown in Figure 2.6. Although the amino acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, including the positions of the three disulfide bonds. These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among species, and insulin from one animal is very likely to be biologically active in other species. Indeed, pig insulin has been widely used to treat human patients (vivo.colostate.edu-insulin_struct.html). Insulin molecules have a tendency to form dimers in solution due to hydrogen bonding between the C-termini of B chains. Additionally, in the presence of zinc ions, insulin dimers associate into hexamers as shown in Figure 2.7.

2.4.7 Stability of insulin

2.4.7.1 Physiological satbility

Pure insulin is known to be very stable and retains its activities for years in a sterile solution (pH 4) at 2°C. The biological activity of a crystalline insulin has been found to stay intact for 2 years at 0°C but shows a 20 % loss after 1 year at 20-25°C.

2.4.7.2 Nonenzymatic degradation

The primary β-amide groups in the C terminal asparagines residue (A_{21}) are particularly sensitive to hydrolysis. Therefore, insulin could be partially degraded to deamido-insulin during the acidic extraction of the pancreas; but the deamido insulin has reportedly retained almost fully the biological activity of insulin.

2.4.7.3 Enzymatic degradation

Native insulin is highly resistant towards hydrolyzing enzymes *in-vitro*, which appears to be particularly related to its zinc content (e.g. leucine aminopeptidase digests only the 'metal-free' insulin). Native insulin is cleaved slowly by trypsin and chymotrypsin only. As soon as one bond has been cleaved, the remainder of the hydrolysis proceeds rapidly. Insulin is attacked by most strongly by subtilisin (Chein, 1996).

2.4.8 Analytical methods for insulin

2.4.8.1 Micro BCA protein assay

Micro-BCA protein assay reagent is highly sensitive reagent for the quantitative colorimetric estimation of total proteins in dilute aqueous solution. This unique reagent system utilizes bicinchonic acid (BCA) as detection reagent for Cu^{+} which is formed when Cu^{2+} is reduced by protein in alkaline environments. The purple coloured reaction product is formed by chelation of two molecules of BCA with one cuprous ion Cu^{+}. This water soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations (Smith, 1985).

2.4.8.2 *High performance liquid chromatography (HPLC)*

HPLC has a very high resolution power and is being increasingly used as an analytical tool to determine the structure and purity of peptides and proteins. However, low sensitivity of

HPLC has limited the direct measurements in certain biological samples like tissue extracts. The HPLC system is an apparatus consisting of a pump, a column, a multisolvent delivery system, a UV-Vis detector alongwith a programmable multiwavelength detector (Oliva et. al., 1996). A reversed-phase C-18 column (Delta-Pack, 300 A) and a mixture of solvents in the ratio of 74:26 of 0.2 M sodium sulphate buffer (pH 2.3) and acetonitrile as the eluent at a flow rate of 1.0 ml/min at room temperature and detection at the wavelength of 214 nm is used for the detection of insulin (Farid et al., 1989).

The chromatogram obtained with the solution of insulin (RS) shows a principal peak and a smaller peak appearing immediately following the principal peak due to the corresponding monodesamido derivatives of insulin. It may be necessary, after application of each solution, to wash the column with equal volumes of acetonitrile and water for sufficient time to ensure elution of interfering substances before injecting the next sample solution. The content of the insulin can be calculated from the peak areas of the principal peak and any peak due to monodesamido derivative of insulin.

2.4.8.3 Radio immuno assay (RIA)

RIA has the advantage of specificity and sensitivity, but it lacks the resolving power of HPLC. RIA techniques exploit the specific association of antibody with a peptide or protein molecules, as the antigen, to determine the peptide and protein at very low concentration in a variety of complex matrices. The ability of proteins to interact with its corresponding antibody is a structural and conformation specific interaction.

The protein or peptide is labeled with radioactive substances like ^{99m}Tc or I^{125}. The radiolabeled samples are then detected by γ-counter. The amount of radioactivity measured gives an account of the amount of the protein or peptide.

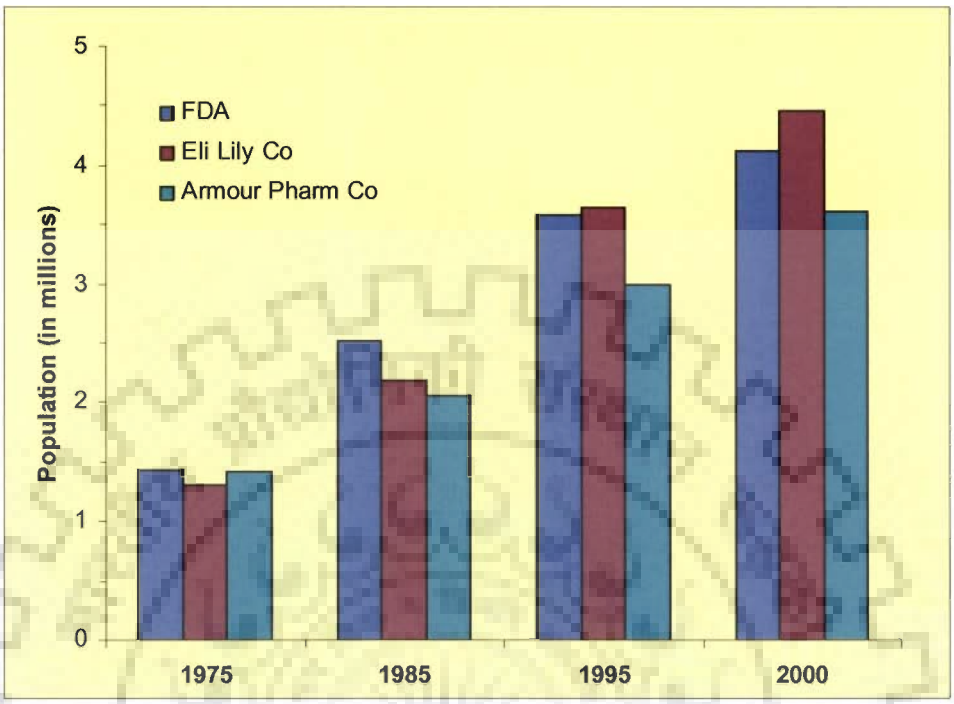

Figure 2.3: Insulin dependent diabetic population

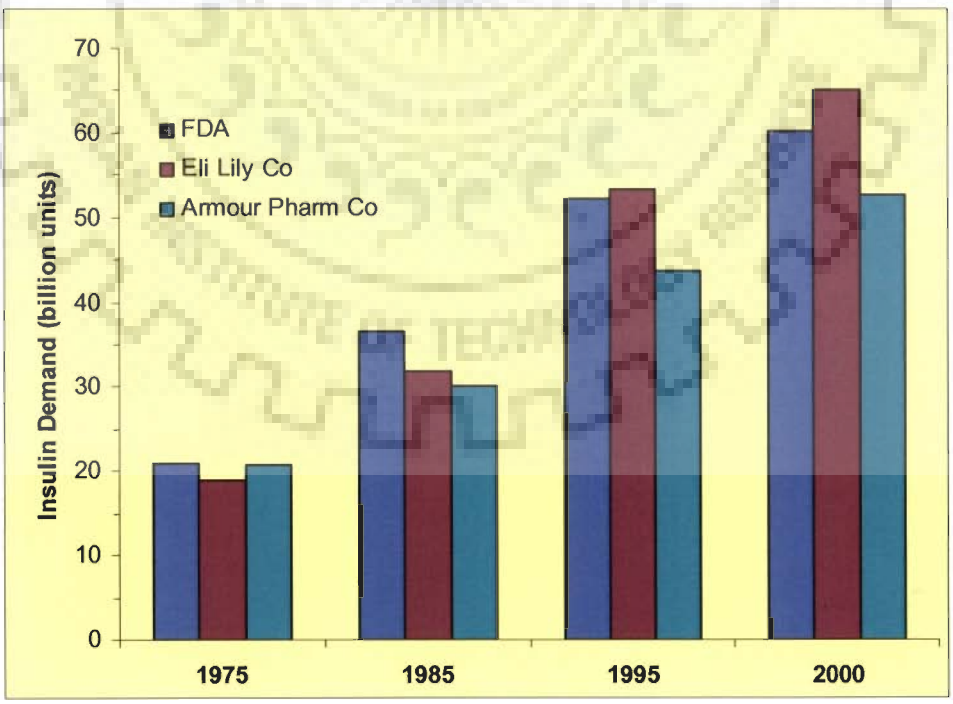

Figure 2.4: Demand for insulin in billion units in USA

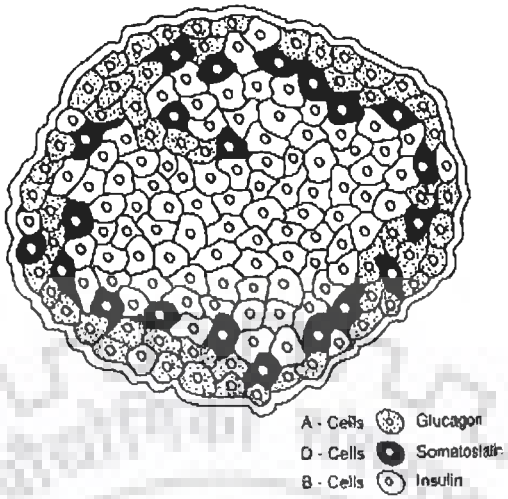

Figure 2.5: Structure of pancreas and arrangement of different types of cells (http://www.vivo.colostate.edu/hbooks/endocrine /pancreas/insulin_struct.html)

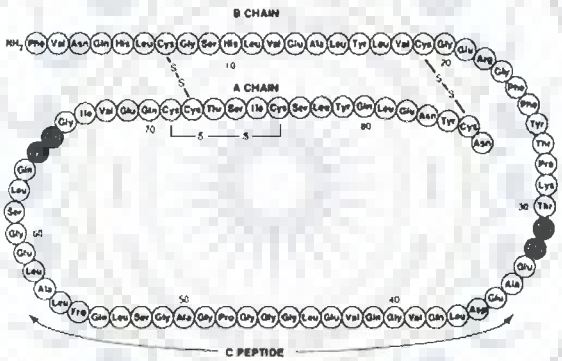

Figure 2.6: Arrangement of different amino acids in insulin synthesized by β-cells of pancreas (http://www.vivo.colostate.edu/hbooks/endocrine /pancreas/insulin_struct.html)

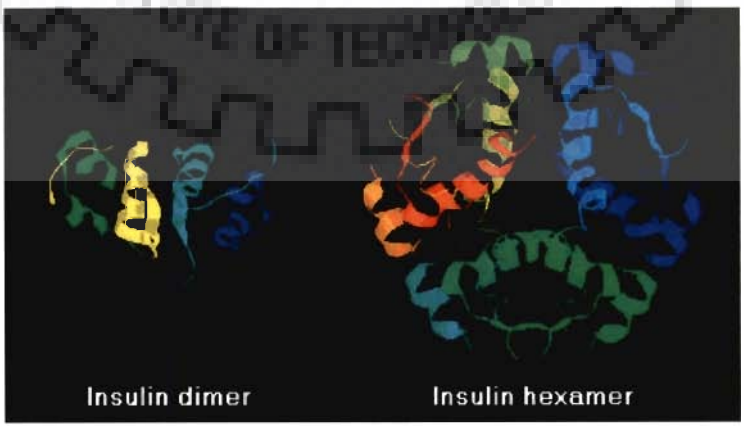

Figure 2.7: Structure of insulin dimer and hexamers

2.5 PHARMACEUTICAL DEVELOPMENT OF INSULIN

Several delivery techniques and systems have been developed for the parenteral controlled and/or sustained delivery of insulin.

2.5.1 Long-acting insulin injectables

An early approach to the development of long-acting insulin preparation included the complexation of insulin with zinc salt and basic proteins; for example, the formulation of protamine-Zn-insulin suspension from protamine, a water soluble basic protein isolated from the sperm of mature testes of fish. Further investigations have applied the controlled crystallization of zinc-insulin complex, without the use of proteins (like protamine, isophane or globin). This approach used a buffer medium with controlled pH in which insulin can be precipitated by zinc ion to yield a water-insoluble Zn-insulin complex in either a crystalline or an amorphous form. The crystallinity of the complex can be controlled by varying the pH of the buffer medium (Haleblian et. al., 1969). The crystalline Zn-insulin complex formed is extremely long active insulin, whereas the amorphous Zn-insulin complex is a moderately long-acting insulin.

Three long acting Zn-insulin preparations, Semilente, Lente and Ultralente, have been formulated and are still widely described today for the treatment of diabetes. These preparations differ in their onset, intensity, and duration of action following subcutaneous administration. The amorphous Zn-insulin complex (Semilente insulin), which forms at pH 6-8, has a rapid onset of action (0.5-1.0 hour) and a moderately long duration of action (12-16 hours) compared to regular insulin (6 hours). On the other hand, the cloudy suspension of crystalline Zn-insulin complex (Ultralente insulin), which is forms at pH 5-6, has a much slower onset (4-8 hours) but a rather long duration of action (> 36 hours). A mixture of crystalline (seven parts) and amorphous (three parts) insulins form a preparation called Lente insulin, which is intermediate in both the onset (1.0-1.5 hours) and duration of action (24

hours) (Petersen et. al., 1959). Attempts have been made to prolong the normoglycemic action of insulin by entrapping it in a liposome-collagen matrix for subcutaneous administration (Weiner et. al., 1985).

2.5.2 New injection devices for insulin delivery

A new jet-type injector (Precijet-50) was developed in the mid eighties (Lindmayer et. al., 1986; Halle et. al., 1986). The features of this device, in addition to being small in size, were simple in design with capability of mixing two types of insulin before injection, which were not available in the earlier model of jet injection technology (Hingson et. al., 1947; Weller et. al., 1966; Chen et. al., 1972). On the other hand, a sprinkler type needle for insulin injection had also been designed (Kuhl et. al., 1986). Another later development was the design of an insulin injection pen called the Novolin Pen, which is a pocket sized apparatus that resembles a fountain pen. When fitted with a disposable needle and a unit-dose ampule of insulin, it becomes a portable, self-contained insulin syringe (Haycock, 1986).

2.5.3 Infusion pumps for insulin delivery

A continuous, subcutaneous insulin infusion device or pump has been in use for almost few decades now (Pickup et. al., 1978; Tamborlane et. al., 1979; Natha, 1982). However, a possible increase in mortality (Teutsch et. al., 1984) morbidity due to mechanical failure (Kitabachi et. al., 1983), and lack of data on the safety of its long-term use (Leichter et. al., 1985) suggest that further long term assessment of the device is needed. The feasibility of intraperitoneal modulated delivery of insulin using an implantable micropump has been investigated in dogs (Sefton et. al., 1979, Sefton et. al., 1984). An implantable controlled-release micropump was also developed for the intraperitoneal modulated delivery of insulin. It is an open-loop control system characterized by operation at two levels: basal delivery for the between meal period and the augmented delivery for short periods following the

ingestion of meals. The rate of delivery is adjusted to meet the insulin requirements of the respective meal. With an adequate supply of insulin to the pump, the concentration gradient from the reservoir produces the delivery of insulin at the basal rate (when no external power source is applied). Augmented delivery is achieved by repeated compression of the foam membrane above the steel piston, which applies a current to the solenoid cell. Further investigations have resulted in the development of a piezoelectrically-controlled micropump (P-CRM) for the programmed delivery of insulin (Walter et. al., 1987). An implant based on chitosan (CS) and hyaluronate (HA) has also been investigated for controlled-release of insulin. Insulin release from the pellet was found to be greatly influenced by the formation polyion complex between chitosan and hyaluronate (Selam et. al., 1999). Using microchip technology, scientists have succeeded in creating a capsule that overcomes biocompatibility problems so that it won't be attacked and destroyed by the body's immune system. Testing for long-term usage and determining the ideal dosage level are the next steps in its development (Blackshear et. al., 1979).

2.5.4 Self-regulating delivery systems

An interesting approach was recently reported with the development of an artificial beta cell that consists of a glucose-sensitive hydrogel membrane for feedback-controlled delivery of insulin (Horbett et. al., 1983; Horbett et. al., 1984). The glucose-sensitive membrane is fabricated by entrapping a glucose oxidase in a hydrogel polymer with pendant amine groups. As glucose diffuses into the polymer, glucose oxidase catalyzes its conversion to gluconic acid (pKa =3.6), thereby lowering the micro-environmental pH in the membrane. The reduced pH results in increased ionization of the pendant amine groups. Electrostatic repulsion between the ionized amino groups increases the swelling and thus the permeability of hydrogel membrane to insulin becomes a function of the glucose concentration surrounding the membrane, and the release of insulin is accelerated by the increase in

glucose level (Albin et. al., 1986). Another potential technique to achieve the self-regulating delivery of insulin in a biochemical approach based on the principle of the competitive and complimentary binding behavior of concanavalin (ConA) with glucose and glycosylated insulin (G-insulin) (Jeong and Feijen, 1984; Jeong and McRea, 1984; Kim et. al., 1984; Sato et. al., 1984; Seminoff et. al., 1986). The ConA-G-insulin complex is encapsulated inside a devise with a polymeric membrane that is permeable to glucose and G-insulin but not to the ConA or its complex. As the glucose level increases, it causes influx of the glucose to the device, resulting in the displacement of G-insulin from the ConA complex with its efflux to the body. The results indicate that the blood levels of pancreatectomized dog can be controlled by the G-insulin delivery device (Chein, 1996).

2.5.5 Various marketed insulin formulations

Some of the marketed insulin formulations alongwith their pharmacokinetic profiles have been listed in Table 2.2.

Table 2.2: Various marketed insulin formulations and their pharmacokinetic profiles

Trade Name	Formulation	Pharmacokinetics
Humulin® R Novolin® R	Zinc-insulin crystalline suspension (Acid regular)	Rapid onset, short duration
Humulin® N Novolin® N	Isophane suspension protamine, zinc crystalline insulin (buffer: water for injection)	Intermediate-acting, slower onset, longer duration than regular insulin
Humulin® 70/30 Novolin® 70/30	70% isophane suspension, 30% zinc crystalline	Intermediate-acting, faster onset, longer duration

Humulin® U	Extended zinc-insulin suspension-all crystalline	Slow-acting, slow onset, longer, less intense duration than R or N forms
Humulin® L Novolin® L	70% zinc-insulin crystalline suspension, 30% amorphous insulin (cloudy suspension)	Intermediate-acting, slower onset, longer duration
Humulin® BR	Zinc-crystalline insulin dissolved in sodium diphosphate buffer	Rapid onset, short duration; used in pumps only

2.6 ROUTES FOR ADMINISTRATION OF INSULIN

The immediate goal of the treatment regimen for diabetes mellitus is to stabilize the blood glucose level alongwith elimination of the high blood glucose symptoms. The long-term goals of treatment are to prolong life, relieve symptoms, and prevent long-term complications such as cardiac diseases and kidney failure (cdc.gov-diabetes-national diabetes fact sheet) Several other routes for insulin administration for the treatment of diabetes mellitus have been investigated and are discussed below.

2.6.1 Parenteral delivery

Currently, subcutaneous insulin injections, three times a day, is the only viable option for treatment of acute diabetes mellitus. The disadvantages associated with injectable insulin therapy are poor patient compliance, due to pain and discomfort during self administration particularly if multiple daily injections are required. Sudden and drastic fall of glucose level associated with the subcutaneous therapeutic regimen fails to deliver insulin in the physiological pattern due to adverse insulin pharmacokinetics and hence normal blood glycemia level is seldom achieved. The parenteral route requires change of site of

administration to avoid inflammation besides predisposing the patient to increased chances of infection. Various alternatives to subcutaneous hypodermic syringe injections have been investigated. Growing attention is being paid to the administration of proteins and peptides by the nasal, ocular, rectal, buccal, pulmonary, transdermal and the oral routes. These methods may be non-invasive or invasive (Drews, 2000, Roller, 1999; Jeandidier et. al.,1999; Bohannon, 1999; Hoffman et. al., 1997; Newhouse, 1999; Patton, 1999; Carino et. al., 1999). Among proteins, insulin delivery has been researched by the nasal, transdermal and by oral routes (Owens et. al., 2003).

2.6.2 Nasal delivery

The nasal route for the administration of proteins and peptides is the only route from the list of non-parenteral delivery systems that has gained regulatory approval. A human insulin inhalation powder has been approved by the U.S. Food and Drug Administration (USFDA) under the name EXHUBRA®. The nasal anatomy has a non ciliated area in the anterior part of the nasal cavity and a ciliated part in the more posterior part of the nose. Therefore, the site of drug deposition becomes important for the retainment of a formulation in the nose (Gizurason et. al., 1991; Hilding, 1963; Proctor et. al., 1977). The rapid onset of action, relatively high bioavailibilities, high vascularity, easy access, and high permeability favour this route of administration. The limiting factors for this route include mucociliary clearance, enzymatic degradation in the mucus layer, and low permeability in the epithelium (Williams and Lemke, 2005; Sayani and Chien, 1996; Senal and Hincal, 2001; fda.gov-2006/021868lbl.pdf; Kissel and Werner, 1998). The physiological conditions existing at the nasal epithelium may alter during various disease conditions, such as allergic rhinitis or nasal blockage, thereby affecting the membrane characteristics. Besides, prolonged use of the formulations containing proteins and peptides may totally disrupt the nasal mucosa and cilia, making this route highly unsuitable for chronic administration of drugs (Williams and

Lemke, 2005). Intranasal administration of starch microspheres and insulin as dry powder has also been reported to decrease the plasma glucose concentration via this route.

2.6.3 Transdermal delivery

Although skin has been used as a site for topical administration for many decades, it is only recently that its use on intact skin as a port for the continuous transdermal delivery has been recognized (Prasad et. al., 2007; Babu and Pandit, 2004; Babu and Pandit, 2005; Verghese and Khar, 1996; Cullander et. al., 1992). Today the transdermal drug delivery system is considered to be one of the successful controlled release technologies in terms of number of approved products that are in the market (Guy, 1996; Nair et. al., 1999; Prausnitz, 2004). The transdermal route of delivery mimics the benefits of intravenous drug infusion while overcoming its limitations of hospitalization and medical supervision of the medication. The transdermal drug delivery systems utilize the skin for the delivery of drug molecules from the surface of skin to the circulatory system. The delivery of drugs transdermally (through the skin) provides several important advantages over traditional oral and intravenous delivery routes (Williams and Barry, 2003). This delivery route provides the possibility of bypassing the hepatogastrointestinal elimination or the 'first pass effect' and achieving better patient compliance. It is considered to be a potential route for delivery of protein based pharmaceuticals as the skin lacks the proteolytic enzymes, which are responsible for the degradation of proteins and peptides. Transdermal iontophoretic delivery is considered useful in the systemic delivery of peptides, which are extremely potent, extremely short acting, and require delivery in a circadian pattern to simulate the physiological rhythm. Insulin has been delivered by this route (Chien et. al., 1989). Various hydrogel patches have also been developed based on polyacrylamide, polyacrylic acid and gelatin for transdermal delivery. These copolymer-based hydrogels have been evaluated for transdermal insulin delivery using iontophoresis and chemical enhancer pre-treatment (Sage, 1993; Banga et. al.

1993; Guy, 1998). Transdermal delivery using ultrasound was shown to shorten the lag time of penetration through the skin. Low frequency ultrasound (~20 kHz) has been reported to increase the permeability of human skin for insulin (Ueda et. al., 1995). The limitations occur with hydrophilic and macromolecular protein and peptide drugs to permeate the skin. In addition, skin is an immunocompetent organ, and irritancy and allergy can cause stringent constraints on transdermal delivery (Sinha et. al., 2007).

2.6.4 Oral Delivery

Among all the non-parenteral routes of protein and peptide administration, the oral route seems to be the most attractive because of the following advantages: ease of administration, avoidance of pain and discomfort leading to high patient compliance, elimination of infections, and the ability to formulate more than one dosage form. The delivery of protein drugs through this route helps in maintaining the blood concentrations for a considerable period of time within the therapeutic window, thus reducing the number of effective dosage required (Wang, 1996). The oral delivery of protein drugs is a worthy objective but an extremely difficult one. However, oral administration has numerous challenges to overcome in order to create an effective system for protein delivery. These include chemical and physical instability of protein drugs, strong hostile acidic environment of the stomach, the abundance of various proteases and peptidases in the gastrointestinal tract, poor penetration capabilities of protein drugs through gastrointestinal membranes, first pass metabolism, and the rapid clearance of proteins in the body. The most formidable, however, are the two barriers in the gastrointestinal tract: the epithelial barrier and the enzymatic barrier.

2.6.4.1 The epithelial barrier

Structurally, the gastrointestinal epithelium consists of a single cell layer. Underlying the epithelium is a layer called the *lamina propria*, which is supported by a layer of smooth

muscle, the *muscularis mucosa*, which is 3 to 10 cell thick in width. Together, the three layers- *epithelium*, *lamina propria* and *muscularis mucosa* are called intestinal mucosa. The epithelium along with *lamina propria*, projects and forms finger or leaf-shaped villi, which increases the surface area by about ten folds. These villi are the sites of electrolyte and nutrient absorption. This brush border is where digestive enzymes are located. Above the epithelial cells, there is the glycocalyx, a filamentous and fuzzy coat, which is weakly acidic and consists of sulfated mucopolysaccharides. Over the glycocalyx is a layer of mucus, which is secreted from goblet cells and consists of mucin, glycoproteins, enzymes, electrolytes, water etc. A protein drug first has to diffuse through two layers, the mucus and the glycocalyx layers, before it reaches the cell surface membrane for subsequent absorption (Wang, 1996).

2.6.4.2 The enzymatic barrier

The enzymatic barrier is considered the most important of the multiple barriers limiting the absorption of protein drugs because of the abundance and variety of protease in the gastrointestinal tract. All protein drugs undergo enzymatic destruction in the gastrointestinal tract. Because of the susceptibility of insulin to undergo enzymatic degradation and poor permeation, this life saving drug still has to be administered by injection though a lot of research is being carried out to devise oral delivery of insulin. Table 2.3 summarizes both the proteases encountered sequentially by protein drugs administered orally and the major sites of protein degradation and their degradation products.

Table 2.3: Various kinds of proteases in the gastrointestinal tract, their major sites of action and degradation products (Wang, 1996)

Categories	Enzymes	Major site of action/ end products
Gastric proteases	Pepsins (aspartic proteases)	Broad activity, hydrolyze many peptide bonds/peptides
Intestinal pancreatic proteases	Trypsin (endopeptidases)	Peptide bonds of basic amino acids/peptides
	α-chymotrypsin (endopeptidase)	Peptide bonds of hydrophobic amino acids/peptides
	Elastase (endopeptidase)	
	Carboxypeptidases	Peptide bonds of smaller and non aromatic amino acids/peptides A:C-terminal amino acid B:C-terminal basic amino acid
Brush border proteases	Aminopeptidases A Aminopeptidases N Aminooligopeptidase Dipeptidylaminopeptidase-IV Carboxypeptidases	Aminopeptidases are N-terminopeptidases, degrading mostly 3 – 10 amino acid residues/ dipeptides and amino acids
Cytosolic proteases	Di-tripeptidases	2-3 aminopeptidase/ amino acids

Despite these challenges, oral route may be a preferred route of administration if it can assure reproducible and predictable bioavailibilities (Bai et. al.,1994). Many different strategies have been attempted to develop a biologically active oral insulin formulation. Protease inhibitors, permeation enhancers, enteric coatings and polymeric microsphere formulations have all been applied towards the development of oral insulin formulation with varying degrees of success (Damgé et. al., 2007). The microsphere delivery is designed to contain biologically active insulin and, after oral administration, protect the protein from

enzymatic degradation in the intestinal tract and facilitate systemic uptake of insulin through gastro-intestinal epithelium.

2.7 BIODEGRADABLE POLYMERS IN DRUG DELIVERY

Polymer based drug delivery systems have been considered for many applications to supplement standard means of medical therapeutics. Commercial success of therapeutic proteins for medication greatly depends on novel technologies to improve and control delivery of drugs. The increased biochemical and structural complexity of proteins compared to conventional drug-based pharmaceuticals makes formulation design for delivery of therapeutic proteins a very challenging and difficult task. This is due to the presence of a few obstacles in oral protein drug delivery. The major challenges include the acidic environment of the stomach and the presence of digestive enzymes in the gastrointestinal tract. Also, the development of delivery systems for therapeutic proteins and their efficacy depends on the biophysical, biochemical and physiological characteristics of protein molecules, including their molecular size, biological half life, immunogenicity, conformational stability, dose requirement, site and rate of administration, pharmacokinetics and pharmacodyanamics. Recent advances in pharmaceutical research have shown that polymeric devices may be utilized for the delivery of very large molecular weight drugs (Atkins, 1997; Barbato et. al., 2001; Benoit et. al., 1999; Bodmeier and Chen, 1989; Buntner et. al., 1996; Buntner et. al., 1998). The main advantage of using microspheres in drug delivery is that it stabilizes the large, fragile structure of the protein to keep it biologically active. The other advantage is that the duration of protein delivery can be made to exceed than that of the conventional drug delivery forms. Also, one can target specific organs and other parts of the body by modifying the size of the microsphere, thus eliminating, or atleast reducing, the adverse side effects.

2.7.1 Biodegradable Polymers

Biodegradable polymers are synthetic or natural polymers which degrade *in-vivo* either enzymatically or non-enzymatically to produce biocompatible or non-toxic bioproducts along with the progressive release of the dissolved or dispersed drug. Biodegradable polymers are preferred because surgical removal of spent device is not required and the clearance of biodegradable polymers from the body is natural and safe. Their release rate can be easily tailored and they degrade in biological fluids to produce biocompatible or non-toxic products, which are removed from the body by normal physiological pathways (Sinha and Trehan, 2003). Widespread advances are being made in the field of natural polymers as well as synthetic absorbable and non-absorbable polymers. Some of the natural and synthetic biodegradable polymers have been listed in Table 2.4.

2.7.1.1 Natural polymers

Natural polymers remain attractive mostly because they are natural products of living organisms, readily available, relatively inexpensive and capable of multitude of chemical modifications. A lot of research on natural polymers for matrices in drug delivery systems have been centered on proteins and polysaccharides. Collagen has been fabricated into wide variety of forms including crosslinked films, meshes, fibers and sponges because of its unique structural properties. Solid ocular inserts have also been prepared from purified animal tissues. Natural polymers as biomaterials offer several advantages: biocompatibility and non-toxicity in most tissues, is readily isolated and purified in large quantities, has well documented structural, physical, chemical and immunological properties and can be processed into a variety of forms. Non-collagenous proteins like albumin, gelatin, casein, fibrinogen in the form of microspheres and nanoparticles continue to be exploited as drug delivery systems.

Extensive research still needs to be done to address many issues before they will have widespread use in clinical situations. These include better understanding of the kinetics of drug release, more effective ways to control burst phenomenon, greater understanding of drug–polymer interactions and their effect on shelf life stability, additional animal studies to determine local tissue response, biodegradation rates and metabolic fate and most importantly well designed clinical studies to assess the efficacy of these polymers in relation to current therapies.

Table 2.4: Examples of various types of polymer categories

Natural Polymers		**Synthetic Polymers**	
Proteins	Polysaccharides	Absorbable	Non-absorbable
Collagen, Albumin, Gelatin	Starch, Cellulose	Polylactide, Polyglycolide, Poly-ε-caprolactone	Silicone rubber, Hydrogels

2.7.1.2 Polyester Polymers

The key features that have attracted investigators to these polymers include largely available toxicological and chemical data, biocompatibility or histocompatibility, predictable biodegradation kinetics, ease of fabrication, commercial availability, variety in copolymers ratios and molecular weights and most importantly, regulatory approval. A broad spectrum of performance characteristics with these polymers includes careful manipulation of four key variables: monomer stereochemistry, co-monomer ratio, polymer chain linearity and polymer molecular weight. The polymer characteristics have been found to play a major role in designing suitable drug delivery devices. Varying crystallinities, range of hydrophilic behaviour and solubility profiles are exhibited by different polymer forms, which ultimately affect the biodegradation and release profiles. Crystalline domain and stereo-irregularity

inhibit the degradation of the polymer. Stereo-irregularity in lactides determines the order of degradation time. Thus degradation time of poly-L-lactide (crystalline and stereo-irregular) is maximum, followed by poly-DL-lactide (amorphous and stereo-irregular) and then by Polyglycolide (crystalline and stereo-regular). (Sinha and Trehan, 2003).

Polyesters are available in a wide range of molecular weights, which influence the ultimate performance characteristics of the delivery system. This affects the biodegradation and resultant release profiles. Higher molecular weight of the polymer results in increasing viscosities of the solutions and in turn affects the entrapment efficiency, microsphere size and sphericity. Varying the co-polymer ratios results in different crystallinities, glass transition temperature (T_g) and hydrophilicities, which in turn affects biodegradation profiles. Polymer chain linearity affects the hydrophilicity of the polymer, which in turn affects the degradation rate. The extent of block or random structure in the copolymer also affects the hydration rate and thus the degradation profile. Degradation of aliphatic polyesters occurs by a random, non enzymatic hydrolytic cleavage of ester linkages, known as bulk erosion mechanism. The nature of degradation can be homogenous or heterogeneous which further determines the release kinetics.

For the aliphatic polyesters, homogeneous degradation is bulk erosion. It occurs throughout the device and rate of water penetration is greater than its conversion to water-soluble fragments. On the other hand, heterogeneous degradation is confined to the surface of the polymer carrier where it is interfaced with the physiological environment. The degradation rate is constant and the ungraded carrier retains its chemical integrity during the process. Biodegradation times vary depending upon other properties which include the molecular weight of the polymer, sequencing and cross-linking within the polymer backbone, surface area of the device, porosity of the matrix, hydrophobicity of the matrix and the reactive groups present.

Extensive research and successful use of polymers of lactic acid and glycolic acid as biodegradable drug delivery systems and as biodegradable sutures led to the discovery of the degradability of poly-ε-caprolactone *in-vivo* and henceforth, the identity of poly-ε-caprolactone as a biodegradable polymer emerged. The homopolymer itself degrades very slowly when compared with polyglycolic acid and polyglycolic acid-co-lactic acid and is most suitable for long-term delivery systems. Poly-ε-caprolactone and its derivatives, with a high permeability to many therapeutic drugs and lack of toxicity are well suited for controlled drug delivery ystemss. Another property of poly-ε-caprolactone, which has stimulated much research, is its exceptional ability to form compatible blends with a variety of other polymers. This adds to the diversity of poly-ε-caprolactone-derived materials that may be used in drug delivery applications.

2.8 DIFFERENT METHODS OF MICROSPHERE PREPARATION

Many different techniques have been used to prepare polymeric microspheres which are described below:

2.8.1 Oil-in-water (o/w) emulsion solvent extraction/evaporation method

An emulsion solvent extraction/evaporation technique can be used for preparing PCL microspheres. Both hydrophobic and hydrophilic drugs like nifedipine and propranolol hydrochloride have been encapsulated by this method. In the solvent evaporation method described by Perez et. al., 2000, the required amount of polymer and drug are dissolved in an organic phase (e.g. methylene chloride) which is emulsified under stirring with 0.25% w/w polyvinyl alcohol (PVA) solution to form an o/w emulsion. Stirring is continued for 3 hours at about 500 rpm, to evaporate the organic phase. The microspheres so formed are filtered and dried (Perez et. al., 2000). PCL microcapsules were also prepared by using a slightly modified emulsion solvent evaporation technique by Barbato et al., 2001. Here, PCL was

dissolved in 10 ml of dichloromethane and emulsified into 100 ml of 4% w/v aqueous PVA solution with the help of a high speed homogenizer at 10,000 rpm. This emulsion was then magnetically stirred at 500 rpm followed by addition of 100 ml of distilled water which increased the diffusion of organic solvent into the external aqueous phase to promote microsphere hardening. The microspheres were collected by centrifugation, washed and dried after completion of solvent evaporation (Barbato et. al., 2001).

2.8.2 Oil-in-oil (o/o) or anhydrous system method

The encapsulation of water soluble drugs by the o/w solvent evaporation method may result in rapid partitioning of the drug from the organic phase into the aqueous phase, resulting in microspheres with little or no drug loading. A modification to the conventional o/w solvent evaporation method has been researched to circumvent this problem. Anhydrous systems or the o/o type are comprised of an organic polymer phase emulsified in an immiscible organic phase resulting in an o/o emulsion. The elimination of water significantly reduces the tendency of the drug to partition into the continuous phase, when the drug is insoluble in external oil (O'Donnell et. al., 1997) Sturesson and coworkers used an o/o system to produce timolol maleate loaded PLGA microspheres (Sturesson et. al., 1993). The drug and polymer was dissolved in acetonitrile and sesame oil was used as the continuous phase. Span 80 was added as an emulsion stabilizer. The PLGA-drug solution was then added to the sesame oil, and the system was vigorously agitated. The system was sonicated to further decrease the particle size. Agitation was continued till acetonitrile evaporated, at which point microspheres were collected. The microspheres were rinsed with hexane to remove the residual oil. Drug release from the microspheres was reported to be triphasic in nature. An initial burst phase was observed due to release of drug located near the microsphere surface. This was followed by a period of slow release which was attributed to degradation of the microsphere and diffusion of the drug out of the microsphere. The third phase, described as a

secondary burst, and was attributed to the increased solubilization and erosion of the polymeric matrix. The initial release of the drug decreased as the polymer concentration in the formulation increased. Similar release profiles have been reported by other researchers (Bodmeier et. al., 1987; Spenlehauer et. al., 1988). The influence of formulation methods on the controlled release of BSA from PLGA microspheres was studied by Wang and his coworkers. Poly (DL-lactide / glycolide) microspheres containing bovine albumin were prepared with and without carbomer 951 (Carbopol 951) by either an o/o, o/w, or w/o/w emulsion techniques and the *in-vitro* albumin release was studied. The particle size of the microspheres was reported to be approximately 500 mm, 25–100 mm, or 10–20 mm for o/o, o/w, or w/o/w emulsion methods, respectively. The greatest burst of albumin release was observed with vacuum dried microspheres formulated by the water-in-oil-in-water method. A higher initial release rate was observed with microspheres prepared by the o/w emulsion method with Carbopol 951 than from those where Carbopol 951 was not used. Albumin was found to be sustained for 54, 36, or 34 days in microspheres prepared by o/o, o/w, or w/o/w emulsion methods, respectively. The investigators concluded that albumin release from microspheres could be controlled by the varying the method of preparation (Wang et. al., 1991). PLGA microspheres containing glycine and its homopeptides (diglycine, triglycine, tetraglycine, and pentaglycine) were also prepared by an anhydrous o/o technique by Pradhan et. al. in 1994 (Pradhan et. al., 1994). Micronized glycine homopeptides suspended in a solution of PLA and acetone served as the inner phase. The external phase consisted of mineral oil containing 0.3% (v/v) sorbitan sesquioleate as an emulsifier. Emulsification was carried out by adding the internal phase to the pre-cooled external phase. The temperature was then elevated to 35 °C to allow evaporation of the solvent. The microsphere suspension was then poured into an excess of *n*-hexane and stirred for an hour. The hardened microspheres were then collected. Formulation parameters like PLA concentration in the dispersed phase, emulsifier concentration and emulsification time was investigated. Release

profiles of glycine and diglycine microspheres indicated that they were released predominantly by a matrix-controlled diffusional process, where tortuosity and porosity of the diffusional path controlled the release rate of the drug. Gentamycin sulphate loaded PLGA microspheres have been obtained by researchers using o/o emulsion technique where the drug was released in over 60 days in a near zero-order fashion (Leel, 1990).

2.8.3 Water-in-oil (w/o) emulsion method

In the water-in-oil emulsion technique, the polymer is dissolved in an organic solvent like acetonitrile. The drug is dissolved/ suspended in this solution of the polymer in the organic solvent. This acts as the dispersed aqueous phase and is emulsified with the continuous oil phase which may be liquid paraffin or vegetable oil containing suitable emulsifiers. Subsequent removal of the organic solvent results in the precipitation of both the polymer and the drug leading to the formation of the microspheres. This technique is found to improve the drug loading properties of water soluble compound. Polyester (PLA) microspheres entrapping mitomycin C have been prepared using this technique (Tsai, 1986).

2.8.4 Water-in-oil-in-water (w/o/w) double emulsion solvent evaporation method

Many protein drugs like nifedipine and propranol HCl (Perez et. al., 2000) and protein drugs like serum albumin (BSA) (Benoit et. al., 1999) have been encapsulated in PCL using the w/o/w double emulsion solvent evaporation method. In the earlier case, aqueous solution of the drug is emulsified with PCL in dichloromethane. The resulting w/o emulsion is further emulsified with water containing PVA as an emulsifier. Stirring for 5 min at 1200 rpm leads the formation of a w/o/w emulsion. The stirring was continued to evaporate the organic solvent leading to formation of microspheres. After decantation, the microparticles were filtered, washed and dried in an oven for 24 hours at 50°C (Perez et. al., 2000). PCL microspheres encapsulating the protein BSA were also prepared by modified w/o/w

emulsion technique (Sah et. al., 1995). Aqueous solution of BSA was emulsified with methylene chloride as the organic solvent containing PCL, by homogenization. Shear rate was set at a particular rpm (11–23 rpm) to prepare w/o emulsion with different shear forces. Double emulsion was finally prepared by pouring the primary w/o emulsion into 4% aqueous PVA solution. This w/o/w emulsion was kept at constant stirring for 30 min. Additional water was slowly added to the emulsion over a period of 30 min. Microcapsules were collected by filtration and dried under vacuum.

The existing methods of emulsification-solvent evaporation techniques for encapsulation of proteins and peptides use an external aqueous medium for the process (Sinha et. al., 2004). Proteins and peptides, due to their hydrophilicity, can preferentially partition out into the external aqueous medium leading to low entrapment efficiency of the protein drug. Thus, in order to prevent such leaching of the drug, a novel method of microencapsulation of proteins and peptides was developed by modifying the w/o/w to w/o/o i.e. water-in-oil-in-oil emulsion technique in which oil was used as processing medium. In w/o/o technique, the hydrophilic proteins would find it unfavorable to diffuse out of the microspheres before they harden, thus increasing the entrapment efficiency of the protein drug (Badri et. al., 1999).

2.8.5 Multiple emulsions: water-in-oil-in-oil (w/o/o) or water-in-oil-in-oil-in-oil (w/o/o/o) method

A multiple emulsion of the w/o/o/o type was developed by Iwata and McGinity (Iwata and McGinity, 1992). Multiphase microspheres of either PLA or PLGA containing water-in-oil (w/o) emulsions were prepared by a multiple emulsion solvent evaporation technique. Acetonitrile was used as the solvent for the polymer and the continuous phase comprised of light mineral oil for the encapsulation procedure. Drug loading efficiencies found for the model water soluble compounds ranged from 80 to 100% of theoretical based on specific preparative conditions. These multiphase microspheres of the w/o/o/o type were observed to

belong to the class of reservoir type drug delivery devices. This was suggested by scanning electron microscopy which showed the transverse cross-sections of the multiphase microspheres depicting cavities in which the w/o emulsion resided. With this type of multiple emulsion system, encapsulation of a primary w/o emulsion within a polymeric microsphere can be carried out as the oil in the primary emulsion prevents contact between the encapsulated protein and the polymer/ solvent systems. The isolation of the internalized protein from the polymer/ solvent system prevents possible denaturization of the protein by the polymer or the solvent. Similarly, the possibility of reactive proteins or drug compounds degrading the polymer is also limited. A multiple emulsion potentiometric dispersion technique for the preparation of multiphase microspheres of poly (DL-lactic-co-glycolic acid) was carried out by O'Donnell and his coworkers (O'Donnell et. al., 1995). Water soluble compounds were dissolved in the aqueous phase and emulsified in soybean oil to form a stable emulsion w/o emulsion. This primary emulsion was dispersed in a solution of PLGA and acetonitrile to form the w/o/o emulsion. The w/o/o emulsion was then dispersed in a hardening solution of light mineral oil using potentiometric dispersion technique to produce microspheres of the w/o/o/o type with a very narrow and selective size distribution. The size of the microspheres was controlled by varying the internal diameter of the conductive infusion tube or by the variation of voltage applied to the conductive tube. Particle size analysis revealed a narrow particle size distribution with 80% of the microspheres made by this method in the 20 to 40 mm range as compared to a wide distribution of 50 to 500 mm for microspheres made by conventional agitation methods. Pentazocine was formulated, by Mishra and Pandit, in multiple w/o emulsion systems for prolonged release (Mishra and Pandit, 1990). Chlorpheniramine maleate was encapsulated with a loading efficiency of 88.9% with the potentiometric method as compared to a loading efficiency of 74.3% for the agitation method (O'Donnell et. al., 1995; O'Donnell et. al., 1997).

2.8.6 Coacervation/ Phase separation by non-solvent addition method

Polymer phase separation in non-aqueous or by addition of polymer is also called coacervation. It is an excellent technique for entrapping water soluble drugs as peptides, proteins or vaccines. This technique involves suspending the drug, either as solid crystals or aqueous solution, in an organic solution of polymer and subsequent phase separation by addition of a second miscible organic solvent in which the solvent is miscible. Leutinizing hormone releasing hormone (LHRH) analogues have been entrapped in PLGA microspheres using this technique (Redding et. al., 1984). Various drugs which have been encapsulated using this technique include diphtheria toxoid (Johansen et. al., 1999), nafarelin acetate (Sanders et. al., 1984), oxytetracycline hydrochloride (Vidmar et. al., 1984), sulphamethazole (Nakano et. al., 1980), pilocarpine hydrochloride (Vidmar et. al., 1985).

2.8.7 Spray drying method

In this technique, the biodegradable polyester is dissolved in a volatile organic solvent, such as dichloromethane or acetone. The drug in solid form, is dispersed in the polymer solution, by high speed homogenization, and this dispersion is atomized in stream of heated air. The solvent evaporates instantaneously from the droplets formed, yielding microspheres in size ranges from 1 to 100 microns depending upon atomizing conditions. The microspheres are collected from air stream by a cyclone separator and residual solvents are removed by vacuum drying. This process can be operated under aseptic conditions, and in closed loop configurations. Important advantages of this technique over other encapsulation techniques are well defined control of particle size, control of drug release properties of the resulting microspheres, established reproducibility and process being tolerant to small changes of polymer specifications. The disadvantages of the spray drying technique include high capital investment, protein encapsulation using this technique requires lyophilisation and homogenization in organic polymer solution. These processing conditions are likely to

induce aggregation and denaturation of sensitive proteins and antigens so that the stability of microencapsulated proteins during processing, release and storage becomes a foremost concern. Some of the drugs encapsulated using this technique include recombinant human erythropoietin (Bittner et. al., 1998), bromocriptine mesylate (Kissel et. al., 1991), diazepam (Guinchedi et. al., 1998), ketoprofen (Giunchedi et. al., 1994), tetracycline hydrochloride (Bittner et. al., 1999).

2.8.8 Solution-enhanced dispersion method

Some microencapsulation techniques have certain shortcomings like use of toxic organic solvents, low encapsulation efficiencies or stability problems. To overcome these drawbacks, a new solution enhanced dispersion method (SED) was used for preparation of polymeric microparticles using supercritical fluid (Bodmeier et al., 1995). These techniques are based on the use of supercritical fluid like carbon dioxide. The following two techniques were used to minimize the use of organic solvents during the preparation of microparticles. Phillips and Stella described a technique, rapid expansion of supercritical solutions (RESS), in which microparticles were prepared by spraying a solution of polymer in mixture of carbon dioxide and organic solvent into the air (Phillips and Stella, 1993). In another method termed as aerosol solvent extraction system (ASES), organic polymer solution was atomized into a vessel containing compressed carbon dioxide. Microparticles were formed after precipitation of the polymer, caused by extraction of organic solvent into carbon dioxide and by carbon dioxide diffusion into droplets. (Bleich et. al., 1993). The later is a more advantageous technique for the preparation of microparticles as the amount of toxic solvents such as dichloromethane is reduced and the properties of carbon dioxide may be adjusted over a continuum throughout the gaseous, supercritical and liquid states by varying the temperature and pressure. The solution-enhanced dispersion by supercritical fluids (SEDS) for the production of polymeric microparticles was also studied by Ghaderi and his co-researchers

(Ghaderi et. al., 1999). They used PCL as one of the polymers for the preparation of microspheres by SEDS. Solutions of the polymers in organic solvents were dispersed and sprayed with supercritical CO_2. Extraction of the organic solvents resulted in the formation of solid microparticles. It was reported that microspheres formed with PCL had diameters ranging from 30 to 210 μm and showed a strong tendency to form films at high pressure. The above stated study led to the conclusion that SEDS is a promising method for production of microparticles from biodegradable polymers since the amounts of highly toxic solvents such as dichloromethane were reduced in the process.

2.8.9 Interfacial deposition method

This technique was used by Uno and his group for preparing ethyl cellulose and polystyrene microspheres containing water. (Uno et. al., 1984). Drug free microspheres of PLA were prepared by Makino and coworkers in order to study the degradation of the polymer (Makino et. al., 1985). For this preparation, they emulsified n-heptane in aqueous Pluronic F_{68} solution (emulsifier) to yield an o/w emulsion. The polymer precipitated at the surface of n-heptane droplets when polymer solution in dichloromethane was added. Concomitant removal of organic solvent resulted in the formation of the microspheres. The disadvantage of this technique is the difficult encapsulation of water-soluble compounds as partitioning of core with the aqueous continuum occurs.

2.8.10 Hot melt microencapsulation method

In this process, the molten polymer was dispersed in a suitable dispersion medium which is then suspended in an immiscible solvent that is heated above the melting point of the polymer. After the emulsion stabilizes, it is slowly cooled to form the microspheres. The solvents used in this process could be silicon oil or olive oil and the low solubility of various drugs in these solvents makes them effective. This technique was initially developed for

photographic applications. This method of microencapsulation is reproducible with respect to yield and particle size distribution. It was especially successful in case of microspheres, which were susceptible to hydrolysis in presence of moisture. Polyanhydide microspheres have been prepared using this technique (Mathiowitz and Langer, 1987). The drawback is the temperatures to which the drug needs to be exposed. Polymers with low melting points could be fabricated into microspheres by this technique. This method cannot be used for encapsulation of protein or peptide drugs since increase in temperatures lead to their degradation.

2.8.11 *In-situ* polymerization method

This process has been patented by Speiser and Hinjsbroek for the preparation of PLA microspheres (Speiser and Hinjsbroek, 1992). A mixture of lactide and estrone was added to dried and degreased silicone oil and heated at 130°C to melt the lactide and estrone was allowed to dissolve in the melted lactide. After dispersion diethyl tin was added as a catalyst, to induce polymerization. Reaction was allowed to proceed for 5 minutes. Estrone embedded in PLA matrix was separated by centrifugation. Their technique produced microspheres from 100-1000 μm size. The disadvantage of this technique is controlling the degree of polymerization in the presence of the drug which is difficult.

2.8.12 Chemical and thermal cross-linking

A crosslinking process is required for the formation of microspheres made from natural polymers like chitosan, gelatin, albumin, dextran and starch. Here, the aqueous phase consists of the solution of the polymer containing the drug. The oil phase is comprised of a vegetable oil or oil-organic solvent mixture containing an oil-soluble emulsifier. A water-in-oil results and the water-soluble polymer is solidified by thermal or chemical crosslinking. It is known that gluteraldehyde forms stable chemical cross-links in albumin. Chitosan

microspheres have also been prepared with gluteraldehyde cross-links (Bodner et al, 2005). But when gluteraldehyde is used, residual solvent amounts and its toxic effects should be determined and taken into account.

2.9 ADVANTAGES OF PCL FOR MICROENCAPSULATION

The majority of the research work in the area of microencapsulation have utilized poly (lactic acid), poly (glycolic acid) or poly(lactic-co-glycolic acid) (PLGA) copolymers for the preparation of polymeric microspheres. The success of these for pharmaceutical applications has further led to the evaluation of aliphatic polyesters such as poly-ε-caprolactone (PCL) as a biodegradable polymer for microencapsulation processes. PCL is a biodegradable, biocompatible and semicrystalline polymer having a very low glass transition temperature. Due to its slow degradation, PCL is ideally suitable for long-term delivery. This has led to its application in the preparation of different delivery systems in the form of microspheres, nanospheres and implants. The major advantages of PCL include its high permeability to small drug molecules, their failure to generate an acidic environment during degradation as compared to polylactides and glycolides, an exceptional ability to form blends with other polymers and the degradation of PCL homopolymer being slow as compared to PLGA and polyglycolic acid-co-lactic acid making it more suitable for long term delivery systems (Koleske, 1990). Various drugs have been encapsulated in PCL for their effective delivery.

2.10 PROPERTIES OF POLY-ε-CAPROLACTONE

Poly-ε-caprolactone is a semicrystalline polymer, melting in the range of 59 to 66°C, depending upon the crystalline size. Because of its low glass transition temperature (T_g) of -60°C, the melt cannot be quenched to a glass. The heat of fusion (ΔH_f) of 100% crystalline poly-ε-caprolactone is reported to be 139.5 J/g, a value that has been used to estimate the crystallinity of poly-ε-caprolactone and its copolymers from differential scanning

calorimetry traces. The number average molecular weight of PCL samples may vary from 10,000 to 100,000 and it is graded according to the molecular weight (M_n). Crystallinity is known to play an important role in determining both permeability and biodegradability because of the accepted fact that the bulk crystalline phase is inaccessible to water and other permeants. Thus, an increase in crystallinity reduces the permeability by both reducing the solute solubility and increasing the tortuosity of the diffusional pathway. The biodegradation rate is reduced by the decrease in accessible ester bonds (Pitt, 1990). The T_g can be varied systemically by copolymerization. That is, the T_g values of copolymers with dilactide increase in proportion to the dilactide content to the Fox equation: (Schindler et. al., 1977).

$$\left(\frac{1}{T_g}\right)_{ab} = \left(\frac{W}{T_g}\right)_a + \left(\frac{W}{T_g}\right)_b \qquad (1)$$

where the subscripts refer to the two constituent homopolymers and W is the weight fraction. Only the polymers with a T_g below the body temperature are in the rubbery state and exhibit a high permeability. This is evident from comparisions of the permeability of polylactic acid with copolymers with increasing ε-caprolactone content.

The solubility parameters of poly-ε-caprolactone are 20.8 and 20.4 $J^{1/2}$ $cm^{-3/2}$ when calculated using Fedors and Hoy parameters respectively. Poly-ε-caprolactone is soluble in a number of solvents at room temperature like THF, chloroform, methyl chloride, dichloromethane, carbon tetrachloride, benzene, toluene etc. It is poorly soluble in acetone, ethyl acetate, acetonitrile and DMF, and insoluble in alcohols, petroleum ether and diethyl ether (Pitt, 1990). The physical properties of PCL are listed in Table 2.5.

Table 2.5: Physical properties of poly-ε-caprolactone (Pitt, 1990)

S. No	Property		Unit	Value
1	1% Secant Modulus		psi	50,000
2	Elongation		%	-750
3	Yield Stress		psi	1,600
4	Tensile strength (initial cross-sectional area)		psi	3,500
5	Melting Point		°C	63
6	Glass Transition Temperature, 1 Hz	i. Partially crystalline ii. Amorphous	°C	-60 -71
7	Density	i. 20°C ii. 30°C iii. 62°C iv. From 30 to -30°C v. From 62 to 100°C	g/cm3	1.149 1.143 1.069 5.6 x 10-4 -6.8 x 10-4
8	Equilibrium moisture content	i. 50% relative humidity ii. 100% relative humidity	%	0.07 0.43

2.10.1 Copolymers of -ε-caprolactone

The synthesis of block and random copolymers of poly-ε-caprolactone are derived from polyethylene glycol, ethylene oxide, polystyrene, polyvinylchloride, chloroprene, THF, substitiuted ε-caprolactone, methyl methacrylate and vinyl acetate. In addition to these, many copolymers have been prepared from oligomers of poly-ε-caprolactone. In particular, a variety of polyester-urethanes have also been synthesized from hydroxy-terminated poly-ε-caprolactone, some of which have achieved commercial status. Graft copolymers with acrylic acid, acrylonitrile, and styrene have been prepared using poly-ε-caprolactone as the backbone polymer (Pitt, 1990).

2.10.2 Blends of PCL with other polymers

The exceptional ability of poly-ε-caprolactone to form blends with many other polymers has stimulated a large amount of research. The potential of such blends for drug delivery has been largely exploited. Polymer blends have been categorized as (i) compatible, exhibiting only a single T_g, (ii) mechanically compatible, exhibiting the T_g values of each component but with superior mechanical properties, and (iii) incompatible, exhibiting the unenhanced properties of phase-separated materials. Based on the mechanical properties, it is suggested that poly-ε-caprolactone-cellulose acetate butyrate blends are compatible. Dynamic mechanical measurements of the T_g of poly-ε-caprolactone-polylactic acid blends indicate that the compatibility may depend on the ratios employed. Both these blends have been used to control the permeability of delivery systems (Pitt, 1990). The permeability of blends of poly-ε-caprolactone with cellulose propionate, cellulose acetate butyrate, polylactic acid, and polylactic acid-co-glycolic acid have been known to be useful for manipulating the rate of release of drugs from microspheres (Chang, 1986). Correspondingly, blending poly-ε-caprolactone with polylactic acid and polylactic acid-co-glycolic acid is a good method of modifying the rate of biodegradation of the composite.

2.10.3 Kinetics of drug release

Early studies by Pitt et al. established that poly-ε-caprolactone and its polymers have a high permeability to low molecular weight drugs (<400D). The permeability, J, of PCL was found to be 0.6×10^{-10} g/cm.sec. The permeabilities of copolymers of ε-caprolactone and dl-lactic acid have been measured and found to be relatively invariant for composition up to 50% lactic acid. The permeability then decreases rapidly to that of the homopolymer of dl-lactic acid, which is 105 times smaller than the value of poly-ε-caprolactone. An analysis of diffusion coefficients of some drugs like progesterone, testosterone, norgestrel, naltrexone, codeine, meperidine in PCL was carried out and the diffusion coefficients were found to be

3.6, 7.3, 4.1, 2.4, 3.8, 2.5 (X 10^9) cm^2/ sec respectively (Pitt et. al., 1987). The high permeability of poly-ε-caprolactone and its copolymers coupled with a controllable induction period prior to polymer weight loss lends itself to the development of delivery devices that are based on diffusion-controlled delivery of the drug during the induction period prior to weight loss. The subsequent biodegradation of the polymer serves the rationale of eliminating the need to recover the spent device.

2.10.4 Biocompatibility

When selecting polymers to be used in pharmaceutical applications, toxicity becomes a major issue that must be considered prior to formulation. The degradation rate of polymer and local tissue clearance are important parameters that need to be determined in order to predict the concentration present in the tissue and the resultant response. The inflammatory response of copolymer PCL and PLA after implantation in male wistar rats was studied (Pitt, 1990). These studies have shown that inflammation was moderate after two weeks of implantation and was greatest around the implanted area. The implantable microspheres of PCL, prepared by solvent evaporation method, were implanted in the brain of wistar rat and the tissue reaction was studied (Menci et al., 1994). Results showed that there was no necrosis which emphasizes the biocompatibility of microspheres with the brain tissue. It was further determined that the inflammatory reactions in bones were less pronounced than in muscles. This inflammatory reaction in muscle might be owing to better vascularization of muscle tissue and a greater amount of implanted material. The inflammatory response may be due to high localized concentration of degradation product and the transport potential of the polymer (Nakamura et. al., 1992; Ekholm et. al., 1999). The PCL microspheres when injected in the body results in activation of neutrophiles and causes localized inflammation. The activation of neutrophiles was measured by chemiluminescence and by measurement of superoxide anion generation. Neutrophiles activation released chemotactic factors leading to

influx of massive number of neutrophiles into the affected site and causing inflammation. Phagocytosis of drug loaded polymeric microspheres by WBCs is the main clearance mechanism by which foreign material is usually eliminated from the body (Jackson et. al., 2000; Tang et. al., 1993). To prevent this phagocytosis, the microsphere surface could be modification by steric stabilization (Jackson et. al., 2000). The effect of poly (epsilon-caprolactone) microspheres on apoptosis and cell cycle of fibroblast was studied using flow cytometry. Results showed that poly (epsilon-caprolactone) microspheres purified in different ways demonstrated different cytocompatibility with well-purified microspheres having good cytocompatibility (Luo et. al., 2003).

2.10.5 Biodegradation profiles

Studies of PCL portray a relatively complete picture of the factors and mechanisms responsible for degradation of polyesters (Pitt and Schindler, 1980; Gilbert et. al., 1982; Pitt and Schindler, 1983). Degradation of PCL is reported to be slow in comparison to polyglycolic acid and other polymers making it suitable for long-term delivery. Biodegradation of this polymer can be enhanced by copolymers like polylactic acid and polyglycolic acid (Koleske, 1978).

Degradation of PCL is a bulk process that can be divided into two phases; first, being molecular weight (M_n) loss up to 5000 due to chain scission and second, being the onset of weight loss (Sinha and Trehan, 2003). Pitt reported that the degradation of PCL is autocatalyzed. The kinetic patterns of PCL degradation are consistent with an autocatalytic process, whereby the liberated carboxylic acid end groups catalyze the hydrolysis, i.e. the cleavage of additional ester groups (Equation. (2)).

$$\text{–COOR} \cdot \cdot \text{HOOC–} + \text{H2O} \rightarrow \text{2-COOH} + \text{ROH} \quad (2)$$

$$\text{COOH} = k(\text{COOH})_0 \text{ (ester)} \quad (3)$$

$$\eta = \eta_0 \exp(-akt) \quad (4)$$

$$M_n = M_n^o \exp(-kt) \quad (5)$$

Assuming the concentration of ester groups is constant during the initial stages of hydrolysis, integration of Equation 3 leads to equations 4 or 5, which are related to the Mark–Houwink relationship (Equation 6)

$$\eta = KM_n^a \quad (6)$$

This equation expresses the observed exponential decline in the intrinsic viscosity (η) and number average molecular weight (M_n). No weight loss is observed during the initial phase of the biodegradation process, which covers a molecular weight (M_n) range of 200,000 to 5000. The second phase of polymer degradation is characterized by a decrease in the rate of chain scission and the onset of weight loss. Weight-loss has been attributed to an increased probability that chain scission of a low molecular weight polymer will produce a fragment small enough to diffuse out of the polymer bulk and the break up of the polymer mass to produce smaller particles with an increased probability of phagocytosis. The decrease in the rate of chain scission is associated with an increase in crystallinity, since cleavage takes place in the amorphous region of the polymer (Sinha and Trehan, 2003). *In-vivo* degradation of microspheres based on PCL and its copolymers have been studied by Chen and his co-researchers. The biodegradation process of PCL was studied by implanting low molecular weight poly (ε-caprolactone- ^{14}C) in rats and measuring the radioactivity in urine, feces, expired air and the residual activity at the implant site. It was observed that absorption was complete in 60 days, although only 60% of the radioactivity was accounted for and only 9 ± 4% of the original radioactivity remained after 120 days. ε-hydroxycaproic acid, derived from complete hydrolysis of the polymer, and tritiated water were the only metabolites detected. The mechanism of bioabsorption was studied by electron microscopic

examination of the tissue at the implant site. This revealed the presence of intracellular polymer particles and demonstrated the role of phagocytosis in the final stage of polymer degradation (Chen et. al., 2000).

2.11 DIFFERENT DRUGS ENCAPSULATED IN PCL MICROSPHERES

Various categories of drugs have been encapsulated in PCL for targeted drug delivery and for controlled drug release. Microspheres of PCL either alone or of PCL copolymers have been prepared to obtain the drug release characteristics (Thombre and Cardinal, 1990). PCL is suitable for controlled drug delivery due to its high permeability to many drugs and non-toxicity (Murthy, 1997). Some of the drugs have been covered below.

2.11.1 Drugs and chemical substances

2.11.1.1 Taxol

PCL microspheres encapsulating the drug, taxol, prepared by solvent evaporation method were tested for angiogenesis and it was found that taxol released from the microspheres induced vascular regression and inhibited angiogenesis. *In-vitro* studies showed 25% of loaded drug was released in 6 weeks from microspheres containing 5% taxol (Dordunoo et. al., 1995).

2.11.1.2 Nifedipine

Nifedipine is an oral calcium-blocking agent used in antihypertensive treatment. It shows complete absorption after oral administration. However, its bioavailability is low due to first-pass hepatic metabolism (Murad, 1990). Solvent evaporation methods using o/w or w/o/w emulsion technique have been used for microencapsulation of lipophilic drug nifedipine. The encapsulation efficiency for nifedipine was 91% and 83% for o/w and w/o/w emulsions respectively. A controlled release profile was obtained for microparticles prepared by o/w

method (Perez et. al., 2000). In vitro drug release studies revealed that varying the manufacturing process of microspheres varied the drug release characteristics of the drug.

2.11.1.3 Propranolol

Propranolol HCl is an effective non-selective β-adrenergic blocking agent that has a widespread use for angina pectoris and hypertension. It also has a short biological half-life of about 2 to 3 hours (Chiao, 1994). After oral administration, it is completely absorbed. However, the systemic availability is relatively low due to metabolism by the liver during its first passage through the portal circulation (Hoffman, 1990), with considerable variation in the plasma level. Double emulsion solvent evaporation method using o/w or w/o/w emulsions have been used for microencapsulation of this hydrophilic drug, propranolol HCl. The encapsulation efficiency was found to be 37% and 57% for o/w and w/o/w emulsions respectively. Perez and his co-researchers revealed that varying the manufacturing process of microspheres varied the drug release characteristics of propranolol HCl (Perez et. al., 2000).

2.11.1.4 Gentamicin

Controlled release techniques propose a new approach for antibiotic delivery by targeting the drug to the site of infection. Antibiotics when administered systemically are not effective in eliminating infection for local drug therapy of arthritis. Sondhof and his coworkeres described the *in-vitro* release kinetics of gentamicin from PLGA and PCL microspheres (50/50), prepared by double emulsion technique, in the synovial fluid. The studies revealed that drug release from PCL microparticles were higher than that from PLGA (Sondhof et. al., 1998).

2.11.1.5 Ketoprofen

Ketoprofen is eliminated rapidly from blood after administration due to its low plasma elimination half-life of 1 to 3 hours. In order to achieve and maintain therapeutic plasma level, it requires to be administered at least twice daily. Microspheres of ketoprofen using PCL and cellulose acetate butyrate were prepared using spray-drying method to overcome this hurdle. It was found that the morphology, particle size distribution and the *in-vitro* release profiles from the microspheres were affected by polymeric composition. However, temperature of the spray drying process was found to have no effect on the above properties (Giunchedi et. al., 1994). Ketoprofen microspheres were also prepared from PCL, hydroxypropylmethylcellulose phthalate (HPMCP50) and PCL, hydroxypropyl methylcellulose phthalate (HPMCP50) blends and studied. The *in-vitro* drug release studies showed that release rate of ketoprofen from PCL microspheres was rapid probably due to porosity of PCL while the release rate of ketoprofen from HPMCP50 was found to be pH dependent (Guzman et. al.,1996).

2.11.1.6 Chlorpromazine

Chlorpromazine loaded microspheres using PCL and polylactide system were prepared by Chang and his research team (Chang et. al., 1986). The *in-vitro* studies demonstrated that microspheres of chlorpromazine prepared by using a combination of PCL and polylactide released drug in biphasic manner consisting of an initial fast release phase followed by slow release phase. It was also found that the mixture of PCL and cellulose acetate butyrate could control the drug release characteristics and the size of microspheres containing chlorpromazine, prepared by solvent evaporation method (Chang et. al., 1987a). Since polymers like poly-methylmethacrylate, polycarbonate formed sticky mass with PCL and prevents recovery of microspheres, microspheres were prepared using a combination of PCL with CAB. The *in-vitro* studies have shown that the drug release pattern changes with a

change in the polymer ratios. Chlorpromazine–HCL and progesterone were also encapsulated in PCL microspheres by an emulsion-solvent evaporation technique and the effect of PVA in the continuous phase on the dissolution properties of the microspheres was studied. Results indicated that the microspheres showed faster dissolution rates than those of the pure drugs. The molecular or colloidal dispersion of drugs in the polymer matrix and the high permeability of the polymer to both the drugs and water could be the possible reasons for the fast drug release (Chang et. al., 1987b).

2.11.1.7 Indomethacin

Indomethacin loaded PCL microspheres were prepared by a melt-dispersion technique without the use of organic solvents. This novel technique was given by Bodmeier and Chen (Bodmeier and Chen, 1989) where the microspheres were prepared by cutting the indomethacin–PCL films into small pieces and dispersing into hot water, containing 0.25% w/v polyvinyl alcohol, at a temperature of 80°C. The temperature being above the melting temperature of PCL caused the polymer to melt, which on homogenization resulted in formation of spherical particles. The toxicity problems of residual solvents could be avoided with this melt-dispersion technique. Solid, free flowing microspheres without drug crystals were obtained after cooling. The drug release from PCL microspheres prepared by the melt method was slower when compared to microspheres prepared by the solvent evaporation method.

2.11.1.8 Cyclosporine

Cyclosporine loaded PCL microspheres were prepared by the solvent evaporation method. Results showed that the stirring speed and the organic phase volume which significantly affected the microspheres size. The microsphere size decreased with increase in the internal phase volume or the stirring rate. A high entrapment percentage (98 ± 0.66%) was obtained

in Cyclosporin microspheres of size 2.5 μm, with the drug dissolved or molecularly dispersed within the dense polymeric matrix of microspheres. This formulation offered the possibility of cyclosporin administration through different routes (Aberturas et. al., 2002). Freeze drying studies showed that microspheres could be successfully lyophilized in the absence of cryoprotectants without significant changes in the drug entrapment.

2.11.1.9 Nitrofurantoin

Nitrofurantoin is an antibacterial agent and was encapsulated in PCL microspheres using the solvent evaporation process. Nitrofurantoin formed crystalline domains dispersed in the polymer matrix. On increasing the drug content, rod like crystals have been observed on the surface. Size distribution and the drug loading of the microparticles were found to modulate the *in-vitro* release rates of nitrofurantoin from the microspheres. Tabletting the microspheres have shown to produce a much slower release rate and that release profile was found to obey the Higuchi equation (Dubernet et. al., 1987).

2.11.1.10 5-Fluoro Uracil

5-FU loaded microspheres, using a triblock copolymer of PCL and ethylene oxide, were prepared by hot melt technique (Martini et al., 2000). Results of the *in-vitro* release studies showed a non linear release kinetics of the drug associated with a pronounced burst release. In another study, 5-FU microspheres were also prepared from copolymer of ε-caprolactone and l-lactide showed a fast initial release followed by a slow release, which stops at a limiting value depending on the copolymer composition (Guerra et. al., 2001). These polymers can be used for delayed drug delivery systems.

2.11.1.11 Steroids

Steroids undergo first pass metabolism when administered orally. To enhance the bioavailability and ensure continuous delivery of these steroids, microspheres were prepared. Microspheres of β-estradiol and progesterone were prepared by solvent evaporation method using co-polymers of l, l-lactide or d, l-lactide and caprolactone (Pitt et. al., 1981). The best uniform release rate of β-estradiol was reported using PCL with the copolymer containing 83–93% of d, l-lactide (Buntner et. al., 1996). The effect of copolymer chain microstructure on progesterone and estradiol release from microspheres was studied by Buntner and it was seen that initial rate of release of progesterone from microspheres containing copolymer of l, l-lactide was higher than that observed in case of microspheres containing d, l-lactide based copolymer. A nearly constant release rate was found when using the copolymer containing 93% of d, l-lactide. Also, in the case of β-estradiol, a more uniform release rate was obtained when d, l-lactide was used with PCL. The *in-vivo* release studies of estradiol in male wistar rats was studied and it was observed that during the first 70 days, the changes in the release rate were small and the release of estradiol was nearly constant (Buntner et. al., 1998). Thus, it was concluded that the copolymers of d, l-lactide and PCL have good potential as materials for controlled release of progesterone and estradiol.

2.11.2 Proteins, peptides and biological substances

2.11.2.1 Bovine serum albumin

In order to understand the relationship amongst morphology, drug distribution and *in-vitro* release profiles for developing controlled release devices, bovine serum albumin (BSA) was encapsulated in PCL microspheres and studied by many researchers (Sinha et. al., 2004). Microspheres fabricated with a low polymer concentration had less defective skin surface and a less tortuous inner matrix which resulted in a more rapid BSA release. It was seen that a low oil-phase volume yielded microspheres with a porous matrix and defective skin

surface, which resulted in a high initial BSA burst as well as a fast release profile. Also, the presence of PVA in the internal water phase enhanced the stabilization of inner water droplets against coalescence. This resulted in a more uniform drug distribution and a slower BSA release. A higher BSA loading gave a larger concentration gradient between the microspheres and the *in-vitro* medium which resulted in lower encapsulation efficiency of the microspheres (Yang et. al., 2001). Spherical microspheres consisting of polymer blends 80:20 PEAD/PCL II and 40:40:20 PEAD/P(HB-HV)/PCL II containing a range of BSA loadings were prepared using a single emulsion technique with solvent evaporation (Atkins, 1997). It was found that 80:20 PEAD/PCL II microspheres had smooth surfaces while 40:40:20 PEAD/P(HB-HV)/PCL II microspheres consisted of a mixture of smooth surfaced, microporous and macroporous microsphere fractions. Irrespective of fabrication polymer, microspheres were produced in high yield (>75%) and BSA incorporation had no significant effect on microsphere size distribution which ranged from 0.6 to 5 μm and from 2.1 to 50μm for 80:20 PEAD/PCL II and 40:40:20 PEAD/P(HB-HV)/PCL II microspheres, respectively. The low encapsulation efficiencies (<14.5%) could be explained due to loss of BSA by partitioning into the aqueous phase. Huatan studied the effect of inclusion of poloxamer 181, a triblock copolymer of poly (ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide), into matrix of entrapped BSA in a novel ternary blend, comprising of high and low molecular weight PCL (Huatan et. al. 1995). It was observed that it retarded the rate of crystallization of PCL, thereby enhancing the particulate sphericity and the regularity. The effect of variables such as protein to polymer ratio, internal phase volume and emulsifier concentration in both the internal and external aqueous phases, on the properties of the microspheres was studied. It was observed that a mean particle size, ranging from 10 to 42 μm, was achieved by altering the internal phase volume of the primary emulsion. A protein entrapment of 11% w/w was observed with a protein to polymer ratio of 1:4. A study based on a 2(3) factorial experiment was designed to compare the BSA loaded PCL microparticles

prepared by hot-melt technique (Lin and Yu (2001). The effect of the particle size of protein, protein/polymer ratio, and hydrophilic PEG on the surface morphology, particle size, yield of PCL microparticles, encapsulation efficiency of BSA, and *in-vitro* release properties were observed. It was seen that the particle size of BSA affected the size, the burst release as well as the cumulative release of protein in these microparticles. The initial loading of BSA in terms of BSA/PCL ratio and the amount of PEG blended with PCL significantly affected all of the properties, except the yield. Another study based on 2(4) factorial experiment was designed by Lin and his research team to investigate the characteristics of bovine serum albumin (BSA) loaded PCL microparticles (Lin et. al. (2001). The influences of polyvinyl pyrrolidone (PVP) concentration, BSA/PCL ratio, w/o/o/o ratio, and PEG/PCL ratio on the surface morphology, particle size, as well as the yield of microparticles, encapsulation efficiency of BSA, and *in-vitro* release properties were evaluated. Surface topography revealed highly porous morphology in all microspheres irrespective of the formulations. Results revealed that the volume ratio of the multi-phases significantly affected the encapsulation efficiency of BSA in PCL microparticles, and the initial amount of BSA encapsulated by PCL in terms of BSA/PCL ratio significantly affected the amount of BSA released at the end of 14 days. In another study, Lin and Huang evaluated the particle morphology and *in-vitro* release of BSA from porous and non-porous PCL-F127 blended microparticles (Lin and Huang, 2001a). Here, the BSA loaded PCL microparticles were prepared by the w/o/o/o emulsion-solvent evaporation method using two types of homogenizer, a Polytron homogenizer and a probe ultrasonicator and the effects of solvent evaporation rate on the crystallinity and the performance of the microparticles were studied. The microparticles prepared with a Polytron homogenizer were quite porous in structure, which created channels for protein to continuously diffuse out, and resulted in sustained and controlled-release characteristics. In addition, the initial burst release of protein from the microparticles was also reduced. The rate of evaporation affected the size of resulting

microparticles although no change was observed in the crystallinity of the final microparticles. The above results led to the conclusion that carefully control of these variables can yield microparticles with desirable release performance. The influence of pluronics on BSA-loaded PCL microparticles prepared by the w/o/o/o solvent evaporation technique with an ultrasonicator was also studied (Lin and Huang (2001b). The morphology revealed spherical shape of microparticles with a rough surface which may be due to crystallization of PCL in the microparticles. The size of microparticles prepared was significantly reduced as the pluronics efficiently prevented microparticles from aggregation. It was seen that incorporation of pluronic F127 significantly increased the encapsulation efficiency and decreased the burst release of BSA from PCL microparticles along with increase in the hydrophilicity of the matrix, which further retained protein in blended microparticles. Youan and his group also prepared BSA loaded PCL microparticles by w/o/w solvent evaporation technique (Youan et al., 1999a). The *in-vitro* release studies of BSA loaded PCL microparticles were carried out in presence or absence of sodium dodecyl sulphate (SDS) and it was observed that the release of BSA from microparticles was significantly faster in medium containing SDS. A slow burst effect from the microspheres followed by a continuous release for 28 days was found.

2.11.2.2 Insulin

In order to maintain constant plasma drug concentrations for the effective control of blood sugar levels over prolonged period of time, PCL microsphere based parenteral depot system for insulin was developed (Shenoy et. al., 2003). This study revealed that biodegradable depot-forming PCL microspheres controlled the release of drug and maintained plasma sugar levels more efficiently than plain insulin injection upon subcutaneous administration. The *in-vitro* release behaviour of insulin from PCL-microparticles was studied by Limin et. al. and the drug release was found to be rapid initially and then slowed down exponentially. The *in-*

vivo studies were carried out in diabetic rats to evaluate the hypoglycemic effects after subcutaneous administration of the microparticles. The blood glucose level in rat serum after administration of insulin containing PCL microparticles was effectively lowered (Limin et. al., 2000). PCL microspheres encapsulating insulin, formulated by double emulsion solvent evaporation technique, was reported by Mukerjee and co-researchers (Mukerjee et. al., 2006). The PCL microspheres obtained were smooth and spherical. A prolonged hypoglycemic effect was observed with these microspheres in experimental animal models. These formulations, with their reduced frequency of administration and better control over drug disposition, may be better than the products currently available for diabetes control.

2.11.2.3 Ribozyme

Ribozymes are the catalytic RNA that bind and cleave specific regions of the target RNA. They are rapidly cleared from plasma so that the effective treatment of proliferative diseases requires repeated administration of these agents to maintain therapeutic ribozyme concentrations. Jackson et al. (Jackson et. al., 2002) encapsulated ribozymes in microsphere formulations in various size ranges using a modified w/o/w emulsion system to allow controlled release of these agents over extended period of time. These formulations released non-degraded ribozymes, *in-vitro*, in a controlled manner.

2.11.2.4 Antigen

PCL has a good permeability to proteins and degrades very slowly. Also, PCL does not generate an acidic environment like PLA and PGA, which can adversely affect the antigenicity of the vaccine and hence, it can be used as a vaccine carrier (Jameela et. al., 1996). A vaccine delivery system against brucellosis was developed with microspheres of poly-epsilon-caprolactone and its blends with PLGA (Murillo et. al., 2002). The antigenic extract in hot saline from Brucella ovis was microencapsulated by the spray drying

technique. Results showed that formulation containing no PCL showed the highest encapsulation efficiency. Release rates were characterized by a high burst effect after 1 hour of incubation, followed by a slow and continuous release. The pH of the medium dropped from 7.4 to 3.5 during release for the formulation based on PLGA while the presence of poly-epsilon-caprolactone attenuated the pH drop. Microspheres prepared with poly-epsilon-caprolactone showed the higher uptake by J744-macrophages and cell respiratory burst. PCL based microparticulate system intended for protein delivery was designed for oral immunization by Youan et. al. (Youan et. al., 1999b). PCL microspheres were prepared containing 3% BSA by a double emulsion technique. Adult wistar rats were used for immunogenicity studies after administration of PCL microspheres. The *in-vitro* release studies showed an initial burst of 10–12% of the protein and about 60.5% of protein release. No change in the pH of the buffer incubated with PCL microspheres was seen over a six-month period, which indicated that protein antigenicity was not affected by encapsulation in PCL. Jameela et al. (Jameela et. al., 1997) also prepared microspheres by the melt encapsulation technique. The *in-vitro* studies showed that the release rate of the protein from microspheres prepared by melt encapsulation technique was slower as compared to release from microspheres prepared by solvent evaporation technique The *in-vivo* studies in rats showed that a single injection of BSA-loaded PCL microspheres generated an immune response comparable in magnitude and time kinetics to that of conventional three-injection schedule. All these research suggests that the PCL microsphere based antigenic formulation can be potential candidates for vaccine delivery.

2.11.2.5 E- & P- selectin

In some diseases, the expression of the endothelial selectins E- and P- appears to be increased. This makes these molecules ideal targets around which targeted drug-delivery systems can be designed. One possible approach for achieving such delivery is to use

polymeric biodegradable microspheres tagged with a humanized monoclonal antibody (MAb) for E- and P-selectin, MAb HuEP5C7.g2. Based on this, Dickerson et al. prepared microspheres from the biodegradable polymer, PCL, using a single emulsion technique and PVA as a stabilizer (Dickerson et. al., 2001). On incubation of the PCL microspheres with HuEP5C7.g2, the adhesion of the resulting HuEP5C7.g2 microspheres to E- and P-selectin under *in-vitro* flow conditions was investigated and it was observed that the HuEP5C7.g2 PCL microspheres exhibited specific adhesion to Chinese hamster ovary cells stably expressing P-selectin (CHO-P) and 4-h IL-1β-activated human umbilical vein endothelial cells (HUVEC). At the same time, HuEP5C7.g2 PCL microspheres exhibited little adhesion to parental CHO cells or unactivated HUVEC. Based on this, it was postulated that the limited attachment efficiency was due to a low level of HuEP5C7.g2 adsorbed to the PCL microspheres. Although the attachment was limited, a significant percentage of the HuEP5C7.g2 PCL microspheres were able to remain adherent at relatively high shear (8 dyn/cm2) which led to the conclusion that HuEP5C7.g2 PCL microspheres exhibited selective limited adhesion to cellular substrate expressing E- and P-selectin.

2.11.2.6 Nerve Growth Factor

Following injury to the central nervous system (CNS), such as after spinal cord injury, nerve growth factor (NGF) may enhance axonal regeneration. Biodegradable polymeric microspheres were prepared from PLGA 50/50, PLGA 85/15, PCL and a blend of PCL/PLGA 50/50 (1:1, w/w) (Cao and Shoichet, 1999). Nerve growth factor loading in the microspheres was varied with highest amount of protein being encapsulated in PCL and minimum in PLGA 50/50. A two-phase release profile was observed for all polymers, where the first phase resulted from release of surface proteins and the second phase resulted predominantly from polymer degradation. The amount and bioactivity of released NGF was

followed over a 91-day period using a NGF-ELISA and PC12 cells, respectively. NGF was found to be bioactive for 91 days.

2.11.2.7 Heparin

Microparticles of pure or blends of biodegradable (PCL and poly (d,l-lactic-co-glycolic acid)) and of positively-charged non-biodegradable (Eudragit RS and RL) polymers entrapping heparin were prepared by a double emulsification and evaporation process. The effect of polymers and some excipients like gelatin A and B, NaCl was observed on the particle size, morphology, heparin encapsulation as well as the *in-vitro* drug release (Jiao et. al., 2002). The use of blends of two polymers in the organic phase was found to modify the drug entrapment as well as the heparin release kinetics compared with the microparticles prepared with a single polymer. Results showed that microparticles prepared with Eudragit RS and RL exhibited higher drug entrapment efficiency but lower drug release within 24 hours as compared to those prepared with PCL and PLAGA.

2.11.2.8 Injectable bovine substitute

PCL microparticles manufactured by a solvent evaporation–extraction process in the size range of 80–200 μm were introduced into the IBS in a 5–50% (v/v) range. Injectability was evaluated by texture analysis. Results revealed that with less than 45% of particles, the material had rheological properties similar to those of the reference IBS, whereas injectability decreased markedly with more than 45% of particles (Sinha et. al., 2004).. The *in-vitro* release analysis showed that this type of triphasic IBS could be efficient for drug delivery systems with osteoconduction properties (Iooss et. al., 2001).

2.11.3 Other forms of PCL used in drug delivery

Many other forms of devices made of PCL are also reported which help in delivering of various therapeutic applications. Some of these are described below.

2.11.3.1 Fibers

Luong-Van and his group suggested that electrospun PCL fibers are a promising candidate for delivery of heparin to the site of vascular injury. Sustained delivery of heparin to the localized adventitial surface of grafted blood vessels has been shown to prevent the vascular smooth muscle cell (VSMC) proliferation that can lead to graft occlusion and failure. In this study heparin was incorporated into electrospun PCL fiber mats for assessment as a controlled delivery device. Fibers with smooth surfaces and no bead defects were spun from polymer solutions with 8%w/v PCL in 7:3 dichloromethane: methanol. Assessment of drug loading and imaging of fluorescently labeled heparin showed homogenous distribution of heparin throughout the fiber mats. A total of approximately half of the encapsulated heparin was released by diffusional control from the heparin/PCL fibers after 14 days. The fibers did not induce an inflammatory response in macrophage cells in vitro and the released heparin was effective in preventing the proliferation of VSMCs in culture (Luong-Van et. al., 2006). In another study, a hydrophilic macromolecule, ovalbumin (OVA), and a lipophilic drug, progesterone, was incorporated in polycaprolactone fibres by gravity spinning using particulate dispersions and co-solutions of PCL and steroid, respectively. Results demonstrated them as potential PCL fibre-based platforms for programmed delivery of bioactive molecules of utility for tissue engineering and drug delivery (Williamson et. al., 2004).

Electrospinning of nanofibers and nanotubes allows generation of fibers with diameters in a wide range around 100 nm. Reneker and his co-researchers showed a new phenomenon where electrospinning a solution of PCL in acetone caused the dramatic appearance of a

fluffy, columnar network of fibers that moved slowly in large loops and long curves. The name 'garland' was given to the columnar networks (Reneker, 2002).

2.11.3.2 Matrices

Microporous PCL matrices were loaded with the aminoglycoside antibiotic, gentamicin sulphate (GS), using the precipitation casting technique by suspension of powder in the PCL solution prior to casting (Chang et al., 2006). Improvements in drug loading from 1.8% to 6.7% w/ w and distribution in the matrices were obtained by pre-cooling the suspension to 4°C. Gradual release of approximately 80% of the GS content occurred over 11 weeks in PBS at 37°C and low amounts of antibiotic were measured up to 20 weeks. GS-loaded PCL matrices retained anti-bacterial activity after immersion in PBS at 37°C over 14 days as demonstrated by inhibition of growth of *S. epidermidis* in culture. These findings recommend further investigation of precipitation-cast PCL matrices for delivery of hydrophilic molecules such as anti-bacterial agents from implanted, inserted or topical devices. Jackson and his group encapsulated ribozymes in injectable polymeric paste formulations to allow the controlled release of these agents over extended periods of time for the treatment of localized disease sites, such as cancer and arthritis (Jackson et. al., 2002). Ribozymes were also effectively encapsulated in poly (L-lactic acid) (PLLA) and poly (lactic-co-glycolic) (PLGA) microspheres. The release rate of ribozymes from PCL pastes could be effectively controlled by altering the loading concentration of ribozymes in the paste. A novel melt-extrusion process was developed by Sprockel and coworkers, to prepare matrix delivery systems. The disks contained drugs suspended in various polymers or polymer/ additive combinations (Sprockel et. al., 1997). Theophylline was incorporated into polyethylene, PCL, polyvinyl alcohol and cellulose acetate butyrate at a 50% drug loading and studied.

2.11.3.3 Bone scaffolds

The use of biodegradable polymers for drug delivery systems excludes the need for a second operation to remove the implant or the carrier. A novel drug delivery system, consisting of PCL or PCL 20% tricalcium phosphate (PCL-TCP) biodegradable scaffolds, fibrin Tisseel sealant and recombinant bone morphogenetic protein-2 (rhBMP-2) was investigated by Rai and his group for bone regeneration. PCL and PCL-TCP-fibrin composites displayed a loading efficiency of 70% and 43%, respectively. PCL-fibrin composites loaded with 10 and 20 mg/ml rhBMP-2 demonstrated a triphasic release profile as quantified by an enzyme-linked immunosorbent assay (ELISA). This consisted of burst releases at 2 h, and days 7 and 16. A biphasic release profile was observed for PCL-TCP-fibrin composites loaded with 10 mg/ml rhBMP-2, consisting of burst releases at 2 hours and day 14 (Rai et. al., 2005). In another experiment, hydroxyapatite (HA) porous scaffold was coated with HA and polycaprolactone (PCL) composites, and antibiotic drug, tetracycline hydrochloride, was entrapped within the coating layer (Kim et. al., 2005). A well-developed porous structure facilitated its usage in a drug delivery system due to its high surface area and blood circulation efficiency. The PCL polymer, as a coating component, was used to improve the brittleness and low strength of the HA scaffold, as well to effectively entrap the drug. To improve the osteoconductivity and bioactivity of the coating layer, HA powder was hybridized with PCL solution to make the HA–PCL composite coating. The *in-vitro* biodegradation of the composite coatings in the phosphate buffer saline solution increased linearly with incubation time and the rate differed with the coating concentration and the HA/PCL ratio; the higher concentration and HA amount caused the increased biodegradation. At short period (<2 hours), about 20–30% drug was released especially due to free drug at the coating surface.

Giavaresi and his group evaluated five novel polymers *in-vivo* after implantation in the rat dorsal subcutis and compared to the reference polycaprolactone (PCL). Poly (cyclohexyl-

sebacate) (PCS), poly (L-lactide-b-1,5-dioxepan-2-one-b-L-lactide) (PLLA-PDXO-PLLA), two 3-hydroxybutyrate-co-3-hydroxyvalerate copolymers (D400G and D600G), and a poly (organo)phosphazene (POS-PheOEt:Imidazole) specimens were histologically evaluated in terms of the inflammatory tissue thickness and vascular density at 4 and 12 weeks from surgery (Giavaresi et al., 2004). These different behaviours to improve neoangiogenesis without severe inflammatory tissue-responses could be further investigated with drugs in order to obtain time-programmable drug delivery systems for musculoskeletal therapy.

2.11.3.4 Joint spacers

Sepsis of a total joint replacement may not respond to treatment with systemic antibiotics. In these circumstances, the treatment is often a two-stage revision of the prosthesis; the infected prosthesis is removed, a period of treatment with an antibiotic-loaded joint spacer ensues, and finally a new total joint is implanted once the infection has subsided.

A PMMA temporary joint spacer is often used. Patient activity causes this temporary implant to wear, releasing potentially damaging PMMA particles into the joint environment. Elfick researched an alternative biodegradable polymer, PCL, was proposed and investigated for use as a temporary joint spacer (Elfick, 2002).

2.11.3.5 Micelles

Drug-loaded methoxy poly (ethylene oxide)-b-poly (ε-caprolactone) (PEO-b-PCL) micellar solutions in isotonic medium were prepared and administered intravenously to healthy *Sprague Dawley* rats. Blood and tissues were harvested and assayed for cyclosporine A (CsA), and resultant pharmacokinetic parameters and tissue distribution of CsA in its polymeric micellar formulation were compared to its commercially available intravenous formulation (Sandimmune®) (Aliabadi et. al., 2005). In the pharmacokinetic assessment, a 6.1 fold increase in the area under the blood concentration versus time curve (AUC) was

observed for CsA when given as polymeric micellar formulation as compared to Sandimmune®. In the biodistribution study, analysis of tissue samples indicated that the mean AUC of CsA in polymeric micelles was lower in liver, spleen and kidney (1.5, 2.1 and 1.4-fold, respectively). Similar to the pharmacokinetic study in these rats, the polymeric micellar formulation gave rise to 5.7 and 4.9-fold increase in the AUC of CsA in blood and plasma, respectively. Results further showed that PEO-b-PCL micelles could effectively solubilize CsA, at the same time confining CsA to the blood circulation and restricting its access to tissues such as kidney, perhaps limiting the onset of toxicity.

Block copolymer micelles were also formed and investigated by Allen and his coresearchers from copolymers of poly (caprolactone)-b poly (ethylene oxide) (PCL-b-PEO) as a drug delivery vehicle for dihydrotestosterone (DHT). The physical parameters of the PCL-b-PEO micelle-incorporated DHT were measured, including the loading capacity of the micelles for DHT, the apparent partition coefficient of DHT between the micelles and the external medium and the kinetics of the release of DHT from the micelle solution. The MTT survival assay was used to assess the *in-vitro* biocompatibility of PCL-b-PEO micelles in HeLa cell cultures. The biological activity of the micelle-incorporated DHT was evaluated in HeLa cells which had been co-transfected with the expression vectors for the androgen receptor and the MMTV-LUC reporter gene. The PCL-b-PEO micelles were found to have a high loading capacity for DHT and the release profile of the drug from the micelle solution was found to be a slow and steady release which continued over a period of one month. The biological activity of the micelle-incorporated DHT was found to be fully retained (Allen et. al., 2000).

2.11.3.6 Oligomers

The effect of the addition of small molecular weight anhydride oligomers to polymer microspheres was investigated. Santos and his group demonstrated increased bioadhesion of

the composite with the addition of oligomers. Blends of low molecular weight anhydride oligomers with thermoplastic poly (fumaric-co-sebacic anhydride) [p (FASA)] and polycaprolactone were examined. The results demonstrated that fumaric anhydride oligomer remained within polymer microspheres for several hours after exposure to phosphate buffer, formed a homogenous crystalline blend, increased bioadhesion as measured on rat intestine, and enhanced drug delivery *in-vitro* as measured by the everted sac technique (Santos et. al., 2003).

2.11.3.7 Composite foams

Biodegradable and biostable composite foams were formed from porous apatite cement infiltrated with ε-caprolactone (CL) or methylmethacrylate (MMA) using a high over vacuum. For CL composites, *in-situ* polymerization was induced using trace water as an initiator and heating for 10 days or for 60 days. CL preparations gave composites with a polycaprolactone (PCL) number average of molecular weight (M_n) up to the maximum of 7.1×10^3 g/mol after 10 days and 16.8×10^3 g/mol after 60 days. Maximum compressive strengths increased from 9MPa to 37 and 64MPa for PCL and PMMA composites, respectively. The water drop contact angle of the PCL composite was 64°, and therefore suitable for cell attachment while the PMMA composite surfaces were more hydrophobic. (Walsh et. al., 2001)

2.11.3.8 Multiphase drug release

Three-dimensional printing, (3DP), is a novel technique for the fabrication of polymeric drug delivery systems. It is an example of a solid free-form fabrication method where both the macro- and microstructure of the device can be controlled since objects are built by addition of very small amounts of matter. The drug concentration profile is first specified in a computer model of the device which is then built using the 3DP process. Complex drug

delivery regimes can be created in this way, such as the release of multiple drugs or multiphasic release of a single drug. In a study by Wu and his group, two dyes were used as model drugs in a matrix of biocompatible polymers. The dye release rate and release time are controlled by either specifying the position of the dye within the device or by controlling the local composition and microstructure with the 3DP process. The mechanism of resorption can also be controlled by manipulating the composition and microstructure of the device during construction. Polyethylene oxide and polycaprolactone were selected as matrix materials and methylene blue and alizarin yellow were used as drug models (Wu et. al., 1996).

2.11.3.9 Tissue engineering

Microporous materials have been produced by gradual precipitation from solutions of poly (e-caprolactone) (PCL) in acetone induced by solvent extraction across a semi-permeable PCL membrane, which is formed *in-situ* at the polymer solution/non-solvent interface. Microparticulates of hydroxyapatite and inulin polysaccharide, respectively, were incorporated in precipitation cast PCL matrices to illustrate potential applications in hard tissue repair and macromolecular drug release. Microporous PCL and HA filled PCL materials were found to provide a favourable surface for attachment and growth of primary human osteoblasts in cell culture. The *in-vitro* degradation characteristics of microporous PCL and inulin /PCL materials in PBS at 37°C were monitored over 45 months. Microporous PCL demonstrated zero weight loss, minor changes in molecular weight characteristics and a fairly constant indentation resistance of around $1MN/m^2$. Inulin-loaded PCL materials exhibited a total weight loss of approximately 17% after 12 months in PBS. The indentation resistance decreased by 50% from an initial value of $28MN/m^2$ in the first 2 months and then remained stable. Precipitation cast materials based on PCL are expected to be useful for formulating long-term, controlled release devices for bioactive molecules such

as growth factors and hormones and extended-residence supports for cell growth and tissue development (Coombes et. al., 2004).

2.12 BIODEGRADABLE MICROSPHERES BASED FORMULATIONS IN MARKET

A list of some of the microsphere formulations in the market has been reported. A key driver for developing these products was to ensure patient compliance. The active pharmaceutical ingredients in these products include small molecules, peptides and proteins.

2.12.1 Decapeptyl® (Ipsen-Beaufour/ Ferring)

It is a sustained release formulation of triptorelin based on lactide/ glycolide microspheres for monthly intramuscular administration. It was developed by Debio R.P. (Valais, Switzerland). After injection, an initial plasma peak is observed during 3 hours, and then triptorelin plasma levels decrease and remain stable at a plateau for 28 days. This preparation was the first sustained release formulation of LHRH agonist allowing continuous release of drug over 30 days for the treatment of prostate cancer, ovarian cancer and breast cancer, endometriosis and other gynecological disorders.

2.12.2 Lupron depot® (TAP)

Lupron Depot is a gonadotropin-releasing hormone (GnRH) agonist. It contains luprorelin in the form of injectable microspheres. It is used to decrease the production of specific hormones, natural chemicals in the body which influence the behaviour of certain cells. Lupron Depot is used for the treatment of specific conditions in men, women and children as it can reduce the production of both male and female hormones. It is also prescribed for the treatment of advanced prostate cancer. The doses available include 1, 3 and 4 month

products. Most common side effects associated with it are hypoestrogenism, vasomotor flushes, headache and vaginal dryness.

2.12.3 Suprecur® MP (Aventis)

It is a controlled release microsphere formulation of buserelin (a GnRH analogue), which enables a long lasting suppression of pituitary and gonad function with one monthly subcutaneous injection. It improves endometriosis, shrinkage of uterine leiomyoma and alleviation of symptoms linked with uterine leiomyoma, namely hypermenorrhea, lower abdominal pain, back pain and anemia.

2.12.4 Sandostatin LAR® depot (Novartis)

Sandostatin LAR® depot is a long acting octreotide loaded microspheres of biodegradable glucose star polymer, D,L-lactide and glycolic acids copolymer. This preparation mimicks the pharmacological properties of the natural hormone somstostatin and is indicated for the long term maintenance therapy in acromegalic patients, in severe diarrhea and flushing episodes associated with metastatic carcinoid tumors and vasoactive intestinal peptide secreting tumors.

2.12.5 Nutropin depot® (Genetech)

This formulation consists of microspheres containing molecules of growth hormone. The microspheres are made of a substance for making surgical threads used for dissolvable stitches. It is indicated for the long term treatment of growth failure due to lack of adequate endogenous growth hormone (hGH or GH) secretion. It is available in once or twice a month dosings.

2.12.6 Trelstar depot® (Pfizer)

Trelstar depot® formulation is a sterile, lyophilized biodegradable microsphere formulation supplied as a single-dose vial containing triptorelin pamoate as the peptide base, poly-d,l-lactide-co-glycolide, mannitol, carboxymethylcellulose and polysorbate 80. It is intended as a monthly dose after mixing with water for injection. Triptorelin is a synthetic decapeptide agonist analog of lutenizing hormone releasing hormone (LHRH or GnRH) and therefore this formulation is indicated in the palliative treatment of advanced prostate cancer.

2.13 DIFFERENT TECHNIQUES OF MICROSPHERE CHARACTERIZATION

Characterization of microspheres is very essential to ensure reproducibility of their *in-vitro* and *in-vivo* drug release profiles. Some of the techniques for the characterization of polymeric microspheres include the following:

2.13.1 Percent yield and percent encapsulation efficiency

Percent yield is a measure of the amount of microspheres obtained from formulation process and is calculated by the formula:

$$Percent\ Yield = \frac{Weight\ of\ microspheres\ obtained}{Weight\ of\ drug\ and\ polymer\ used\ for\ microsphere\ preparation} \times 100$$

Encapsulation efficiency refers to the amount of drug encapsulated inside the microspheres by a particular preparation technique. For its determination, the drug is extracted out of the microsphere in some suitable solvent and the amount of the drug is then estimated by UV spectroscopy at a particular wavelength or by an assay procedure specific to the drug. In another process, the microspheres are dissolved in a solvent (in which both the polymer and the drug are soluble) provided that the polymer does not interfere in the analysis at the

wavelength chosen for the drug analysis. The percent encapsulation efficiency (EE) is calculated using the formula:

$$Percent\ Encapsulation\ Efficiency = \frac{Drug(encapsulated)}{Drug(total)} \times 100$$

2.13.2 Particle size analysis

The most commonly used techniques for determination of particle size are optical microscopy, laser light scattering techniques, coulter analysis and photon correlation spectroscopy, the later being used in case of very small particle size. The three statistically acceptable measures of size in optical microscopy are Martin's diameter, Feret's diameter and Projected area diameter. Optical microscopy is very time consuming and is restricted to measuring particle in the size range of 3 to 150 μm only. Another limitation of optical microscopy, for wide size distribution, is the small depth of focus which results in viewing only some particles in a particular view at any one time.

The Coulter technique was presented as an automatic method for counting and sizing blood cells and was soon applied to many kinds of materials (Miller et. al, 2005). A coulter counter is capable of counting and sizing thousands of particles in a few seconds. The large number of particles analyzed greatly improves the accuracy of a number- based distribution. Further, it improves the accuracy when data are converted to a volume distribution. The concentration of suspended particles should be controlled as passage of two particles through the orifice at the same time, leads to over-sizing. This limitation becomes significant at higher concentrations.

Instruments based on light scattering principles for particle size analysis have become popular as they allow quick and absolute determination of particle size, without any need for calibration. There are three major light scattering methods which use laser and are based on

different principles. These are Static Light Scattering (SLS), Laser Diffraction and Dynamic Light Scattering (DLS) (Bodner et. al, 2005). All these methods require dilute concentrations where interaction between particles and secondary scattering are minimized, although this must be balanced against the requirement for enough sample to obtain sufficient signal to noise ratio. The Laser Diffraction and SLS are also referred to as total intensity light scattering (TILS) method (Aboofazeli, 2000). Laser Diffraction uses time averaged measurements of scattered light flux, whereas DLS measures fluctuations in the intensity of scattered light and relate them to size dependent parameters such as diffusion coefficients. Particle size distributions of 0.1-1000 μm can easily be measured with laser diffraction based analyzers.

2.13.3 Surface morphology analysis

Surface characterization of microspheres is very critical to the formulation as it provides important information about the microstructure, topography, texture, porosity, physical state, nature of diffusion barriers and polymer erosion.

Scanning Electron Microscopy (SEM) has a high resolution of 3 nm and is one of the standard techniques for microsphere surface characterization. SEM allows investigation of microsphere surfaces, texture, and morphology of fractured or sectioned surfaces. The biggest advantage of SEM over other techniques is its versatility.

2.13.4 Atomic force microscopy

The Atomic Force Microscope (AFM), invented by Binnig et al. in 1986, is a powerful instrument for studying the structures and properties of nanoscale molecules (Binnig et. al., 1986). Atomic force microscopy is one of the most advanced techniques for studying of surface characteristics of micro and nanoparticles as AFM is successful in providing three-dimensional information. It is often used to study biological systems because it does not

require the sample surface to be electrically conductive and can be used in liquid as well as gaseous environments. It enables to obtain details of topography, size, and shape of a nanostructured surface (wikipedia-atomic_force_microscope). It has been used to obtain high-resolution images of cells, proteins, and nucleic acids (Sekiguchi et. al., 2003; Hinterdorfer et. al., 1997; Hinterdorfer et. al., 2000; Kienberger et. al., 2000; Schmidt et. al., 1999).

2.13.5 Confocal laser scanning microscopy

Light microscopy and scanning electron microscopy are the most frequently used visualization techniques for microparticles and microcapsules. The conventional light microscopy is impeded by the scattered or emitted light from structures outside the optical focal plane. Scanning electron microscopy does not allow visualization of the inner structures of objects and usually requires a relatively complex sample preparation before analysis. Confocal Laser Scanning Microscopy (CLSM) provides a significant advantage to optical microscopy and SEM. It has the capacity for noninvasive, serial optical sectioning of intact, dense, living samples with a minimum of sample preparation, provided the material is sufficiently translucent. A sample may be needed to be treated with fluorescent dyes to make objects visible as CLSM is dependent on fluorescence. By collecting several coplanar cross sections, a three-dimensional reconstruction of the inspected objects can be performed. Computational image analysis, thereby, allows the visualization and quantification of structures not only on the surface, but also inside the material. With all these advantages, CLSM has considerable potential for investigating the structure and following the dynamic processes within pharmaceutical drug delivery systems. (Lamprecht et. al., 2000).

2.13.6 Thermal analysis

Calorimetric techniques are very useful for the determination of various thermal parameters, which allow a better understanding of drug-polymer interactions, drug-excipient interactions and thermal denaturation of polymers. These techniques include Differential Thermal Analysis (DTA), Differential Scanning Calorimetry (DSC), Modulated Differential Scanning Calorimetry (MDSC), Thermo Gravimetric Analysis (TGA), isoperibol calorimetry and heat conduction calorimetry. Amongst them, DSC is the most widely used. It measures the heat capacity of the system as a function of temperature. Following the change in heat capacity of the sample as a function of temperature allows for the detection of any phase transitions.

2.13.7 Residual solvent analysis

Residual solvents are the organic chemical solvents that are used in the production of drug formulations or are produced during the synthesis of drug substances in the pharmaceutical industry. Usually, many of these volatile organic solvents cannot be completely removed by standard manufacturing procedures and are left behind at low concentrations. High levels of these residual solvents become a risk to human health because of their toxicity. They may generate occurrence of odor and colour changes in the final pharmaceutical product. The ICH and the USP Pharmacopoeia recommendations certain permissible limits for each class of solvents that may be present in any microsphere formulation which need to be met for clearing the FDA approval.

2.14 TECHNIQUES FOR EVALUATION OF MICROSPHERES

2.14.1 *In-vitro* release studies

The possible mechanisms of drug release from the polymeric microspheres include initial release from microsphere surface, release through the pores dependent on microsphere structure, diffusion through intact polymer barrier, which is dependent on intrinsic polymer properties and core solubility, diffusion through a water swollen barrier dependent on

polymer hydrophilicity, which in turn depends upon the polymer molecular weight, polymer erosion and bulk degradation, where release is affected by the rate of erosion and the hydrolysis of polymer chains, leading to pore formation in matrix.

2.14.2 *In-vivo* studies

In order to determine the efficacy of the formulation, the biological activity of the drug encapsulated and the in-vivo release of the drug from the microspheres needs to be ascertained. This is determined by carrying out experiments in animal models like *Sprague-Dawley* rats and male New Zealand rabbits under different conditions.

2.15 PHARMACOSCINTIGRAPHY AND TOXICITY STUDIES

2.15.1 Pharmacoscintigraphy

Nuclear medicine is defined as the branch of medicine which utilizes the nuclear properties of radioactive and stable nuclides for diagnostic evaluation of the anatomic and/or physiological conditions of the body and provides therapy with radioactive substances. Radiometric detection of orally administered drugs labeled with a suitable radiotracer is believed to be very useful to determine the path and morphology of drugs and to determine the efficiency of formulation to deliver the drug at a desired place in the gastrointestinal tract. The usefulness of radiopharmaceuticals for diagnostic or therapeutic applications is a function of both the radioisotope and the pharmaceutical moiety used. For diagnostic applications, the isotopes used should undergo γ-decay, since nuclear medicine cameras mostly detect γ-radiations. The half life should be just enough to allow the procedure to be performed and should not unduly impart radiation burden to the patient. Before being used for imaging, it is also necessary to establish the suitability of a radiopharmaceutical in terms of percent labeling and stability of the radioactive compound especially in presence of constituents of the serum with which it may come in contact during the course of imaging.

(Morandeau et. al., 2006; Mittal et. al., 2005; Kumari et. al., 2004; Chuttani et al., 2003; Bhattacharya et. al., 2001; Jaggi et. al., 1991).

2.15.2 Toxicity studies

After the development of any novel drug or its delivery system, preclinical toxicological evaluation is carried out (Badithe et. al., 2007). There are various types of toxicity studies like the conventional acute toxicity (LD50) and the repetitive dose toxicity etc. Toxicity studies are usually followed by a detailed histopathological evaluation of various tissues and organs of the animals to determine any adverse effect of the formulation on the tissues or the organs studied before carrying out the clinical studies for the formulation.

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CHAPTER 3

PREPARATION AND CHARACTERIZATION OF POLY-ε-CAPROLACTONE MICROSPHERES

3.1 PREAMBLE

The major aim of drug therapy is to achieve a steady-state blood or tissue level within the therapeutically effective and non-toxic range for an extended period of time. This ideal drug therapy requires spatial placement, which is targeting a drug to the specific organ or tissue and temporal delivery, which is controlling the rate of drug release to the target tissue. It is known that the conventional oral drug administration neither maintains drug blood levels within the therapeutic range for extended period of time, nor do they assure controlled drug release or target specificity. To maintain desired drug levels in the therapeutic range for a longer period of time, a drug may need to be administered repetitively using calculated dosing intervals. This results in saw tooth kinetics which is observed as a sharp increase of local drug concentration for relatively short periods at the expected therapeutic level, followed by a sharp drop and diminution until the drug is re-administered. This type of kinetics result in potential problems characterized by large fluctuations of drug concentrations in blood, frequent dosing intervals for drugs with short biological half-life and patient compliance. These problems can be resolved with use of polymeric microsphere based drug delivery systems. By delivering therapeutic molecules at a desired rate for a prolonged period of time, these delivery systems sustain optimal drug concentrations (Mathiowitz et. al., 1988; Mathiowitz et. al., 1990; Tamada and Langer, 1992), protect the bioactive drug molecule (Castellanos et. al., 2001) and increase patient compliance by effectively reducing the drug administration frequency.

One of the enduring features of drug delivery technology is the central role that polymers play in control of drug release and fabrication of drug delivery devices. Aliphatic polyester polymers are an important biomedical material because they are biodegradable and possess

good biocompatibility. Therefore, they find wide applications as surgical sutures, drug delivery carriers and in tissue engineering (Frazza and Schmitt, 1971; Coombes et. al., 2004).

Among biodegradable polymers, aliphatic polyesters have been used as a carrier material for drug encapsulated microspheres and nanoparticles. The advantages associated with the microsphere based delivery are improved therapeutic effect, prolonged biological activity, controlled drug release rate and decrease in the administration frequency. Further, the carrier may decompose after drug release and form nontoxic and low molecular weight species which can be metabolized or absorbed by the organism. Poly-ε-caprolactone (PCL) is one of the biocompatible and biodegradable aliphatic polyester polymers that degrades slowly and does not generate an acid environment unlike the other polyester polymers, polylactide (PLA) or polyglycolide (PLG). Although the permeability of macromolecules in PCL is low, such low permeability may be sufficient for drug delivery (Benoit et. al., 1999). Other advantages of PCL include its *in-vitro* stability and low cost.

The choice of a precise method of encapsulation is of enormous importance for the proper entrapment and drug release. Among many incorporation methods, the double emulsion technique has been used for higher microencapsulation of hydrophilic drugs and proteins within microparticles (Alex and Bodmeier 1989; Cohen et. al., 1991).

The objective of our work was to formulate a method for the efficient encapsulation of insulin in microspheres of a relatively new biodegradable polymer, poly-ε-caprolactone, using a water-in-oil-in-water (w/o/w) double emulsion solvent evaporation technique. The characterization of the microspheres thus obtained, was carried out in terms of percent yield, percent encapsulation efficiency, surface morphology and particle size. The optimization of this preparation process was further carried out by varying some of the influencing process parameters.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Insulin Human Injection USP, 40 and 100 IHU/ml (Eli Lily and Company, USA), Poly-ε-caprolactone (M_n 42,500; Sigma-Aldrich Chemical Company, USA), Hydroxypropyl methylcellulose (M_w 10,000; Sigma-Aldrich Chemical Company, USA) Polyvinyl Alcohol (M_w 30,000; Fluka, USA), Dichloromethane (Merck, Mumbai, India), Chloroform (S.D. Fine-Chem Ltd., India), Ethyl Acetate (S.D. Fine-Chem Ltd., India), Micro BCA Protein Estimation Kit (PIERCE®, USA). Double adhesive tapes were used for SEM studies. Double distilled water was used for all the experiments.

3.2.2 Preformulation studies

3.2.2.1 Establishing analytical procedure for estimation of insulin by micro BCA assay

The micro BCA protein assay is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of protein. Analysis of dilute protein samples, 0.5-20 µg/ml, have been reported with the micro BCA assay. This method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment (Smith, 1985). A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increase in protein concentrations. The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA (Wiechelman et. al., 1988).

The micro BCA protein estimation assay consists of three solutions; (1) micro BCA reagent A containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH, (2) micro BCA reagent B containing 4% bicinchoninic acid in water and (3) micro

BCA and reagent C containing 4% cupric sulfate, pentahydrate in water. For analysis, the three solutions (A, B and C) are mixed in the ratio of 25:24:1 and this final solution acts as the working reagent. The sample to be analyzed is mixed with this working reagent in equal parts and mixed well. It is then incubated at 60°C in a water bath for 1 hour. The sample is then cooled to room temperature and absorbance of the sample is measured by a UV-visible spectrophotometer (Cary-100 Bio, Varian, USA) at 562 nm. This entire protocol is illustrated in a schematic diagram in Figure 3.1.

3.2.2.2 Spectrophotometric standard plot of insulin

The standard plots of insulin were prepared in both water and phosphate buffer saline (pH 7.4). All standardization experiments were carried out in triplicate. The slope was calculated from the regressed line obtained from the plot between concentration and absorbance at 562 nm wavelength.

3.2.2.2.1 Spectrophotometric standard plot of insulin in water

A known amount of drug (10 mg) was dissolved in 100 ml of distilled water to make a stock solution of 100µg/ml. Suitable dilutions were made to obtain working standards in the range of 1-10 µg/ml and corresponding absorbance was determined at 562 nm (Appendix IA).

3.2.2.2.2 Spectrophotometric standard plot of insulin in phosphate buffer saline, pH 7.4

A known amount of drug (10 mg) was dissolved in 100 ml of phosphate buffer saline (pH 7.4) to make a stock solution of 100µg/ml. Suitable dilutions were made to obtain working standards in the range of 1-10 µg/ml and corresponding absorbance was determined at 562 nm (Appendix IB).

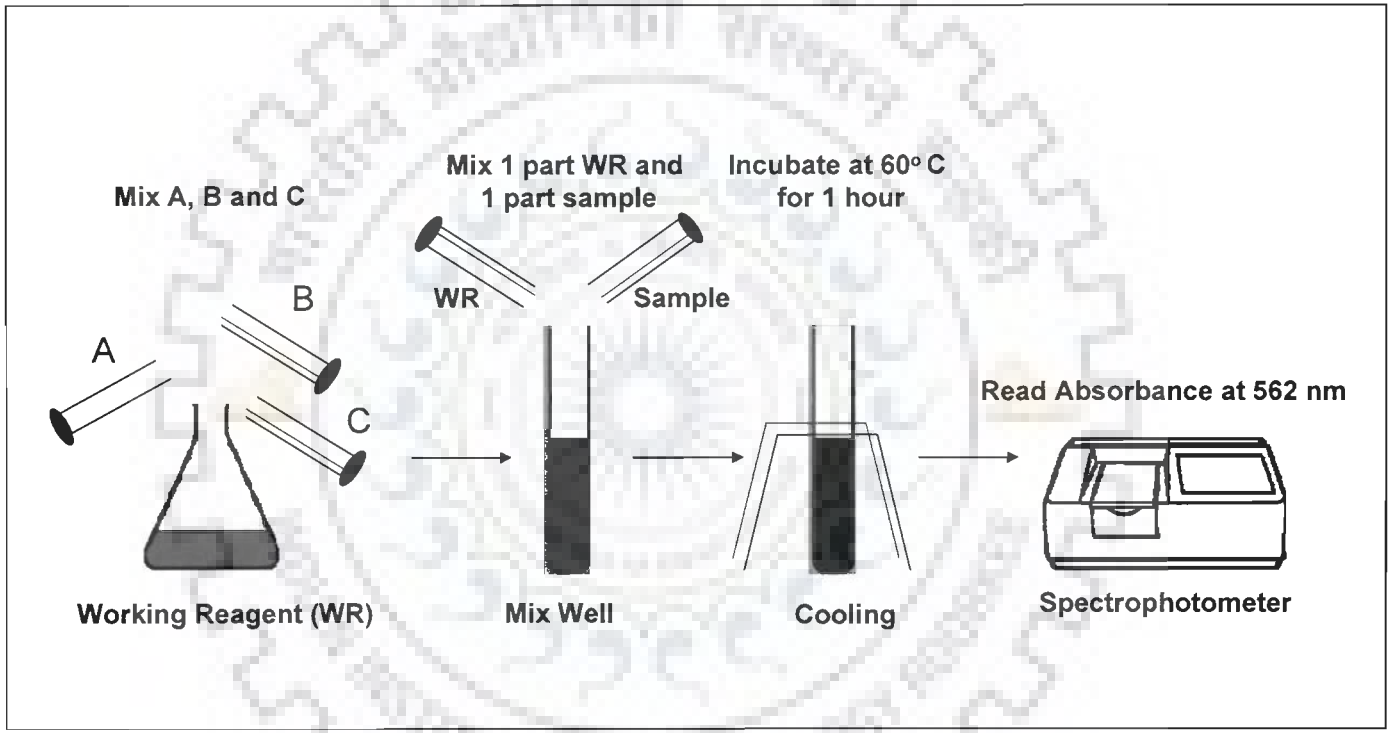

Figure 3.1 Schematic diagram of micro BCA protein assay protocol

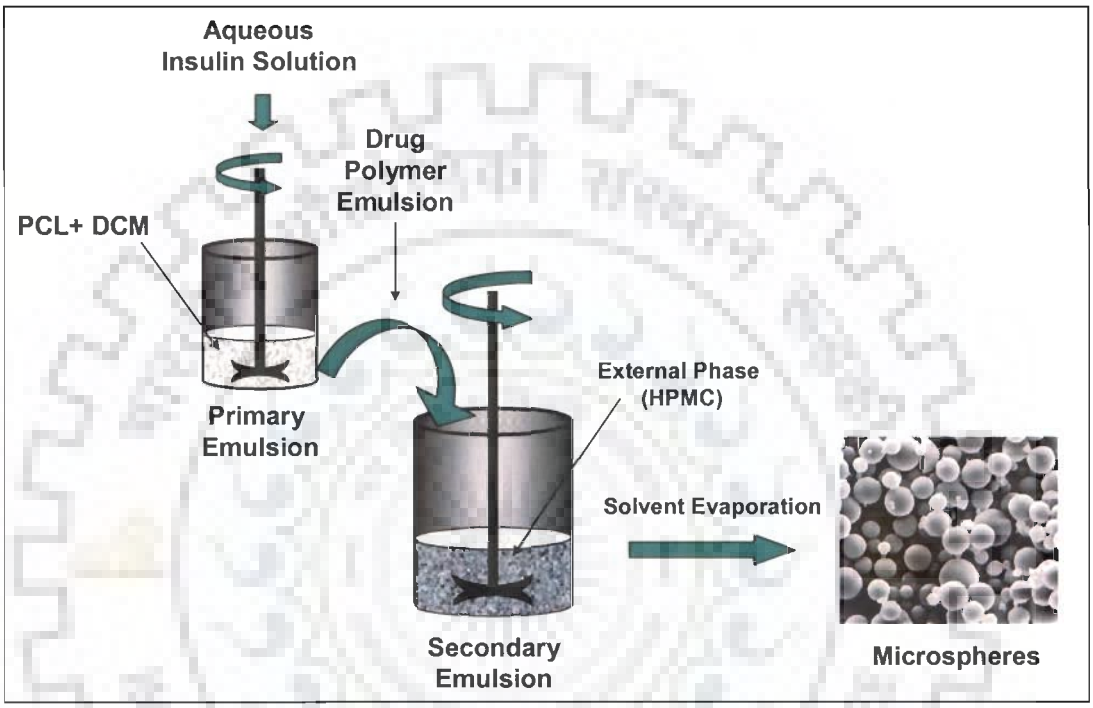

Figure 3.2 Schematic diagram of w/o/w double emulsion solvent evaporation technique

3.2.3 Preparation of poly-ε-caprolactone microspheres

The water-in-oil-in-water (w/o/w) double emulsion solvent evaporation technique was chosen for the preparation of the microspheres due to the solubility of insulin in water. PCL was chosen as the biodegradable polymer for the microencapsulation process. The preparation procedure was partially modified using the protocol of Sinha (Sinha and Trehan, 2003) and Attivi (Attivi et. al., 2005). The schematic diagram of the double emulsion solvent evaporation technique is shown in Figure 3.2.

An aqueous insulin solution (100 IHU/ml, 3 ml) was emulsified in the organic solvent, dichloromethane (10 ml) containing 1% concentration of PCL by stirring at 6000 rpm for 2 minutes, using a mechanical stirrer (RQT-124A, Remi Motors, India). The water-in-oil emulsion, thus formed, was thereafter added to 30 ml of external aqueous phase containing 1.0% solution of the stabilizer, HPMC, and again stirred at 4500 rpm for 150 minutes, resulting in the formation of the final w/o/w double emulsion. To facilitate solvent evaporation, the final emulsion was magnetically stirred for about five hours. After complete evaporation of the organic solvent, the polymer hardens trapping the encapsulated protein drug, insulin, forming the desired microspheres. The microspheres, thus formed, were isolated by centrifugation at 3000 rpm for 5-10 minutes. The microspheres were then washed three times with deionized distilled water, to remove any free drug or stabilizer by vacuum filtration, and finally, dried at room temperature. The dried microspheres were stored at 4°C. The microspheres obtained from this technique were then characterized.

3.2.4 Characterization of poly-ε-caprolactone microspheres

The microstructure of the microsphere is greatly affected by the formulation conditions and it, in turn, affects the microsphere characteristics like drug entrapment, stability of the drug entrapped and the drug release profiles (Yang et. al., 2001). Therefore, characterization of microspheres becomes very essential. The PCL microspheres obtained by

double emulsion solvent evaporation technique were characterized by measuring the percent yield, percent insulin encapsulation efficiency, determining the surface morphology and their particle size.

3.2.4.1 Determination of percent yield

The dried PCL microspheres were weighed to calculate the yield using the following formula:

$$Percent\ Yield = \frac{Weight\ of\ microspheres\ obtained}{Weight\ of\ drug\ and\ polymer\ used\ for\ microsphere\ preparation} \times 100$$

3.2.4.2 Determination of percent encapsulation efficiency

Controlling or increasing the encapsulation efficiency is desirable as it can prevent loss of the therapeutic protein drug and can also help in extending the duration and dosage of treatment. The encapsulation efficiency of the microspheres was determined by mixing the PCL microspheres (10mg) with 1 ml of dichloromethane. Water (5 ml) was added into the above solution for the extraction of insulin. The solution was mixed by a vortex mixer (Spinix, Tarson) and allowed to stand for a few minutes. After the layers separated, the upper layer (water) was analyzed for insulin. Micro BCA protein estimation kit (PIERCE®, USA) was used for the spectrophotometric estimation (Katare et. al., 2005) and the amount of drug present was calculated from calibration curves of concentration versus absorbance already prepared with known standards of the drug using the same kit (Appendix IA). The amount of the drug encapsulated and the percent encapsulation efficiency of the microspheres is given by the formula:

$$Percent\ Encapsulation\ Efficiency = \frac{Drug(encapsulated)}{Drug(total)} \times 100$$

3.2.4.3 Determination of surface morphology

The Scanning Electron Microscopy (SEM) is one of the excellent techniques for surface characterization of the microspheres. It is very useful for the physical observation and evaluation of the shape and surface morphology of microspheres. The scanning electron microscope is a type of electron microscope that creates various images by focusing a high energy beam of electrons onto the surface of a sample and detecting signals from the interaction of the incident electrons with the sample's surface. The type of signals gathered in a SEM varies and can include secondary electrons, characteristic x-rays, and back scattered electrons. In a SEM, these signals come not only from the primary beam impinging upon the sample, but from other interactions within the sample near the surface. The SEM is capable of producing high-resolution images of a sample surface in its primary use mode, secondary electron imaging. Due to the manner in which this image is created, SEM images have great depth of field yielding a characteristic appearance useful for understanding the surface structure of a sample. This great depth of field and the wide range of magnifications are the most familiar imaging mode for specimens in the SEM. Characteristic x-rays are emitted when the primary beam causes the ejection of inner shell electrons from the sample and are used to tell the elemental composition of the sample. The back-scattered electrons emitted from the sample may be used alone to form an image or in conjunction with the characteristic x-rays as atomic number contrast clues to the elemental composition of the sample. SEM allows analysis of the microsphere surfaces, texture and morphological features of fractured or sectioned surfaces at a high resolution. The spatial resolution of the SEM depends on the size of the electron spot, which in turn depends on both the wavelength of the electrons and the magnetic electron-optical system which produces the scanning beam (Wikipedia-scanning_electron_microscope).

The surface morphology of the microspheres was investigated using scanning electron microscope (Leo, VP-435, UK), shown in Figure 3.3. The dry samples of microspheres were

mounted on metal stubs using double sided adhesive tapes. They were then, sputter coated with gold particles under reduced pressure conditions and observed under the scanning electron microscope (Mukerjee et. al., 2007). The accelerating voltage was kept constant at 15 KV.

To determine the influence of release medium on the microspheres with time, experiments were carried out for a period 60 days and any changes on microsphere morphology and surface characteristics were studied. For this, the microspheres were placed in the release medium (PBS, pH 7.4) and samples of microspheres were collected at different time intervals of 0, 7, 15, 30, 45 and 60 days and scanned by SEM.

3.2.4.4 Particle size analysis

The particle size of a microsphere is a key parameter and may affect other parameters like rate of dissolution, rate of drug release and the dose of delivery. The mean diameter of the microspheres was evaluated by laser diffractometry using Brookhaven particle size analyzer (Brookhaven Instruments, UK), shown in Figure 3.4. Microspheres were dispersed in distilled water in an ultrasonic disperser for 1 minute to bring about disaggregation and analyzed.

The polymer concentration and stabilizer concentration has been found to influence the particle size of the microspheres. Hence the effect of polymer concentration and stabilizer along with its concentration, on the particle size of the microspheres was further studied.

3.2.5 Effect of variation of formulation parameters

3.2.5.1 Effect of different solvents on microsphere formulation

Using the above formulation process, experiments were designed using different organic phase (dichloromethane, chloroform or ethyl acetate) keeping the external aqueous phase as 1% solution of HPMC and the internal aqueous phase as insulin in water for injection (100

IHU/ml) constant. The entire set of experiments was then repeated with 1% solution of PVA as the external aqueous phase. This led to a total of six experiments, α1 to α6.

3.2.5.2 Effect of different stabilizers on microsphere formulation

The effect of each of the two stabilizers, HPMC or PVA, in the preparation of insulin loaded poly-ε-caprolactone microspheres was studied. The microspheres prepared with each of these stabilizers, batches (α1-α4), were characterized for their particle size and surface morphology.

3.2.5.3 Effect of different stabilizers and their concentrations on microsphere formulation

Experiments were designed using different stabilizers (HPMC or PVA) at various concentrations in the external aqueous phase for the preparation of the poly-ε-caprolactone microspheres. Different concentration of 0.5%, 1.0% and 1.5% solution of HPMC were used as the external phase for the preparation of the microspheres using the double emulsion solvent evaporation technique, keeping the polymer concentration at 1% and internal aqueous phase as insulin in water for injection (100 IHU/ml) constant. The entire set of experiments was then repeated with 0.5%, 1.0% and 1.5% solution of PVA as the external aqueous phase. This led to a total of six experiments, L1 to L6.

3.2.5.4 Effect of varying the polymer concentration on microsphere formulation

Experiments were designed using different concentrations of 0.5%, 1.0%, 1.5% and 2.0% of the polymer, PCL, for the preparation of the polymeric microspheres using the double emulsion solvent evaporation technique. The internal aqueous phase was kept as insulin in water for injection (100 IHU/ml) and the stabilizer concentration was kept at 1% for all the experiments. This led to a total of four experiments, P1 to P4.

3.2.5.5 Effect of varying the drug concentration in the internal aqueous phase on microsphere formulation

Experiments were designed using two different concentrations of the drug, insulin (100 IHU/ml and 40 IHU/ml), in the internal aqueous phase for the preparation of the polymeric microspheres using the double emulsion solvent evaporation technique. The stabilizer concentration and the polymer concentration were kept constant at 1%. This resulted in two experiments, D1 and D2.

3.3 RESULTS AND DISCUSSION

3.3.1 Preparation of poly-ε-caprolactone microspheres

The poly-ε-caprolactone microspheres were successfully prepared by the double emulsion solvent evaporation technique. Microspheres of biodegradable polymers encapsulating various drugs have been formulated using this technique by many research groups (Damgé et. al., 2007; Jiao et. al., 2002; Coccoli et. al., 2007; Yang et. al., 2001; Srinivasan et. al., 2005). Yang et. al., 2001 and Coccoli et. al., 2007, reported this method for the encapsulation of model protein drug, BSA in PCL microparticles while Jiao et. al., 2002, reported the same technique for heparin loaded polymeric microspheres of PCL, PLGA and polymethacrylates. This method was used for encapsulation hydrophilic non-protein based drugs like propranolol HCl by Pe´rez and his group (Pe´rez et. al., 2000).

Figure 3.3: Scanning Electron Microscope (Leo, VP-435)

Figure 3.4: Particle Size Analyzer (Brookhaven Instruments Corporation)

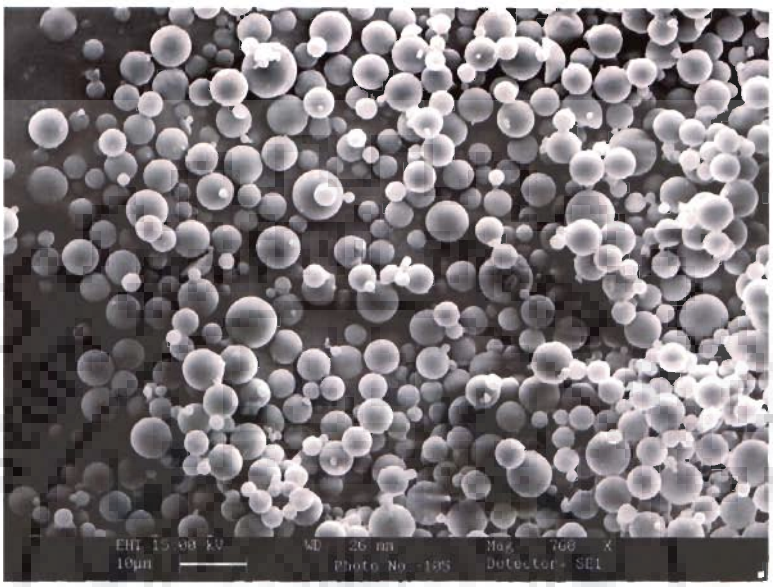

Figure 3.5: Poly-ε-caprolactone (PCL) microspheres

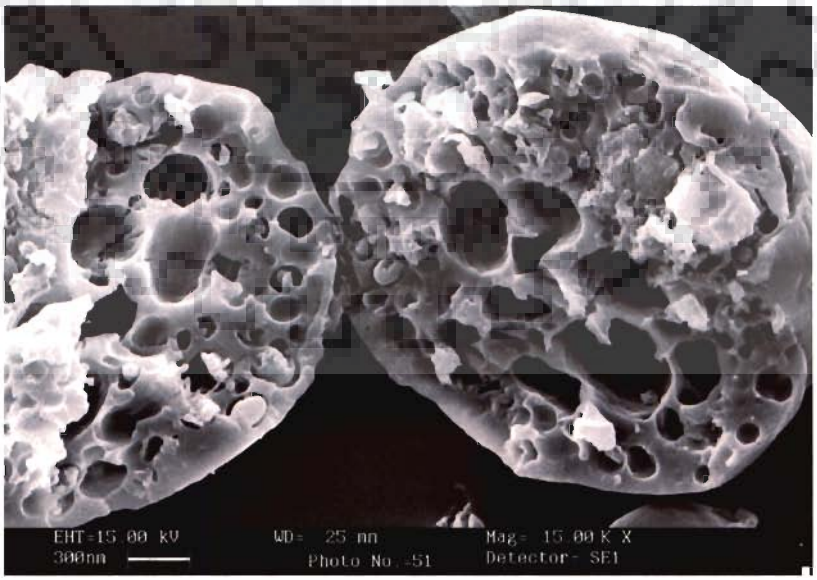

Figure 3.6: Cross section of a PCL microsphere revealing the internal morphology

3.3.2 Characterization of the microspheres

3.3.2.1 Determination of percent yield and percent encapsulation

The percent yield of microspheres prepared by the w/o/w double emulsion solvent evaporation technique, with dicloromethane as the organic solvent, 1.0% polymer (poly-ε-caprolactone) and 1.0% HPMC as the stabilizer was found to be high at 92.2 ± 0.26%. A comparable yield of 72-85 % was reported for PCL microspheres prepared by w/o/o double emulsion technique by Ramesh and his group (Ramesh et. al., 2002).

The percent insulin encapsulation efficiency of the above batch of microspheres prepared by the w/o/w double emulsion solvent evaporation technique was found to be moderate at 65.72 ± 0.13%. The hydrophilic nature of insulin and the difference in osmotic pressure between the internal and external aqueous phases could be responsible for the moderate entrapment efficiency observed with these microspheres. The solubility of insulin in the external aqueous phase and its high volume compared to that of the internal aqueous phase (w/o/w double emulsion) may have caused the leaching of the drug from the internal into the continuous external aqueous phase. This leaching process is believed to happen mainly during the first minutes of emulsification since the polymer precipitates rapidly thereby decreasing leaching (Bodmeier and McGinity, 1988). Similar moderate drug encapsulations have been reported by other researchers. An encapsulation of 56.6% has been reported for hydrophilic drug propranolol HCL in PCL microspheres (Pe´rez et. al., 2000). Coccoli et. al., 2007, reported a percent encapsulation efficiency of 60-63% for BSA in PCL microspheres prepared by the w/o/w method using 1% PVA as the stabilizer (Coccoli et. al., 2007). An encapsulation efficiency of 53% has been reported for heparin loaded PCL-Eudragit blend microspheres (Jiao et. al., 2002). Dhanaraju et. al., 2003 showed a similar 46.3 to 57.7% entrapment of levonorgestrol and ethinylestradiol in PCL microspheres formed by w/o/w emulsion technique (Dhanaraju et. al., 2003). Kim et al, 2005, reported a 61.3%

encapsulation of felodipine in PCL microspheres when prepared by o/w method and a 58.1% encapsulation when prepared by o/o method (Kim et. al., 2005).

3.3.2.2 Determination of surface morphology

Researchers have used the scanning electron microscopy to study and understand the morphology, the surface characteristics and the inner structure examination of the microspheres. In our study, the SEM scan revealed smooth, spherical microspheres for the above obtained batch, as shown in Figure 3.5. Other research groups have also used SEM to characterize their formulations and reported the formation of spherical microspheres (Coccoli et. al., 2007; Yang et. al., 2001; Pe´rez et. al., 2000; Srinivasan et. al., 2005).

The internal morphology of the microsphere, as seen from a cross-sectioned microsphere, Figure 3.6, revealed a porous inner structure which is a characteristic of w/o/w emulsion (Pe´rez et. al., 2000). The oil droplets of the double emulsion contain several aqueous droplets, resulting from the primary emulsion. The aqueous droplets are the precursors of pores and are a result of the phase separations occurring in the organic phase during hardening of the microspheres (Ch. Schugens et. al., 1994).

The results of the studies regarding the influence of release medium on changes in the microsphere morphology and surface characteristics with time are shown in Figure 3.7a to 3.7f. It is observed that the microspheres retain their morphology till the end of the study (60 days). However, some cracks may be seen after a month and there is slight change visible in the surface morphology of the microspheres after about 45 days due to the appearance of wrinkles. This may be explained due to the slow degradation characteristics of poly-ε-caprolactone. No degradation of the poly-ε-caprolactone microspheres till 60 days has also been reported by Yang and his co-researchers (Yang et. al., 2001).

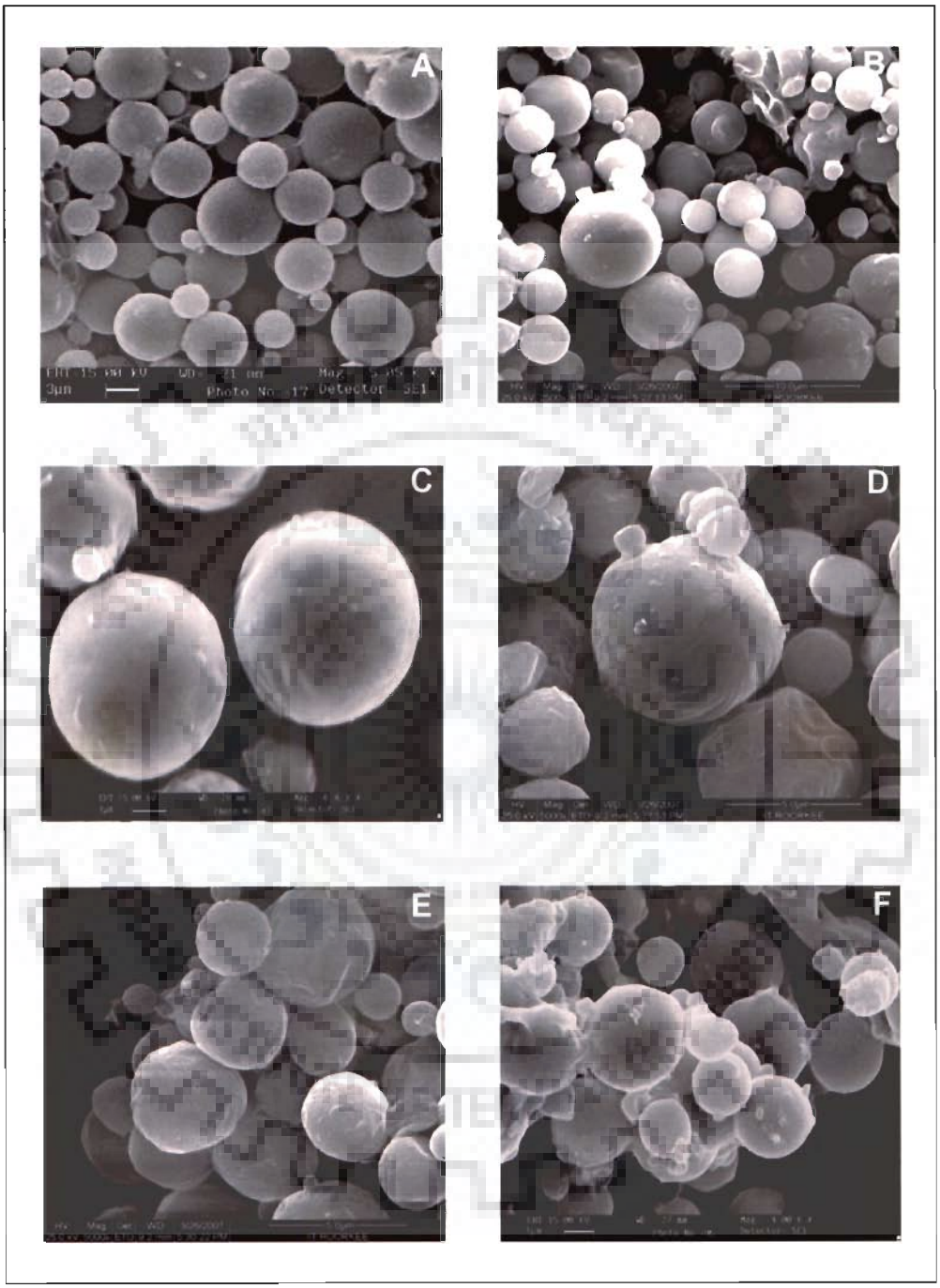

Figure 3.7: Effect of release medium on morphology and surface characteristics of PCL microspheres with time (A:0 day; B:7 days; C:15 days D:30 days; E:45 days; F:60 days)

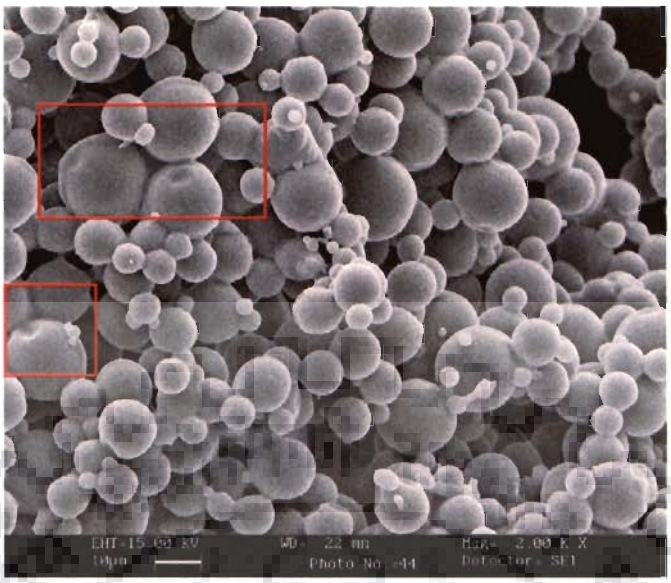

Figure 3.8: PCL microspheres prepared with chloroform as solvent with HPMC as stabilizer

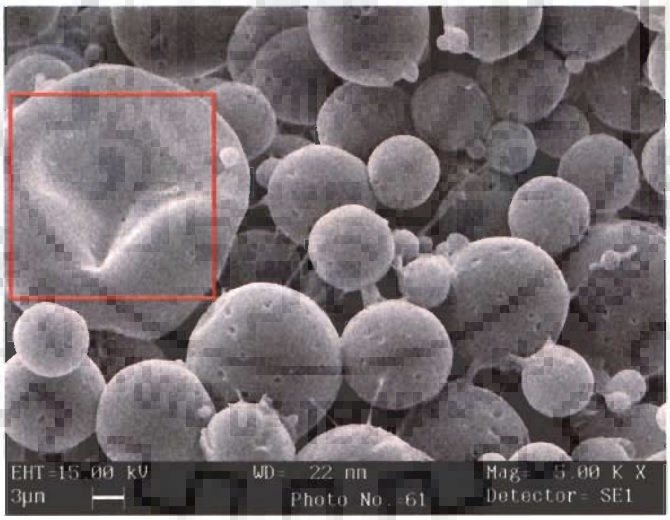

Figure 3.9: PCL microspheres prepared with chloroform as solvent with PVA as stabilizer

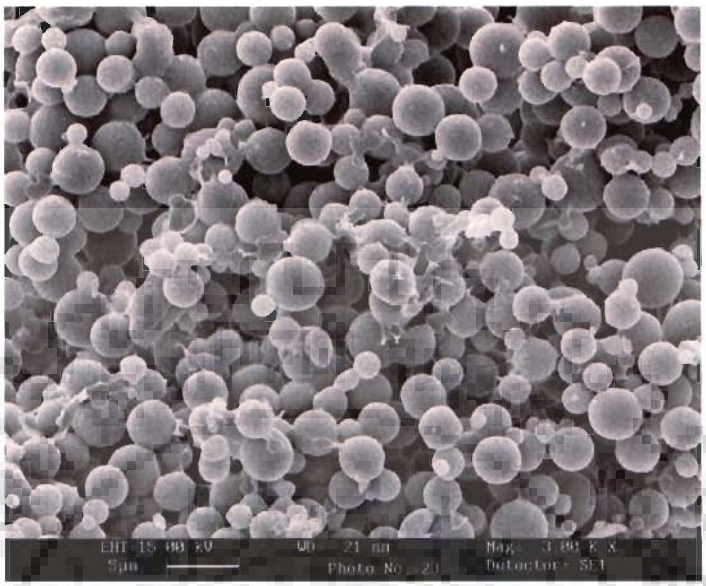

Figure 3.10: PCL microspheres prepared with dichloromethane as solvent with HPMC as stabilizer

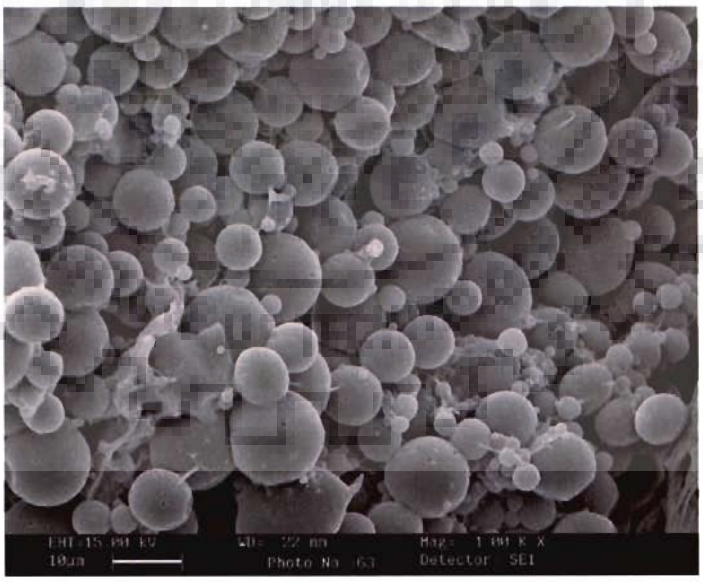

Figure 3.11: PCL microspheres prepared with dichloromethane as solvent with PVA as stabilizer

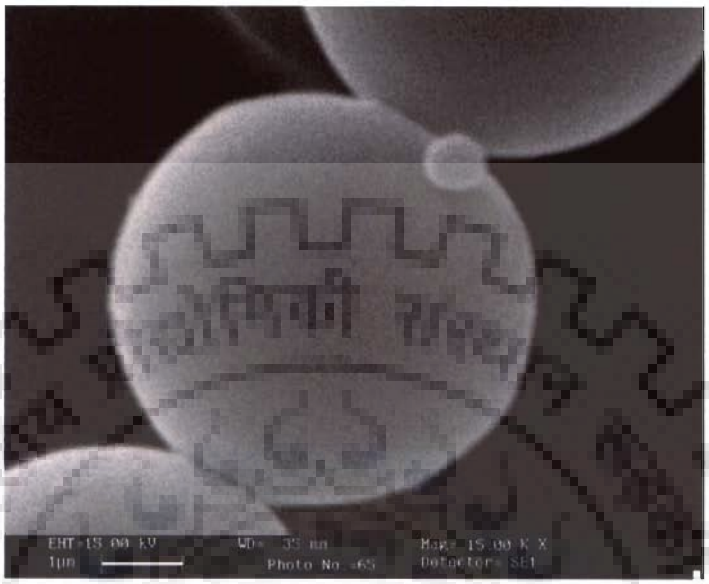

Figure 3.12: Smooth PCL microspheres prepared with HPMC as stabilizer

Figure 3.13: PCL microspheres prepared with PVA as stabilizer showing pits

3.3.2.3 Determination of particle size

The particle size of the microspheres was evaluated by laser diffractometry and results showed that the microspheres formed with 1% PCL and 1% HPMC were in the size range of 2-3μm which are much smaller as compared to the particle size reported by other scientists. A particle size range of 10-65μm was reported for PCL microspheres prepared by w/o/o emulsion technique by Ramesh and his group (Ramesh et al., 2002). Microspheres of PLA and PLGA prepared by w/o/w emulsion were reported in the size range of 10-30 μm by Srinivasan (Srinivasan et. al., 2005) while Kim et al., 2005, reported a particle size of 12.69 μm of PCL microspheres prepared by o/o emulsion method (Kim et. al., 2005). A particle size of 302-477 μm was reported for PCL microspheres entrapping propranolol HCl by the w/o/w emulsion technique (Pe´rez et. al., 2000). A size range of 98.2 to 121.5 μm was observed for microspheres prepared with different PVA concentrations by Yang and his co-workers (Yang et. al., 2001).

3.3.3 Effect of variation of formulation parameters

3.3.3.1 Effect of different solvents on microsphere formulation

Our studies showed that when chloroform was used as a solvent for the middle organic phase, batches α3 and α4, few of the microspheres obtained showed collapsed walls, as shown in Figures 3.8 and 3.9. The microspheres prepared by dichloromethane as the organic phase, batches α1 and α2, were spherical with no collapsed walls, as illustrated in Figures 3.10 and 3.11. However, no microsphere formation was observed with ethyl acetate as the organic phase, batches α5 and α6.

Table 3.1: Different solvent & stabilizer combinations for microsphere formulation

Batch	Organic Phase	Stabilizer	%Yield	Mean Size (μm)
α1	Dichloromethane	HPMC	92.0 ± 0.4	2.2
α2	Dichloromethane	PVA	86.2 ± 1.2	10.2
α3	Chloroform	HPMC	82.7 ± 1.4	14.4
α4	Chloroform	PVA	75.1 ± 2.3	18.1
α5	Ethyl acetate	HPMC	No microspheres obtained	--
α6	Ethyl acetate	PVA	No microspheres obtained	--

The yield was found to be in the range of 86-92% with dichloromethane as the solvent in the organic phase (batches α1 and α2) as compared to 75-83% with chloroform, batch α3 and α4 (Table 3.1). It was also observed that microspheres prepared with dichloromethane were smaller in size (2-10μm) as compared to those prepared with chloroform (14-18μm).

Since microspheres prepared with dichloromethane showed better yield, smaller size and good morphology, it was selected as the solvent of choice over chloroform and ethyl acetate for the organic phase for future preparation of the PCL microspheres for further studies. Dichloromethane has also been used by many scientists for the formulation of microspheres of various biodegradable polymers like PLGA, PCL and poly methacrylates and their blends for the encapsulation of various drugs by single or multiple emulsion techniques (Damgé et. al., 2007; Pe´rez et. al., 2000; Jiao et. al., 2002; Kim et. al., 2005; Coccoli et. al., 2007).

3.3.3.2 Effect of different stabilizers on microsphere formulation

All four batches (α1-α4) showed formation of spherical microspheres with varying smoothness of surface as revealed by the scanning electron microscopy scans. Microspheres prepared with HPMC as the external aqueous phase were very smooth and spherical, as

shown in Figure 3.12. When PVA was used as the stabilizer in the external aqueous phase, microspheres formed were spherical with distinct pits, as shown in Figures 3.13. These pits indicate the microspheres have porous outer surfaces (Yang et. al., 2001). This is similar to the work of Somavarapu et al., 2005, who reported pitted microspheres when prepared with PVA as the stabilizer (Somavarapu et. al., 2005). No such pits were observed in the microspheres prepared using HPMC, as shown in Figure 3.12. Wang and his coworkers reported the formation of such smooth and spherical microspheres using PLGA alongwith the *in-vitro* and *in-vivo* controlled release studies of levonorgestrel from biodegradable poly (d, l-lactide-co-glycolide) microspheres (Wang et. al., 2005).

In our experiments, the mean diameters of all the batches of microspheres were between 2.2μm-18.1μm (Table 3.1). Smaller microspheres were formed with HPMC as the stabilizer (2.2 μm) as compared to PVA (10.2 μm).

3.3.3.3 Effect of different stabilizer and their concentrations on microsphere formulation

The effect of different stabilizers and their concentrations on microsphere formulation in terms of percent yield, percent encapsulation efficiency and particle size are listed in Table 3.2. The percent yields of all the batches, L1 to L6, were found to vary from 78.3 ± 0.08 % to 92.2 ± 0.26%. The percent encapsulation was found to be in the range of 41.81 ± 2.14 % to 65.72 ± 0.13% for all batches.

Table 3.2: Effect of different stabilizers and their concentrations on percent yield, percent entrapment and particle size of the microspheres

Batch	Stabilizer	Stabilizer Concentration	% Yield	% EE	Mean Size (μm)
L1	HPMC	0.5 %	89.1 ± 0.15	53.70 ± 0.17	2.53
L2	HPMC	1.0 %	92.2 ± 0.26	65.72 ± 0.13	2.33
L3	HPMC	1.5 %	85.8 ± 0.09	42.41 ± 0.19	3.67
L4	PVA	0.5 %	80.4 ± 1.12	48.22 ± 1.22	11.83
L5	PVA	1.0 %	86.2 ± 0.16	56.35 ± 0.17	10.26
L6	PVA	1.5 %	78.3 ± 0.08	41.81 ± 2.14	13.35

Our experimental results showed that microspheres prepared with HPMC as the stabilizer showed higher percent yield and encapsulation than those prepared with PVA as the stabilizer in the external aqueous phase. The batch L2 (corresponding to 1.0% HPMC) showed the highest entrapment and batch L6 (corresponding to 1.5% PVA) showed the least. It was seen that when the stabilizer concentration was increased from 0.5% to 1.0%, there was an increase in the percent encapsulation. This may be explained as at low stabilizer concentration, there is a formation of a less stable emulsion. This results in the aqueous drug leaching to the external aqueous phase during the formation of the secondary emulsion facilitating the insulin-containing droplets to diffuse out, resulting in lower entrapment (Yang et. al., 2001). When the stabilizer concentration is increased from 1.0% to 1.5%, we again see a drop in the encapsulation efficiency. This may be attributed to higher concentrations of stabilizers leading to a very viscous phase which is not able to cover and protect the organic and inner aqueous phase during the formation of the double emulsion and thereby resulting in low entrapment of the hydrophilic drug.

Stabilizer concentration in the external phase has also been reported to be an important parameter that influences the size of the microsphere (Jelery et. al., 1993; Carrio et. al.,

1995; Yeh et. al., 1995; Celebi et. al., 1996). In the present set of experiments, the microspheres were fabricated at 0.5%, 1.0% and 1.5% stabilizer (HPMC and PVA) concentration so as to examine the effect of the stabilizer concentration. The sizes of microspheres of all the batches are shown in Table 3.2. Smallest microsphere size of 2.33μm was obtained with a concentration of 1.0% of HPMC in the external aqueous phase. It was found that as the stabilizer concentration was increased from 0.5% to 1.0%, smaller microspheres were obtained for both the stabilizers, as seen in Table 3.2. This can be explained as, more the stabilizer concentration, more the stabilizer molecules covered the interface between organic phase and external aqueous phase. An improved protection of the droplets from coalescence was obtained, consequently leading to smaller emulsion droplets than at lower stabilizer concentrations. Since microspheres were formed from the emulsion droplets after the solvent evaporation, their size is dependent upon the size and the stability of the emulsion droplets (Lamprecht et. al., 2000). The microspheres prepared with 1.5% stabilizer concentration were found to be bigger in size than those prepared with 1.0% concentration of the stabilizer due to increased viscosity at higher stabilizer concentration. Therefore, it was concluded that 1% of the stabilizer served as the optimum concentration as no further reduction of microsphere size was seen when the stabilizer concentration was increased from 1.0% to 1.5% of stabilizer concentration (Mukerjee and Pruthi, 2007).

The use of HPMC as the stabilizer led to smaller microspheres (2.3-3.6μm) and better entrapment than obtained by using PVA (10.2-13.3 μm). This may be explained by the high molecular weight of PVA. The presence of PVA, as compared to HPMC, in the external water phase may increase the viscosity of the double emulsion, resulting in an increased difficulty in breaking up the emulsion into smaller droplets. Thus, this yields bigger microspheres than obtained by HPMC as observed.

The surface morphology of all the batches of microspheres was also studied by scanning electron microscopy. The SEM scans of the microspheres of batches L1 to L6 are shown in

Figures 3.14 to 3.19. Scans of all the batches revealed the formation of spherical microspheres. As discussed earlier, microspheres prepared with HPMC as the external aqueous phase were smooth and spherical. But distinct pits were observed microspheres when PVA was used as the stabilizer in the external aqueous phase indicating a porous outer surface. This is similar to the work of Somavarapu et al., 2005, and Yang et al, 2001, who reported pitted microspheres with PVA (Somavarapu et. al., 2005; Yang et. al, 2001). Also, batches prepared with the optimum stabilizer concentration of 1% (L2 and L5) showed less microsphere aggregation and more uniform particle size. Batches prepared with higher stabilizer concentration resulted in increased aggregation among the microspheres. This may be as a result of the more viscous solution of the stabilizer in the external aqueous phase at higher concentration.

3.3.3.4 Effect of varying the polymer concentration on microsphere formulation

The polymer concentration is also one of the key factors influencing the microsphere size and release characteristics. Our results showed that by increasing the polymer concentration from 0.5% to 2.0% in the organic phase, an increase of the size of microspheres from 2.04 μm to 30.11μm, was observed (Table 3.3). These results correlated with those previously observed by other researchers (Ogawa et. al, 1988; Yang et. al, 2001; Coccoli et. al., 2007). Indeed, they reported that an increase in polymer concentration led to an increase in the viscosity of the first emulsion and consequently aggravated the dispersion of the inner aqueous phase in the organic phase. This first emulsion subsequently led to less efficient reduction of the emulsion droplet size during the second emulsification step leading to bigger microspheres.

The surface morphology of the microspheres was studied using SEM and the scans of all the batches, P1 to P4, are shown in Figures 3.20 to 3.23. It is clearly seen from the scans that as

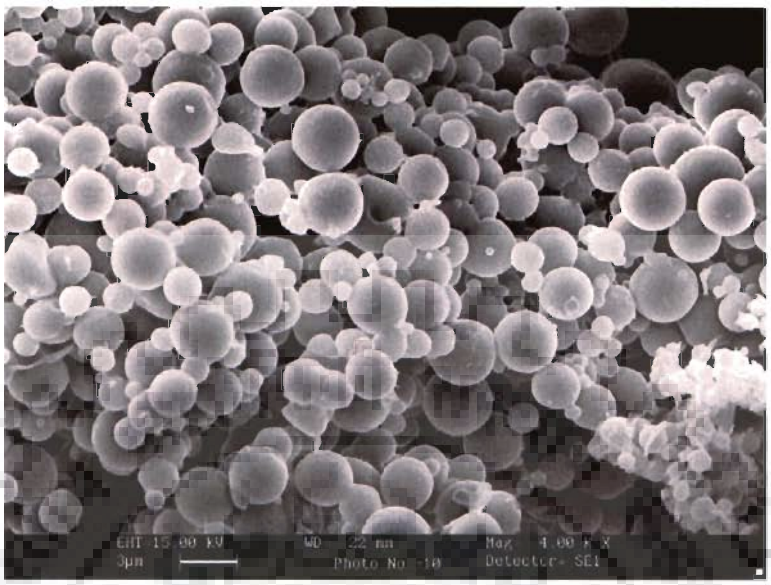

Figure 3.14: PCL microspheres prepared with 0.5% HPMC: Batch L1

Figure 3.15: PCL microspheres prepared with 1.0% HPMC: Batch L2

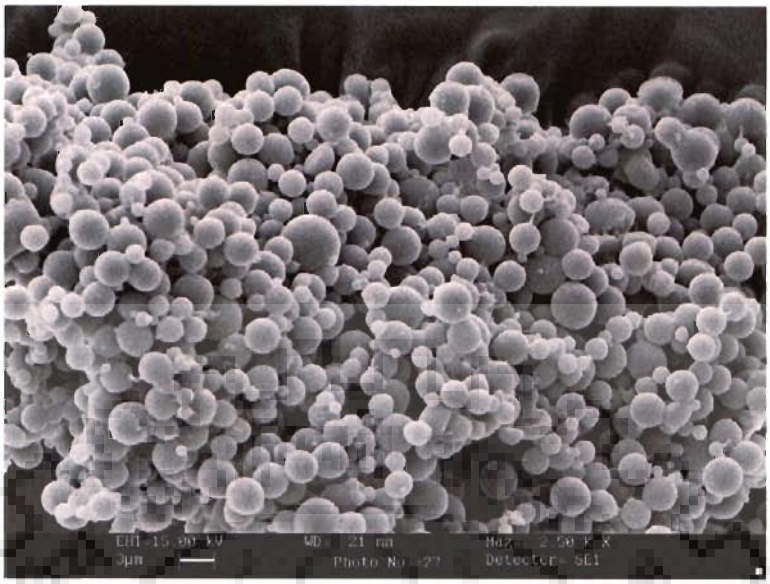

Figure 3.16: PCL microspheres prepared with 1.5% HPMC: Batch L3

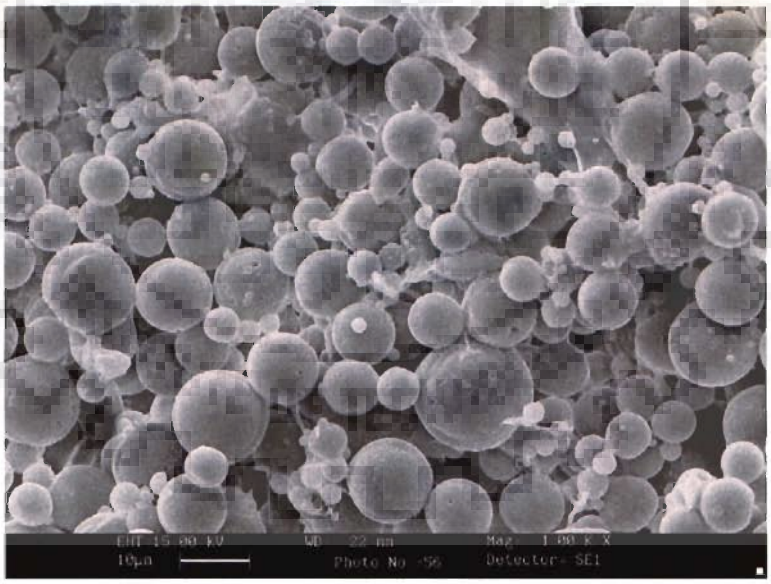

Figure 3.17: PCL microspheres prepared with 0.5% PVA: Batch L4

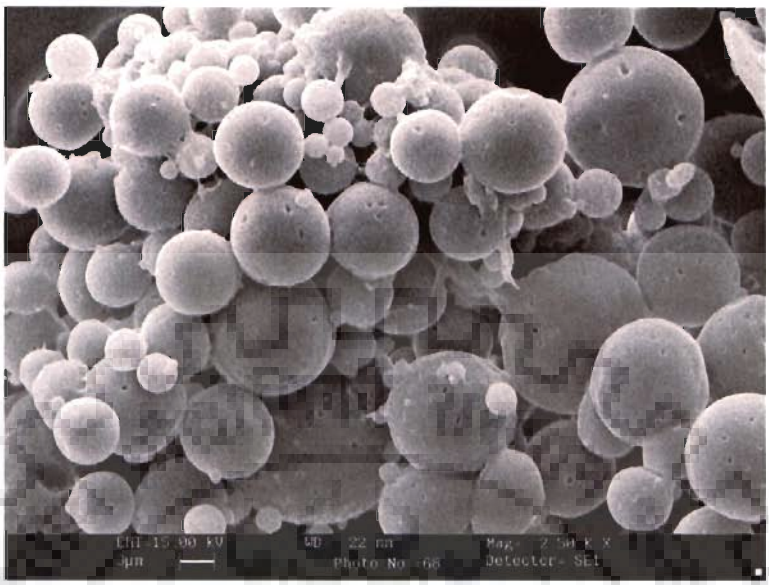

Figure 3.18: PCL microspheres prepared with 1.0% PVA: Batch L5

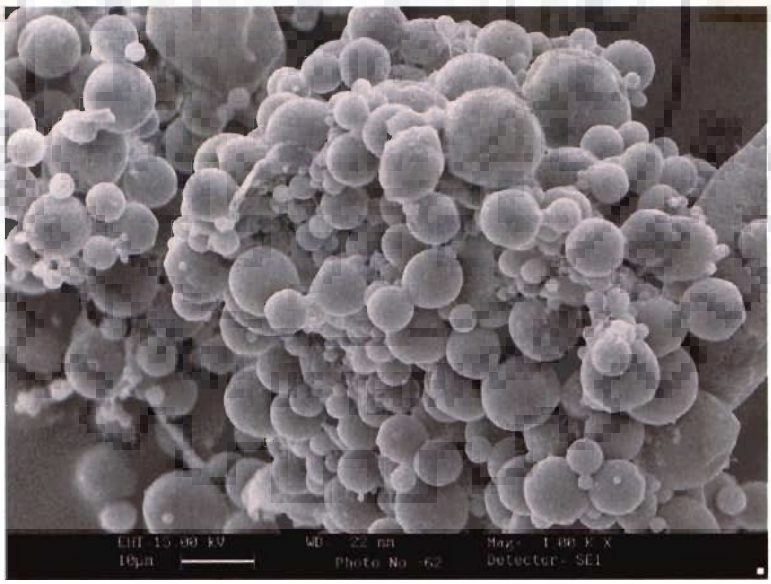

Figure 3.19: PCL microspheres prepared with 1.5% PVA: Batch L6

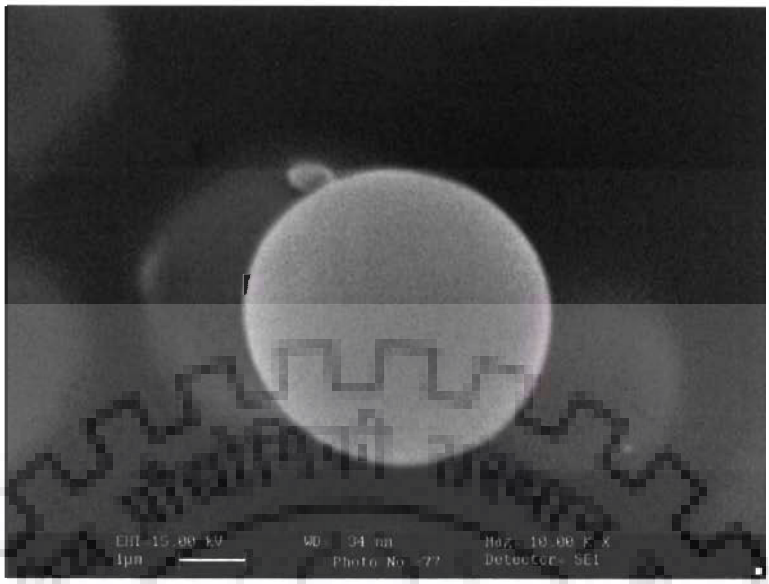

Figure 3.20: Microsphere prepared with 0.5% PCL: Batch P1

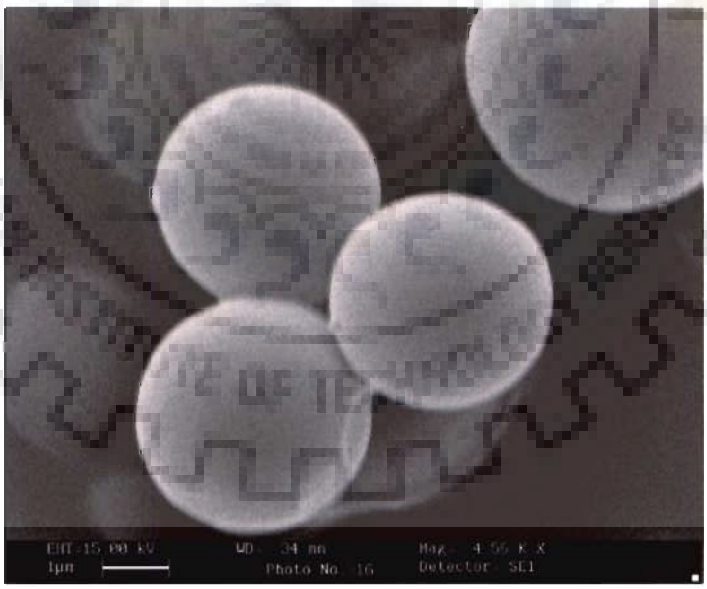

Figure 3.21: Microsphere prepared with 1.0% PCL: Batch P2

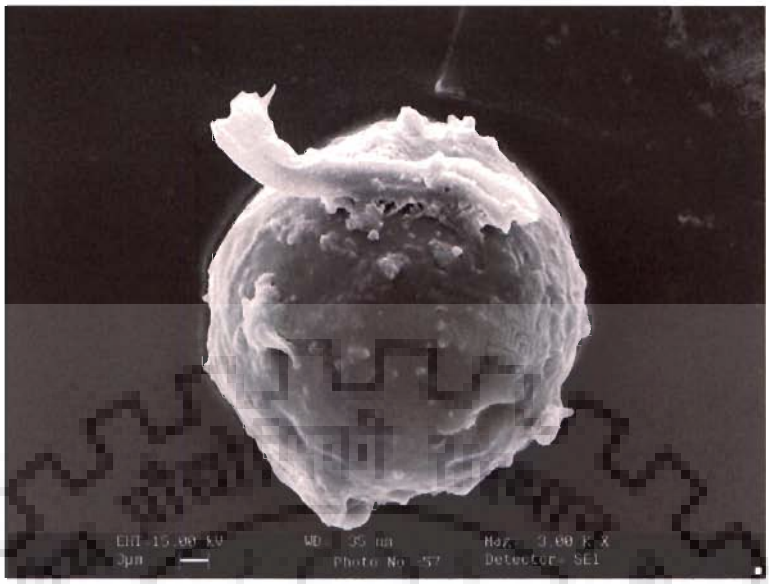

Figure 3.22: Microsphere prepared with 1.5% PCL: Batch P3

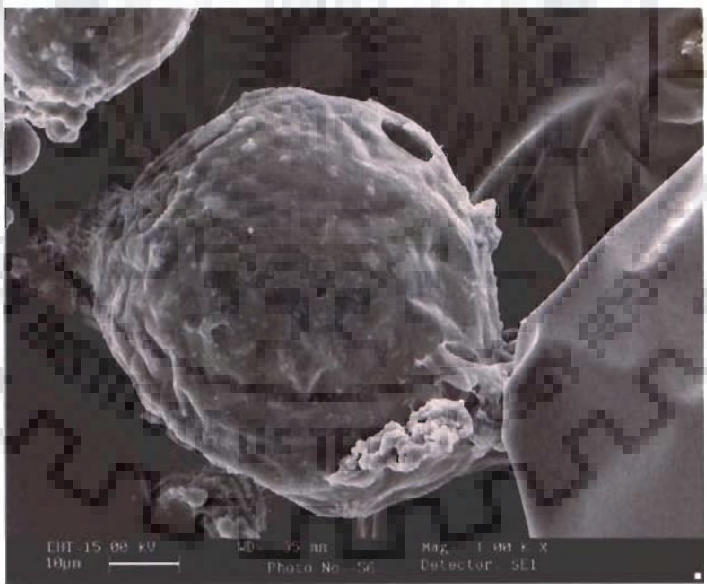

Figure 3.23: Microsphere prepared with 2.0% PCL: Batch P4

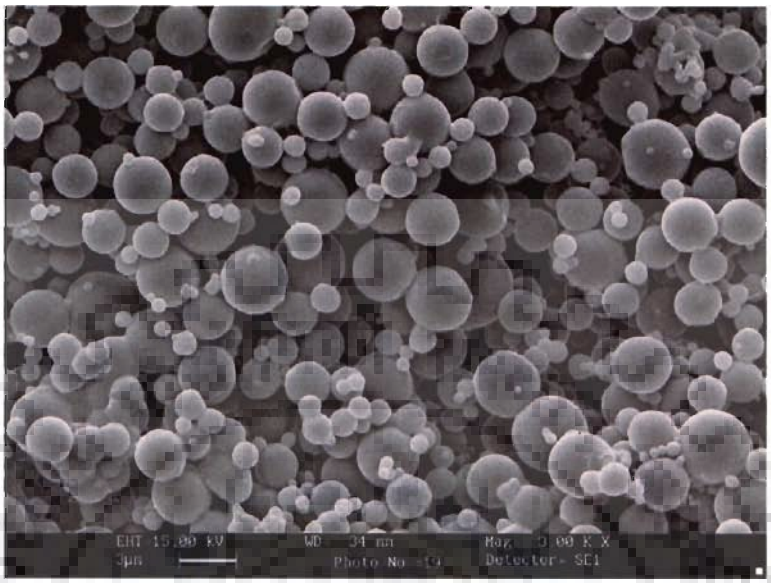

Figure 3.24: Microsphere prepared with insulin concentration, 100 IHU/ml: Batch D1

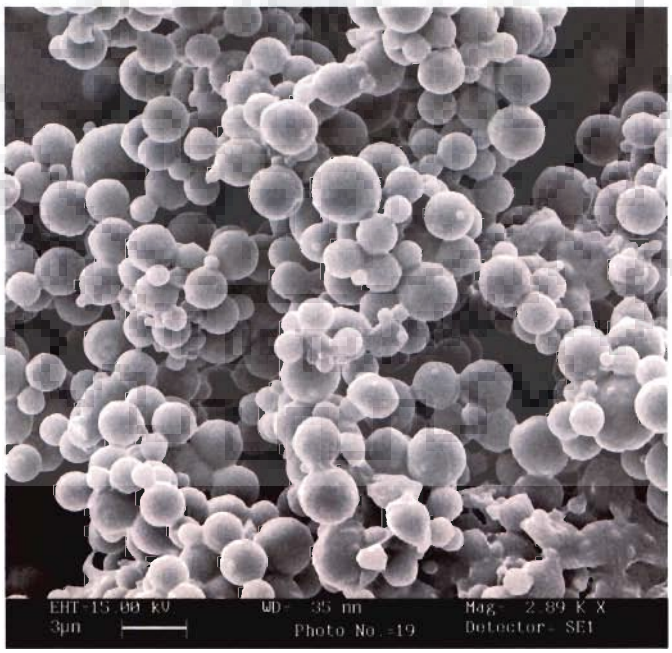

Figure 3.25: Microsphere prepared with insulin concentration, 40 IHU/ml: Batch D2

Table 3.3: Effect of varying the polymer concentration on percent yield, percent entrapment and particle size of the microspheres

Batch	%Yield	%EE	Mean Size (μm)
P1	83.4 ± 0.12	42.45 ± 0.34	2.04
P2*	92.2 ± 0.26	65.72 ± 0.13	2.33
P3	71.5 ± 0.42	34.51 ± 0.24	17.18
P4	66.1 ± 0.35	21.56 ± 0.48	30.11

* Batch P2 has the same formulation parameters as batch L2

the polymer concentration is increased beyond 1.0%, there is loss of smoothness and shape of the microspheres (Figures 3.22 to 3.23). However, there was no identifiable difference in the surface morphology of the microspheres prepared with 0.5% and 1.0% of polymer concentration (Figures 3.20 to 3.21).

3.3.3.5 Effect of varying the drug concentration in the internal aqueous phase on microsphere formulation

The effects of varying the drug concentration in the internal aqueous phase during the microsphere formulation in terms of percent yield, percent encapsulation efficiency and particle size are shown in Table 3.4.

Table 3.4: Effect of varying the drug concentration on percent yield, percent entrapment and particle size of the microspheres

Batch	% Yield	% EE	Mean Size (μm)
D1*	92.2 ± 0.26	65.72 ± 0.13	2.33
D2	90.1 ± 0.31	37.25 ± 0.22	2.39

* Batch D1 has the same formulation parameters as batch L2 and P2

It is clearly seen from our experiments that there was a significant decrease in the insulin encapsulation efficiency with decrease in the drug concentration used for the internal aqueous phase during the double emulsion solvent evaporation technique. This can be explained by the fact that for the same amount of internal aqueous phase encapsulated by a formulation process, higher concentration of drug solution used would result in higher percent encapsulation of the drug. However, there were no marked changes observed in the percent yield, surface morphology (Figures 3.24 and 3.25) and particle size of the microspheres between the batches, D1 and D2.

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Chapter 4

ADVANCED CHARACTERIZATION STUDIES

4.1 PREAMBLE

In the study so far, we developed insulin loaded biodegradable microspheres of poly-ε-caprolactone (PCL), a polymer commonly used for the manufacturing of resorbable threads, using the double emulsion technique which is mostly used for encapsulation of peptides and proteins. These biodegradable PCL microspheres used for oral drug delivery need to be characterized both for *in-vitro* and *in-vivo* results along with their degree of reproducibility. Once the final batch was obtained after studying the effect of various formulational parameters, advanced characterization techniques like confocal laser scanning microscopy, atomic force microscopy, differential scanning calorimetric studies and gas chromatography were further carried out to study the drug distribution within the microspheres, surface topography, drug-polymer interaction and residual solvent analysis respectively.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Insulin loaded PCL microspheres, Insulin Human Injection USP, 100 IHU/ml (Eli Lily and Company, USA), Poly-ε-caprolactone (M_n 42,500; Sigma-Aldrich Chemical Company, USA), Hydroxypropyl methylcellulose (M_w 10,000; Sigma-Aldrich Chemical Company, USA), Dichloromethane (Merck, Mumbai, India), Nile Red (Acros Organics, Belgium), Fluorescein isothiocyante (Himedia, India), Dimethylsulphoxide (S.D. Fine-Chem Ltd., India), Ethanolamine (S.D. Fine-Chem Ltd., India), Dimethylformammide (S.D. Fine-Chem Ltd., India). Micro BCA Protein Estimation Kit (PIERCE®, USA), Cellulose membrane dialysis tube with 10000 Dalton pore (Himedia, India), Carbonate/ bicarbonate buffer

solution (pH 9.0) 0.1M. Double adhesive tapes were used for AFM studies. Double distilled water was used for all the experiments.

4.2.2 Drug distribution studies within the microsphere by confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a technique mostly used for obtaining high-resolution optical images. The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens. Confocal microscopy also provides a substantial improvement in lateral resolution and the capacity for direct, noninvasive, serial optical sectioning of intact, thick specimens with a minimum of sample preparation. Because CLSM depends on fluorescence, a sample usually needs to be treated with fluorescent dyes to make objects visible. The actual dye concentration can be low to minimize the disturbances of biological systems. (Wikipedia-confocal_laser_scanning_microscopy).

For studying the drug distribution within the microspheres, the polymer, PCL, was labeled with nile red and insulin was labeled with FITC. Microspheres were prepared using the labeled insulin and polymer by the double emulsion solvent evaporation technique (same procedure as reported in chapter 3) and observed under a confocal laser scanning microscope, shown in Figure 4.1. Further, experiments were designed where either PCL labeled with nile red or insulin labeled with FITC was used for the microsphere preparation.

4.2.2.1 Fluorescence labeling of the organic phase by nile red

Nile red was dissolved in dichloromethane and this was used as the organic phase for the formation of the primary water-in-oil emulsion in the double emulsion procedure for obtaining the microspheres. As nile red has a strong susceptibility to fluorescence bleaching, a concentration of not less than 1 mg/ml was used.

Figure 4.1: Confocal Laser Scanning Microscope (Carl Zeiss LSM 510 Axiovert 2)

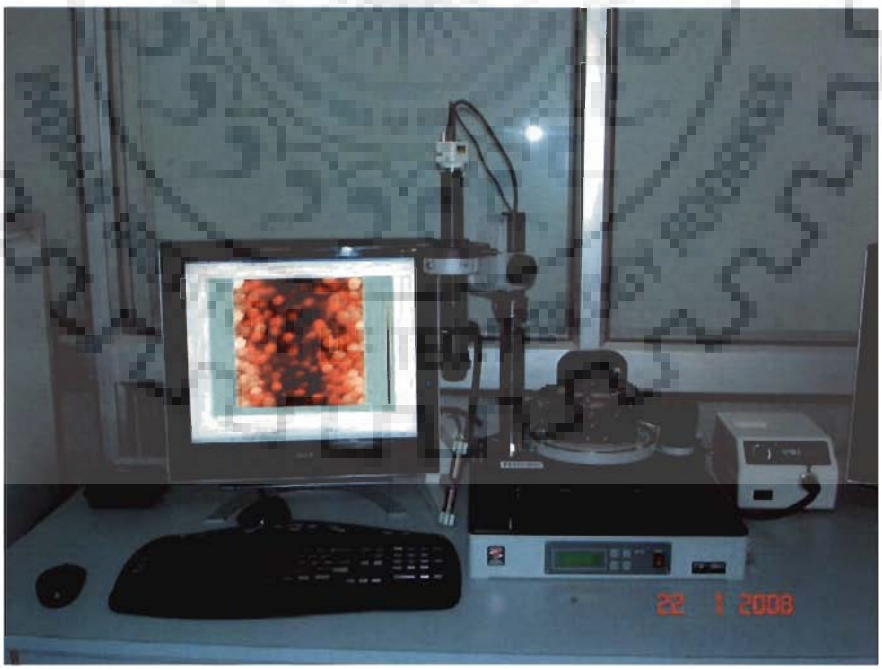

Figure 4.2: Atomic Force Microscope (NT-MDT, Ntegra)

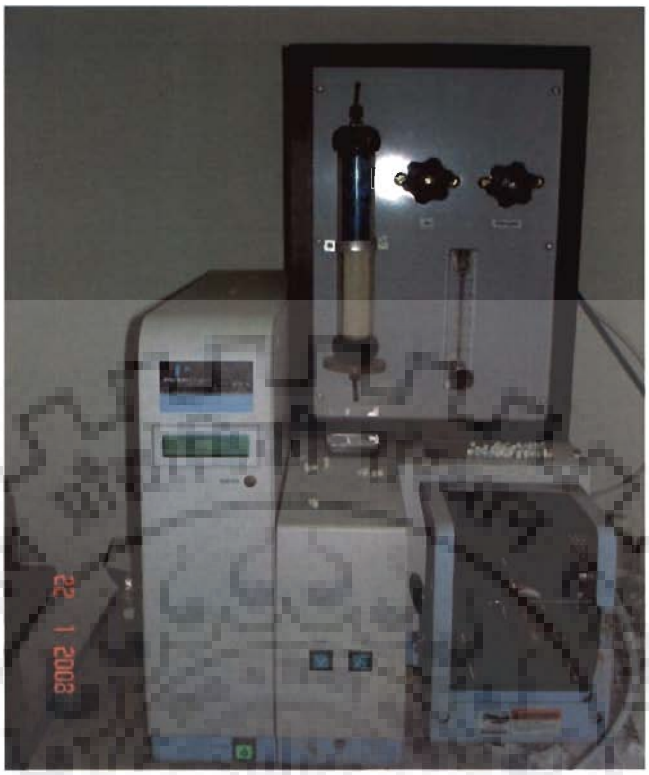

Figure 4.3: Differential Scanning Calorimeter (Pyris Diamond, Perkin Elemer)

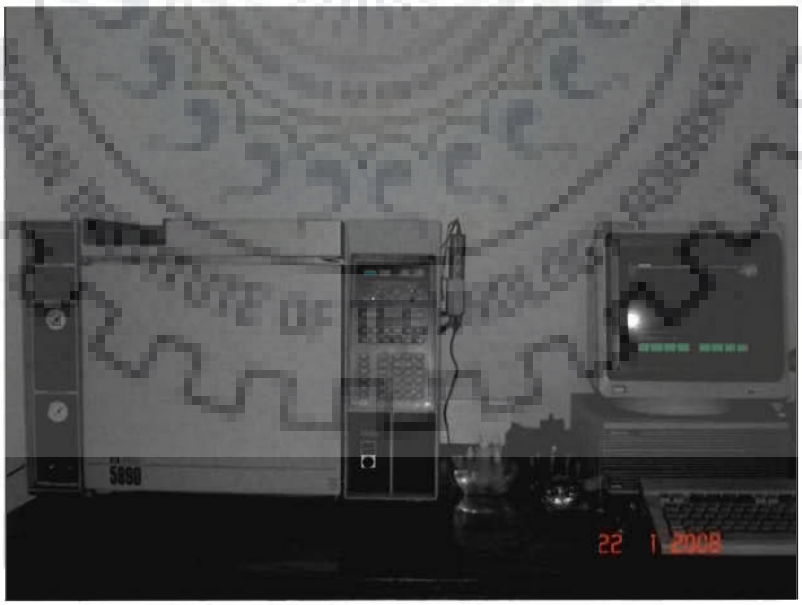

Figure 4.4: Gas Chromatograph (5890A, Hewlett Packard)

4.2.2.2 Fluorescence labeling of insulin by FITC

Based on the method of protein labeling by Schreiber et. al., 1983, the covalent labeling protocol was adapted to our requirements. The aqueous protein solution was adjusted at pH 8-9 by 0.1M carbonate/ bicarbonate buffer solution (pH 9.0). The fluorescent dye, Fluorescein isothiocyante, (FITC) was dissolved in DMSO at a concentration of 1 mg/ml. The solution was kept on ice in dark, covered with foil, at room temperature for atleast 5 hours. The reaction was then stopped by adding 20µl ethanolamine. Free FITC was removed by dialysis (dialysis tube with a 10, 000 Dalton pore size) against distilled water until no diffusion of the free marker was observed (Schreiber et. al., 1983).

A confocal laser scanning microscope (Carl Zeiss LSM 510 Axiovert 2) was used to determine the drug distribution within the PCL microspheres. The laser was adjusted in the green/red fluorescence mode, which yielded two excitation wavelengths at 488 and 514 nm. Green and red fluorescence images were obtained from two separate channels, with the option of a third picture from the transmitted light detector. The final pictures were composed from mixer A (red fluorescence) and mixer B (green fluorescence) to visualize the marked structures and also the signal from the ordinary transmission light microscope (mixer C -transmitted light) within the same image.

4.2.3 Atomic force microscopy

The Atomic Force Microscope (AFM) is a very high-resolution type of scanning probe microscope, with demonstrated resolution of fractions of a nanometer. The surface morphology of the microspheres was investigated using atomic force microscope (NT-MDT, Ntegra, Russia), shown in Figure 4.2. The dry samples of microspheres (500 µg) were mounted on a glass slide, rubbed in with another glass slide and then viewed. The topography images were recorded in the semi-contact mode with cantilever of about 0.01 N/m nominal spring constant in air at room temperature. The image size was 5 µm × 5

μm at 1 Hz lateral scan rate. The height calibration of the AFM was performed with a standard silicon gratings set (NSG10; Silicon, NT-MDT).

4.2.4 Drug polymer interaction studies

Differential Scanning Calorimetric (DSC) studies of the drug, polymer and microspheres were carried out to define the physical state of the drug in this carrier and the possibility of any interaction between the drug and the polymer in the microspheres. A characteristic of a pure substance is a defined melting point or melting range. This property is used to determine the purity of the drug substance and also the compatibility of various substances in the same dosage form. If not pure, a substance will exhibit a change in the melting point or range.

DSC curves were recorded on a scanning calorimeter, shown in Figure 4.3, equipped with a thermal analysis data system (Pyris Diamond, Perkin Elemer, USA). The instrument was calibrated using alumina powder as the standard. A small amount of microsphere samples was placed in hermetically sealed aluminum pans and heated from 25°C to 150°C at a heat flow rate of 10°C/min under nitrogen spurge. For the DSC of insulin, the temperature range kept was 0°C to 150°C.

4.2.5 Residual solvent analysis

For any microsphere preparation to gain US FDA approval, it is necessary to consider regulatory requirements of the residual solvent content in the microspheres. Since microsphere formulation requires the use of an organic solvent such as dichloromethane for polymer dissolution, they may pose significant health risks if present in high concentrations on long term exposure.

Based on the recommendations of the ICH and the USP Pharmacopoeia, FDA limits for dichloromethane, which is a class II solvent, is 600 ppm. The estimation of dichloromethane

in the PCL microspheres was carried out according to the procedure reported for the same solvent in PCL and PLA microspheres by Sinha and Trehan in 2006 (Sinha and Trehan, 2006). To determine the amount of dichloromethane in our microsphere formulation, a known weight of microspheres were dissolved in dimethylformamide and then directly injected in a gas chromatograph (5890A, Hewlett Packard, USA), shown in Figure 4.4, with a HP-1 column, 30m X 0.32mm (Dimethyl poly siloxane) and flame ionization detector.

4.3 RESULTS AND DISCUSSION

4.3.1 Drug distribution studies within the microsphere

Morphology and CLSM allows visualization and characterization of structures not only on the surface, but also inside the particles, provided the materials are sufficiently transparent and can be fluorescently labeled. Unlike conventional light microscopy, CLSM also enables the user to qualitatively determine localization as well as amount of the inner drug phase without having to first establish complex analytical procedures. Therefore, CLSM provides a good approach to explore the internal structure of the microspheres and drug distribution as a nondestructive visualization technique.

CLSM permited us to depict and identify all fluorescently labeled compounds at light microscopical resolution. Figure 4.5a and 4.5b clearly shows the microspheres with FITC labeled insulin (shown in green) entrapped inside the PCL polymer microsphere shell (labeled red with nile red). Figure 4.6 shows red PCL microspheres with dark core inside. This is due to nile red labeled outer PCL shell entrapping insulin. On the other hand, Figure 4.7 shows only the internal phase of a microsphere where insulin labeled by FITC is visible in green, inside the polymer shell which is not visible, due to unlabeled polymer. Coccoli, et al, 2007 also reported this technique for the determination of the distribution of BSA, labeled with rhodamine, within PCL microspheres (Coccoli, et al, 2007).

4.3.2 Atomic force microscopy

In principle, AFM can provide higher resolution than SEM. It has been shown to give true atomic resolution in ultra-high vacuum. The AFM consists of a microscale cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface. The cantilever is typically made of silicon or silicon nitride with a tip radius of curvature of the order of nanometers. The atom at the apex of the tip senses individual atoms on the underlying surface when it forms incipient chemical bonds with each atom. Because these chemical interactions subtly alter the tip's vibration frequency, they can be detected and mapped. The AFM can be used to image atoms and structures on a variety of surfaces. The only disadvantage of AFM compared with SEM is the image size and the depth of field (Wikipedia-atomic_force_microscopy).

The AFM scan in Figure 4.8 reveals spherical microspheres in semi contact mode. The microspheres were seen as three dimensional spherical structures and complete sphericity was revealed as compared to SEM scans which did not establish good three dimensional visualization. Similar results have also been reported by Lamprecht et. al., 2000, for PCL micro and nanoparticles (Lamprecht et. al., 2000).

4.3.3 Drug polymer interaction studies

DSC studies are the most widely used calorimetric techniques for the determination of various thermal parameters, which allow a better understanding of drug-polymer interactions, drug-excipient interaction and thermal denaturation of polymers. DSC is also widely used in industrial settings as a quality control instrument due to its applicability in evaluating sample purity and for studying polymer curing (Wikipedia-differential_scanning_calorimetry).

DSC is a thermo-analytical technique in which the 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 sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned. DSC may be used to observe more subtle phase changes, such as glass transitions. In our experiments, we compared the DSC curves of unloaded PCL microspheres and insulin loaded PCL microspheres, Figure 4.9 and 4.10. The melting temperature, T_m, for the unloaded and insulin loaded PCL microspheres was found to be 59.7°C and 62°C respectively confirming no major change in the T_m of the polymer, since polymers usually exhibit a melting range instead of a sharp melting point. DSC curves of pure polymer (PCL) and HPMC are shown in Figure 4.11 and 4.12. The T_m of PCL polymer was found to be 66°C, which falls in the melting range of the polymer. The DSC curve of insulin is shown in Figure 4.13. The DSC curves of insulin and HPMC shows no interaction with polymer in the given temperature range. DSC studies were carried out by other scientists who also reported similar T_m of PCL polymer, unloaded PCL microspheres and drug encapsulated PCL microspheres (Pe´rez et. al., 2000).

4.3.4 Residual solvent analysis

Residual solvents are classified in three categories based on their toxicity levels and the degree to which they can be considered an environmental hazard. Class I solvents are the most toxic and should be absolutely avoided. Some examples of this class include the benzene, carbon tetrachloride etc. The concentration limits for this class of solvents is generally between 2-8 ppm except for 1,1,1-trichloroethane for which the limit is 1500 ppm. Class II solvents are considered to be less toxic but they should also be limited in pharmaceutical products. This class includes solvents like dichloromethane, acetonitrile etc. and their limits vary from 50-4000 ppm. Class III solvents are the lowest in risk and include

solvents like acetic acid, pentane etc. They require only GMP based testing and are limited to 5000 ppm or 0.5% w/w.

Regulatory agencies often require that the manufacturer should attempt to reduce residual solvents to the maximum extent. It has also been found that high levels of the residual solvents in the formulation may have a direct impact on the stability of the encapsulated drug. It has been reported that higher concentrations of residual ethanol may reduce the glass transition temperature of polymer causing microspheres to agglomerate under storage and may result in poor injectability (Niu and Chiu, 1998).

Determination of the residual solvent concentrations can be carried out by many analytical techniques. Specific residual solvent analysis is done by gas chromatography (GC). GC has the ability to separate and identify component solvents and is able to record low detection limits when the appropriate detector is used. Flame ionization detector (FID) is the most widely used detector for gas chromatography because of its low detection limits, wide linear dynamic range and reliability for trace organic compounds, especially to hydrocarbons (Wikipedia-gas_chromatography).

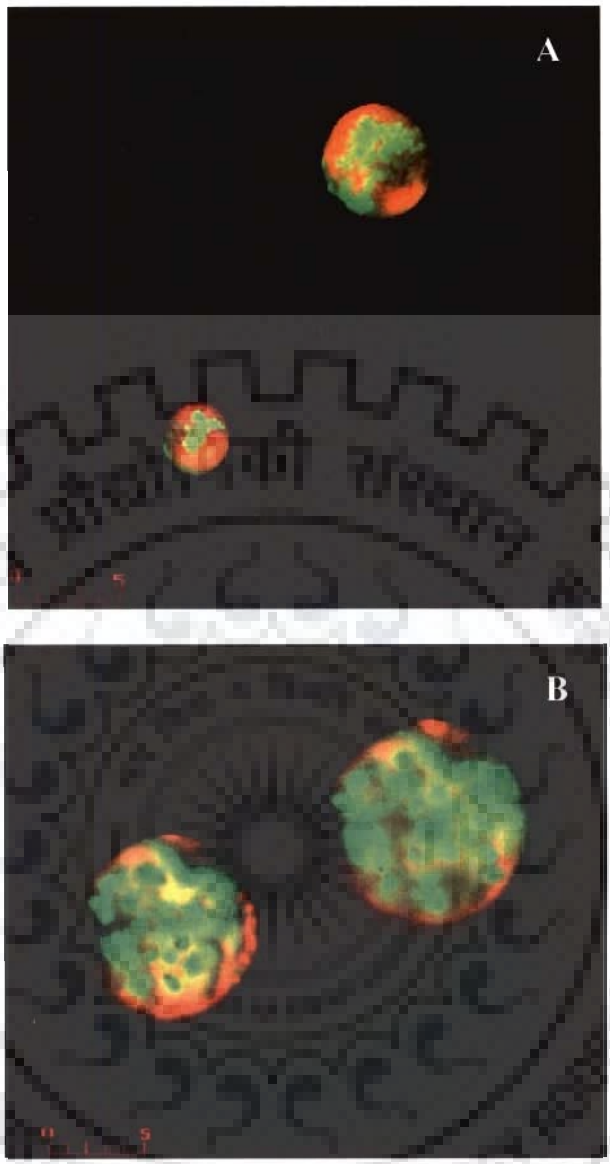

Figure 4.5: Confocal scans of PCL microspheres: FITC labeled insulin, shown in green, entrapped inside the polymer PCL shell, labeled red with Nile Red

[Scale bar is shown in μm]

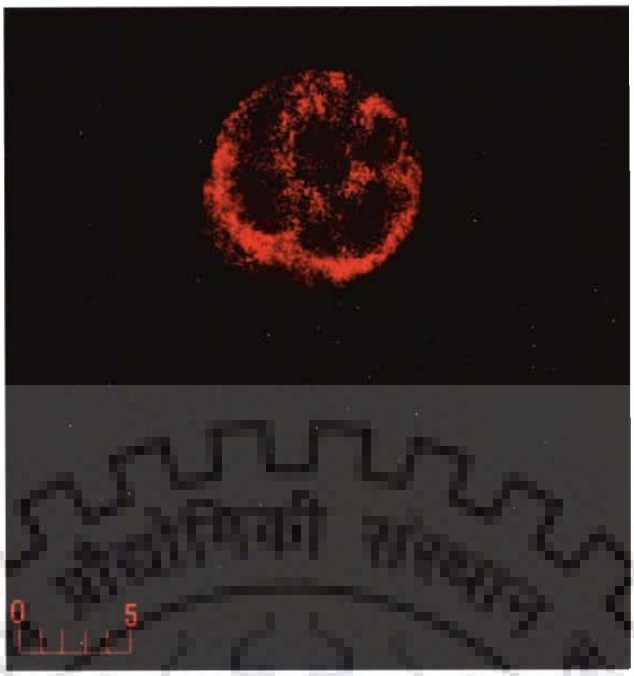

Figure 4.6: Confocal scan of a PCL microsphere with the polymer labeled with Nile Red [Scale bar is shown in μm]

Figure 4.7: Confocal scan of PCL microspheres with insulin labeled with FITC (appearing green) inside the unlabeled polymer shell [Scale bar is shown in μm]

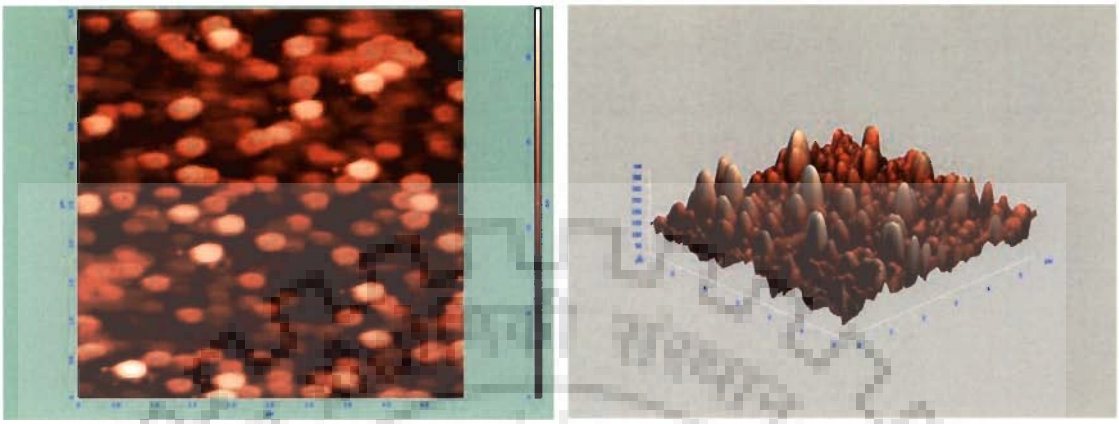

Figure 4.8: Atomic force microscope scan of PCL microspheres with its 3-D image

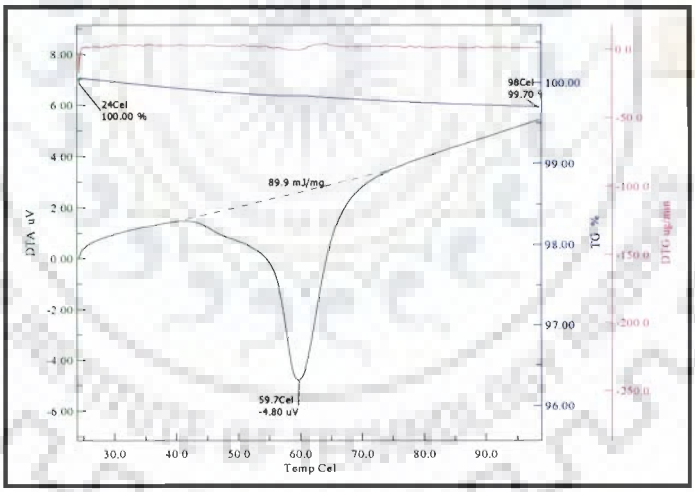

Figure 4.9: Differential scanning calorimetric curves of unloaded PCL microspheres:

T_m=59.7°C

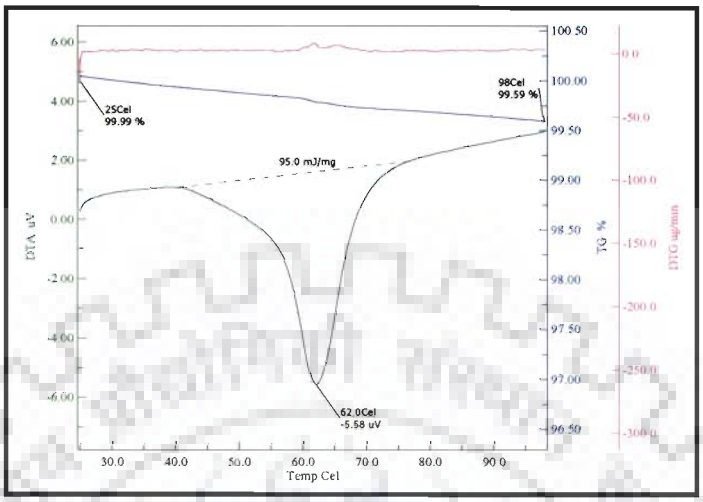

Figure 4.10: Differential scanning calorimetric curves of insulin loaded PCL Microspheres: T_m=62°C

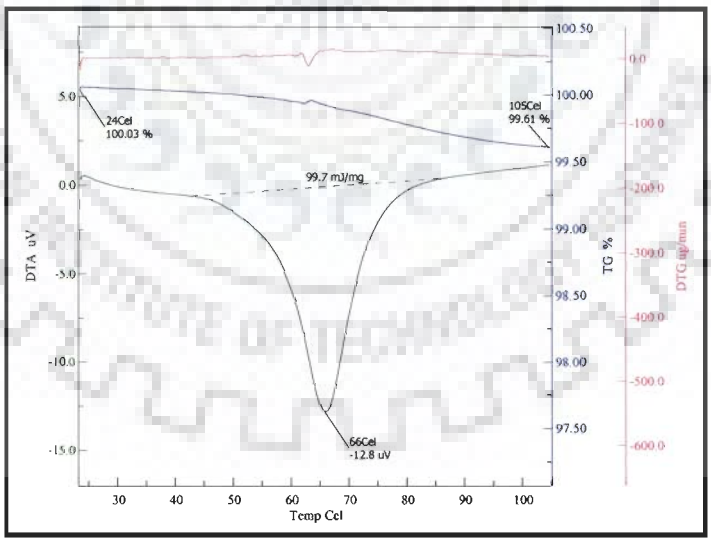

Figure 4.11: Differential scanning calorimetric curves of the polymer, PCL: T_m=66°C

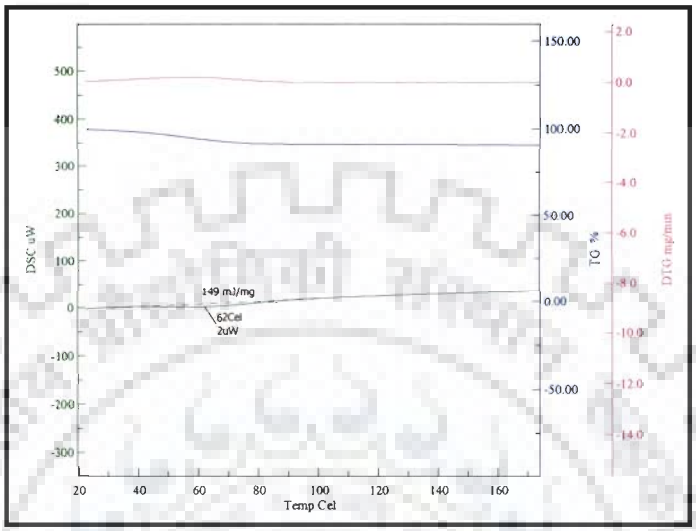

Figure 4.12: Differential scanning calorimetric curves of the stabilizer, HPMC

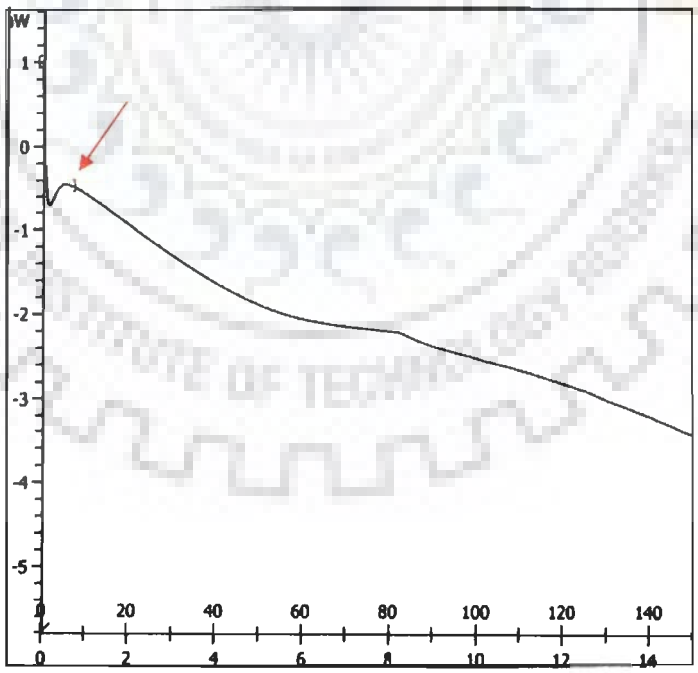

Figure 4.13: Differential scanning calorimetric curve of insulin

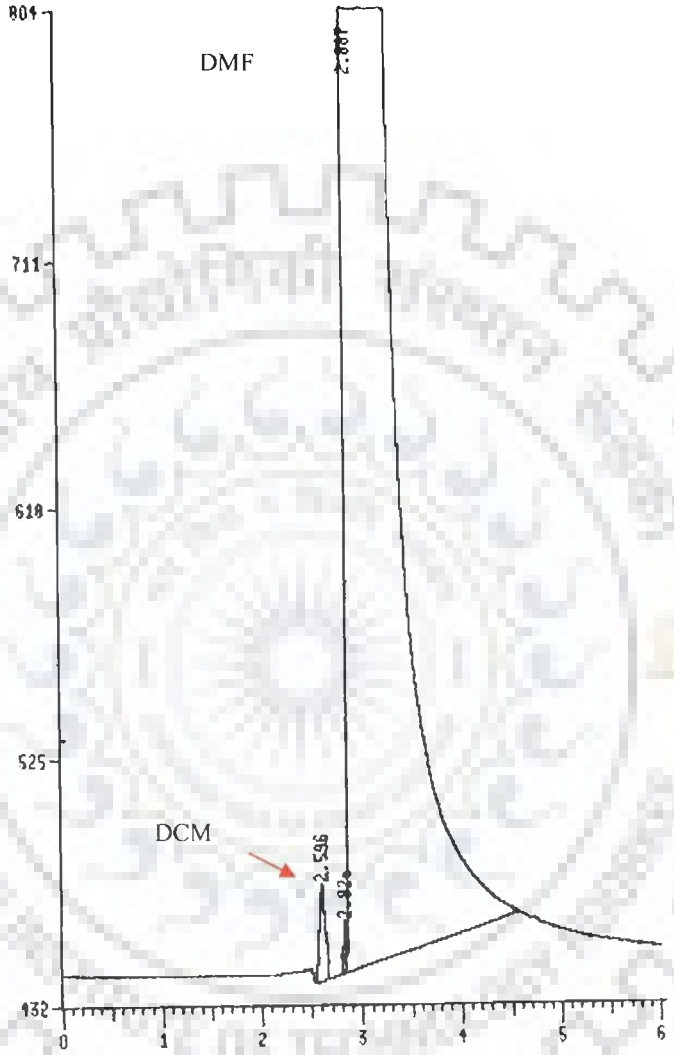

Figure 4.14: Gas chromatograph showing the residual amount of dichloromethane in the PCL microspheres

The gas chrpmatograph of the residual solvent, dichloromethane, is shown in Figure 4.14. The FDA limit for dicholoromethane, which falls in the category of Class II solvents, is 600 ppm based on the ICH and USP pharmacopoeia recommendations. The residual dichloromethane content in the microsphere formulation was found to be 238 ppm, which is well below the pharmacopoeial limits. The microsphere formulation is therefore safe for administration. Similar results have also been reported by Sinha and Trehan for dichloromethane used for the preparation of PCL and PLA microspheres (Sinha and Trehan, 2006).

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Chapter 5

EVALUATION OF MICROSPHERES

5.1 PREAMBLE

The poly-ε-caprolactone (PCL) microspheres obtained need to be evaluated for their *in-vitro* and *in-vivo* insulin release profiles. The possible mechanisms of release include initial release from microspheres surface, release through the pores, diffusion through the intact polymer barrier, diffusion through a water swollen barrier, polymer erosion and bulk degradation. All these mechanisms together play a part in release process. The release of drug from the biodegradable microspheres is dependent both on diffusion through the polymer matrix and on polymer degradation. The release of core material from such systems is dependent on diffusivity through the polymer barrier, solubility of core in bulk phase, size of drug molecule and distribution of core throughout the matrix etc. Nature of polymer is also known to play a major role in release processes. If the polymer degradation is considerable during the desired release time, the release rate may be unpredictable and erratic due to breakdown of microspheres. Theoretically, release from biodegradable microspheres has been classified into four different categories.

5.1.1 Degradation controlled monolithic system

Here, the drug is strongly attached to the polymer matrix and is released only with the degradation of the matrix. Diffusion is slow as compared to degradation which can be homogeneous or heterogeneous. In case of heterogeneous degradation, release from sphere is governed by the equation:

$$\frac{M_t}{M_\infty} = 1 - \left[1 - \frac{t}{t_\infty}\right]^3$$

Where M_t is amount of agent released at time t, M_∞ is the amount of agent released at infinite time, and t and t_∞ are times for erosion

5.1.2 Diffusion controlled monolithic system

Diffusion of drug is prominent as compared to degradation of the polymer matrix. Degradation of polymer matrix affects the rate of release. Rate of release depends on whether the polymer degrades by homogeneous or heterogeneous mechanisms.

5.1.3 Diffusion controlled reservoir system

In this case, the drug diffuses through the rate controlling membrane and the membrane erodes only after complete delivery. Drug release is unaffected by degradation of matrix.

5.1.4 Erodible poly-agent system

Here the active agent is chemically attached to the matrix and rate of biodegradation of matrix is slow as compared to rate of hydrolysis of drug polymer bond.

The nature of core also influences the release kinetics either by increasing polymer degradation or by physically binding with the polymer chain. Any form of drug polymer interaction may lead to changes in the release rate. Additives such as plasticizers decrease T_g, which leads to increased diffusion rates. Release from PLA and PLGA microspheres is dependent both on diffusion and polymer degradation (Johnson et al, 1996). The release from the PCL microspheres is also dependent on diffusion through the polymer matrix and polymer degradation though it is more diffusion dependent due to the slow degradation characteristics of the polymer (Yang et. al., 2001). Designing the morphology and drug entrapment of the polymeric microspheres is critical to obtain a desired release profile, especially for cases where the polymer has a low degradation rate. Maintaining the biological efficacy of insulin in PCL microspheres is important for successful formulation.

For this, the hypoglycemic effect brought about by the insulin loaded PCL microspheres after oral administration was also determined in diabetic animal models.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Insulin loaded PCL microspheres, Alloxan (Sigma-Aldrich Chemical Company, USA), Phosphate buffer saline (pH 7.4), Micro BCA Protein Estimation Kit (PIERCE®, USA). Double distilled water was used for all the experiments.

Experimental Animals: Sprague-Dawley rats weighing 280-330 gm (15-16 weeks old) and Male New Zealand rabbits weighing 2.5-3.0 kg were provided by experimental animal facility of INMAS, DRDO, Delhi. The study protocol was reviewed and approved by institutional animal ethics committee.

5.2.2 *In-vitro* release studies

The *in-vitro* studies were designed in three sets to study the effect of stabilizers (HPMC and PVA) and their concentrations (0.5%, 1.0% and 1.5%), polymer concentration (0.5%, 1.0%, 1.5% and 2.0 %) and drug concentration (100IHU/ml and 40 IHU/ml) on the release profiles of insulin from the PCL microspheres.

In-vitro release of insulin was determined by placing 10mg of microspheres from each batch, in 5ml of release medium (Phosphate buffer saline, pH 7.4) in 15ml glass vials. The samples were kept in an orbital shaker (SGM-300, Gallenkamp, Sanyo) maintained at 37°C ± 0.5°C stirring at 50 rpm. At pre-specified time intervals, 0.5ml aliquot of the release medium was removed and replaced with fresh PBS. These samples were then centrifuged at 3000 rpm at room temperature. Drug content in the supernant was then analyzed by using the micro BCA protein assay protocol. The protein estimation by using micro BCA assay has

also been reported by Katare (Katare et. al., 2005) wherein he estimated the content of tetanus toxoid entrapped within polylactide particles.

5.3.3 Comparison of hypoglycemic effect of free subcutaneous insulin and oral insulin microspheres in *Sprague-Dawley* rats

A comparative study was carried out to ascertain the role of insulin-loaded PCL microspheres for the oral delivery of insulin in animals. In order to determine and compare the hypoglycemic effects, insulin-loaded polymeric microspheres were administered orally to the first group of overnight fasted diabetic rats. Aqueous free insulin (10 IU/kg) (Damgé et. al., 2007) was administered subcutaneously to the second group of rats. Blood samples from the tail vein were collected before administration and at different time intervals from 0.5 hours to 24 hours after administration. Plasma glucose concentrations were measured by AccuChek® blood glucometer (Roche, Germany). Changes in blood glucose level versus time profiles in the diabetic mice were recorded.

5.3.4 *In-vivo* studies in *Sprague-Dawley* rats

Insulin loaded PCL microspheres were used for *in-vivo* oral release studies. Diabetes was induced in the experimental animals by injecting a single dose of alloxan (140 mg/kg body weight) dissolved in saline water (0.9% NaCl solution in distilled water) intraperitoneally and their blood glucose levels were checked for diabetic conditions after 48 hours. Animals with blood glucose level ≥300 mg/dl were selected for further studies (Rerup, 1970; Srinivasan and Ramarao, 2007). Diabetic rats were selected randomly and divided into two groups as the animal studies were carried out in two phases. Insulin loaded polymeric microspheres were administered orally by force feeding.

5.3.4.1 Effect of oral insulin loaded microspheres in fasted-diabetic Sprague-Dawley rats

In the first phase, diabetic animals were fasted overnight prior to oral administration of insulin loaded microspheres and divided into four groups. Animals of first group were taken as control and fed with PCL microspheres without insulin loading. In second, third and fourth groups, animals were fed with insulin loaded PCL microspheres. The dose of polymeric microspheres loaded with insulin given to second, third and fourth groups of rats was 30 IU/kg, 60 IU/kg and 90 IU/kg animal body weight and their blood glucose levels were measured at regular time interval up to 24 hours.

5.3.4.2 Effect of oral insulin loaded microspheres in fed-diabetic Sprague-Dawley rats

In the second phase of experiments, animals were allowed to move freely and fed properly to simulate the natural conditions and the desired blood glucose level was maintained using an appropriate dose of insulin loaded PCL microspheres for up to eight days. Animals of the first group were taken as control and fed with PCL microspheres without insulin loading. In the second group, animals were fed with insulin loaded PCL microspheres with insulin concentration of 90 IU/kg animal body weight. Blood samples were collected from the tail vein of rats at specific time interval after oral administration of insulin loaded microspheres and blood glucose level was measured using AccuChek® blood glucometer (Roche, Germany). The reduction in blood glucose concentration at a particular time was obtained using the equation below and the blood glucose concentration versus time curves were plotted.

$$\text{Glycemia (\% Basal Value)} = \left[\frac{F - P_t}{F}\right] \times 100$$

Where, F is the fasting glucose level, P_t is the plasma glucose level at time (t) after oral administration of the insulin loaded microspheres.

5.3.5 *In-vivo* studies in male New Zealand rabbits

Male New Zealand rabbits weighing 2.5-3.0 kg were selected for the experiments. Diabetes was induced in the experimental rabbits by injecting a single dose of alloxan (150 mg/kg body weight) dissolved in sterilized water intravenously and their glucose levels were checked for diabetic conditions after 48 hours (Srinivasan and Ramarao, 2007). Animals with glucose levels above ≥300mg/dl were only used in subsequent studies (Bendayan et. al., 1994). Diabetic rabbits were selected randomly and divided into two groups as the animal studies were again carried out in two phases. Insulin loaded PCL microspheres were administered orally by force feeding.

5.3.5.1 Effect of oral microspheres in fasted-diabetic male New Zealand rabbits

In the first phase, diabetic rabbits were fasted overnight prior to oral administration. Animals of the first group were taken as control and fed with PCL microspheres without insulin. In the second group, animals were fed with 90 IU/kg insulin loaded PCL microspheres. The blood glucose level of all the animals was checked at regular time interval up to 24 hours.

5.3.5.2 Effect of oral insulin loaded microspheres in fed-diabetic male New Zealand rabbits

In the second phase of experiments, diabetic rabbits were selected randomly and allowed to move freely and fed properly to simulate the natural conditions and the desired blood glucose level was maintained using an appropriate dose of insulin loaded PCL microspheres. Animals of the first group were taken as control and fed with PCL microspheres without insulin loading. In the second group, animals were fed with insulin loaded PCL microspheres with insulin concentration of 90 IU/kg animal body weight. Blood samples were collected from the ear vein of rabbits at specific time intervals after oral administration of insulin loaded microspheres and blood glucose level was measured using AccuChek® blood

glucometer (Roche, Germany). The reduction in blood glucose concentration at a particular time was obtained using the equation below and the blood glucose concentration versus time curves were plotted.

$$\text{Glycemia (\% Basal Value)} = \left[\frac{F - P_t}{F}\right] \times 100$$

Where, F is the fasting glucose level and P_t is the plasma glucose level at time (t) after oral administration of the capsule.

5.4 RESULTS AND DISCUSSION

5.4.1 *In-vitro* release studies

We observe that the microspheres are characterized by a more significant initial release, followed by a slower release. The initial burst release (amounting to about 10% to 14%) is attributed to the drug desorption from the particle surface. This is then followed by the gradual release phase, resulting from the diffusion of the drug dispersed into the polymer matrix contributing to approximately 60% release in 30 days. After the accessible protein molecules have diffused, there is a significant reduction in release rate. This may be explained by the slow degradation in the PCL microspheres.

The encapsulated protein may be loosely bounded to the surface, embedded in the surface layer, or trapped within the microsphere matrix. The protein loosely bounded to the surface and embedded in the surface layer gives rise to the initial release after the burst effect. However, the release of protein trapped within the microsphere matrix may depend on the diffusion from the microsphere as well as the degradation of the microsphere matrix. We found that the degradation of the PCL microspheres almost does not occur even after 60 days of release. As a result, the predominant mechanism of insulin release from these microspheres is found to be diffusion-dependent. This result is comparable to the results

reported by Lamprecht, Yang and Pe´rez and their co-researchers for PCL nanospheres and microspheres (Lamprecht et. al., 2000; Yang et. al., 2001; Pe´rez et. al., 2000).

5.4.1.1 Effect of stabilizers and their concentrations on the in-vitro release profiles

The *in-vitro* release profiles of the batches L1 to L6 are shown in the Figure 5.1. Our experimental results indicate that microspheres fabricated with a low HPMC or PVA concentration in the external water phase have a more rapid insulin release. This may be explained as at a low stabilizer concentration, a less stable emulsion together with possible convective mass transfer at inner surface during the mixing facilitate insulin-containing droplets to diffuse outward. Thus, insulin may be distributed more within the exterior of microspheres than within the interior. Yang et. al., 2001 also reported similar results for different PVA concentrations in external aqueous phase preparation of PCL microspheres (Yang et. al., 2001).

Also, it was observed that the encapsulation efficiency and drug release profile showed some correlation. Higher the entrapment more was the release. A higher drug encapsulation resulted in a larger concentration gradient between the microspheres and the *in-vitro* medium. This further resulted in a faster initial burst and a more rapid release profile. This may explain the lower release rates of batches L3, L4 and L6 which have lower percent drug encapsulation. The particle size may also play a role in the rate of drug release as bigger the particle, more is the diffusion path that the drug needs to cover for being released into the release medium, thus resulting in decreased rate of release. This may explain the low release profile of batch L5 when compared to the release from batch L2, which has the same concentration of stabilizer and comparable percent efficiency but bigger particle size.

5.4.1.2 Effect of polymer concentration on the in-vitro release profiles

The *in-vitro* release profiles of the batches P1 to P4 are shown in the Figure 5.2. Our experimental results show that microspheres fabricated with higher polymer concentration

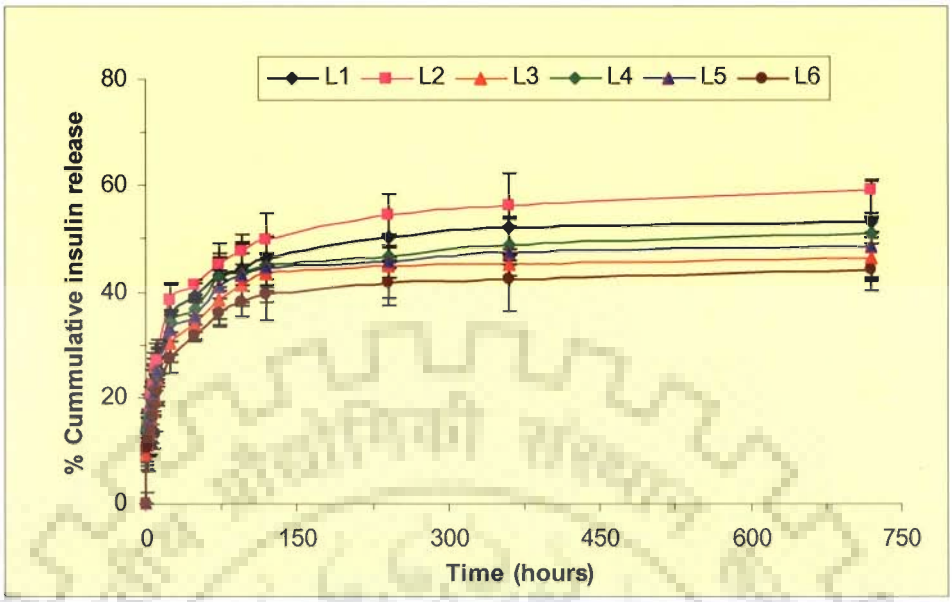

Figure 5.1: Effect of stabilizers and their concentrations on the *in-vitro* release profiles of PCL microspheres; L1: 0.5% HPMC, L2: 1.0% HPMC, L3: 1.5% HPMC, L4: 0.5% PVA, L5: 1.0% PVA and L6: 1.5% PVA. PCL concentration was kept constant at 1% and drug concentration was kept constant at 100 IHU/ml. Results are expressed as means ± S.D. *n=3*

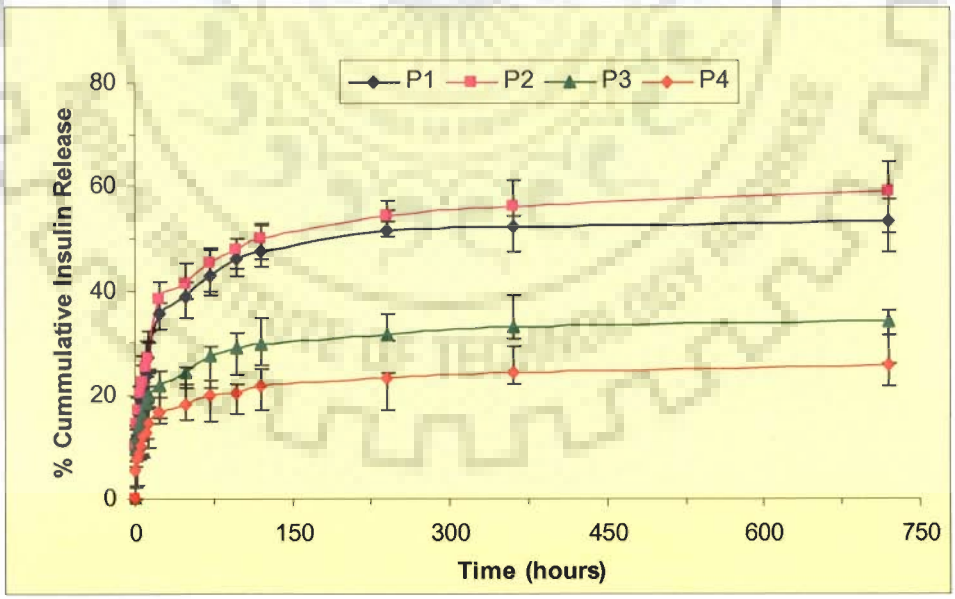

Figure 5.2: Effect of polymer concentration on the *in-vitro* release profiles of PCL microspheres; P1: 0.5% PCL, P2: 1.0% PCL, P3: 1.5% PCL and P4: 2.0% PCL. HPMC concentration was kept constant at 1% and drug concentration was kept constant at 100 IHU/ml. Results are expressed as means ± S.D. *n=3*

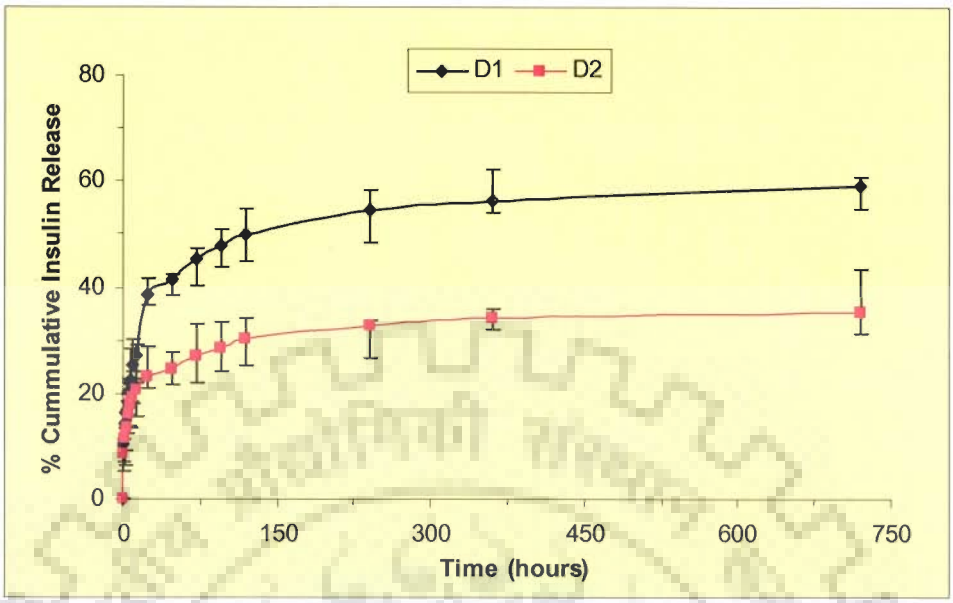

Figure 5.3: Effect of drug concentration on the *in-vitro* release profiles of PCL microspheres; D1: 100 IHU/ml, D2: 40 IHU/ml. PCL and HPMC concentration was kept constant at 1%. Results are expressed as means ± S.D. *n=3*

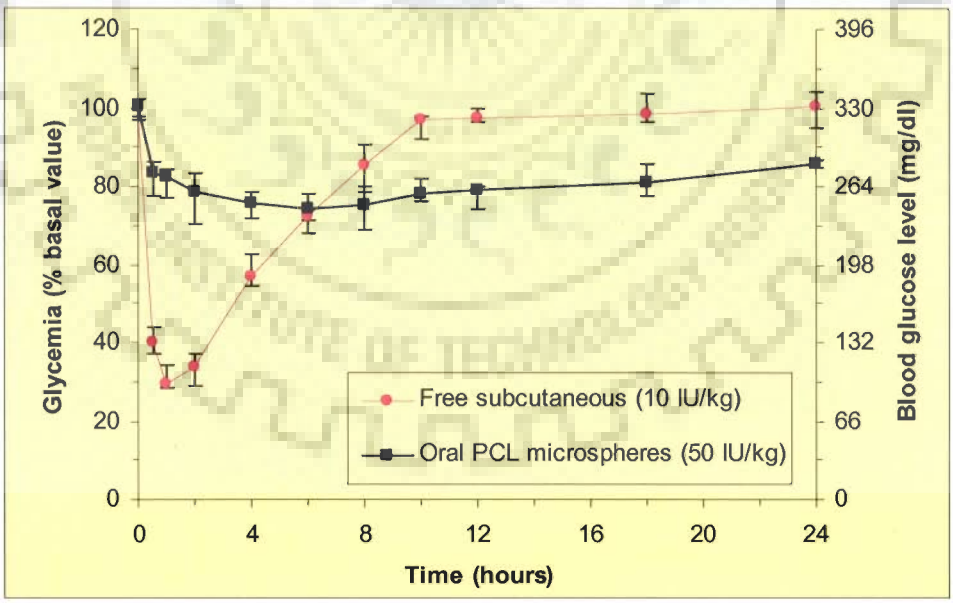

Figure 5.4: Comparison of hypoglycemic effect of free subcutaneous insulin and oral insulin loaded PCL microspheres in fasted diabetic *Sprague-Dawley* rats, ($p<0.001$); Results are expressed as means ± S.D. (6 animals per group)

(1.5% and 2.0%) showed the least drug release, depicted by batches P3 and P4. An increase in the amount of polymer gives rise to microspheres with a dense, less porous polymeric phase, thereby inhibiting the insulin release. Microspheres fabricated with a low polymer concentration have less tortuous inner matrix which resulted in a more rapid insulin release. The microspheres prepared with the lower polymer concentration also have a more porous matrix compared with the microspheres formulated from the higher concentration. This may be due to two factors: (1) the internal water droplets in the low polymer concentration solution tend to coalesce together more easily, leading to bigger pores and a less tortuous network, (2) the high polymer concentration solution coagulates faster during the second emulsion and yields a tighter structure because of chain entanglement (Yang et. al., 2001). Consequently, a low polymer concentration yields microspheres with a more rapid release profile as seen in Figure 5.2. Another reason for the slow release rate of batches P3 and P4 are their bigger particle size. The increase in size of the microspheres with increasing polymer concentration arises from the more viscous or concentrated polymer solution which reduces the dispersion of the inner aqueous phase in the organic phase during the microsphere formation.

5.4.1.3 Effect of drug concentration on the in-vitro release profiles

Figure 5.3 shows the *in-vitro* release profiles of our batches D1 and D2. The release profiles and the initial bursts can be co-related to the degree of percent insulin entrapment. A higher insulin percent encapsulation resulted in a larger concentration gradient between the microspheres and the *in-vitro* medium. Since the gradient is the driving force for insulin diffusion, a high insulin encapsulation within the microsphere led to a higher initial burst and a more rapid release rate as seen in the case of batch D1. Moreover, at a high percent encapsulation, there may be more insulin distributed near the surface area of microspheres

(Yang et. al., 2001). This further led to the greater initial release. As the insulin released, it left more pores and interconnecting channels for the release of the remaining insulin.

5.4.2 Comparison of hypoglycemic effect of free subcutaneous insulin and oral insulin loaded microspheres in *Sprague-Dawley* rats

The decrease in glycemia after the subcutaneous injection of free, non encapsulated insulin (10 IU/kg) was compared with the glycemic decrease brought about by insulin entrapped PCL microspheres (50 IU/kg) after oral administration. The initial decrease in glycemia was faster for subcutaneous administration of free insulin, than for insulin encapsulated microspheres administered orally, as seen in Figure 5.4. In case of the subcutaneous administration of insulin, the glycemia decreased significantly after 30 minutes, the maximal decrease of 70% ($p<0.001$) being reached after 1 hour. After this, glycemia increased progressively and returned to control values after 10 hours. When insulin entrapped microspheres were administered orally, the glycemia decreased to a lesser extent but its effect was observed between 2 to 12 hours post administration, with the maximal decrease (25%, $p<0.001$) noted from 4 to 8 hours. The effect lasted for up to 12 hours. No decrease in glycemia was observed with oral administration of free non-encapsulated insulin.

It was therefore observed from Figure 5.4 that increase in glycemia was observed after 2 hours in case of subcutaneous injection of non encapsulated aqueous insulin as compared to 8 hours in case of encapsulated insulin in polymeric microspheres. Thus, encapsulation of insulin prolonged its biological effect by almost 6 hours as compared to subcutaneous administration. Since non encapsulated insulin did not reduce glycemia after oral administration, it is suggested that PCL microspheres could protect insulin from enzymatic degradation in the gastrointestinal tract and allowed its absorption in a bioactive form.

5.4.3 Effect of oral insulin loaded microspheres in fasted-diabetic *Sprague-Dawley* rats

The results observed in this study emphasized the ability of insulin-loaded microspheres to reduce glycemia with prolonged action in diabetic rats after oral administration. When these microspheres were administered orally by force-feeding in overnight fasted diabetic rats, they decreased glycemia in a dose-dependent manner in comparison with rats treated with empty microspheres as seen in Figure 5.5.

Control animals fed with PCL microspheres without insulin showed almost no change in the blood glucose level during experiments. The lowest concentration of insulin loaded microspheres (30 IU/kg) was found to be almost as ineffective as the PCL microspheres administered without insulin. But the 90 IU/kg insulin loaded microspheres appreciably reduced glycemia by about 42% ($p<0.05$) after 4 hours and 45% ($p<0.05$) after 6 hours of the administration. This decrease in glycemia was maintained up to 10 hours (35%). Analogous results were observed with 60 IU/kg insulin loaded microspheres but the decrease of glycemia was less pronounced, being about 31% ($p<0.05$) after 4 hours and 33% ($p<0.05$) after 6 hours. A slight reduction of glycemia (9%) was still noted after 24 hours with the concentration of 90 IU/kg insulin but not with 60 IU/kg insulin loaded microspheres. One way analysis of variance (ANOVA) followed by the Dunnett's method was carried out and results showed $p<0.05$, thus implying that there was statistically significant difference amongst the set of formulations studied.

Therefore, there was a dose dependent reduction in glycemia in the diabetic animals with the hypoglycemic effect being more pronounced with 90 IU/kg than the 60 IU/kg concentrations of insulin loaded microspheres. Prolongation of the hypoglycemic effect was also observed as it was seen to last from 4 hours to 10 hours with both the above concentrations of insulin loaded microspheres. Slight reduction of glycemia was also observed at 24 hours with 90 IU/kg concentration of insulin loaded microspheres. An

aqueous insulin solution administered orally to diabetic rats by catheter did not affect glycemia, no matter what the dose (30, 60 and 90 IU/kg) administered was. Thus, it can be inferred that insulin entrapped in microspheres remained biologically active and acted in a dose dependent manner after oral administration to fasted diabetic rats.

As an initial fast release of drug, also known as the burst effect, occurring within an hour of administration is often associated with polymeric microspheres (Hoffart et. al., 2002; Attivi et. al., 2005), the hypoglycemic effect observed between 4 hours to 10 hours could be explained by the diffusion depended slow release of the protein encapsulated from the polymeric microspheres. Similar results have also been reported with Eudragit® RS and PCL blended nanoparticles (Damgé et. al., 2007) and with poly-alkylcyanoacrylate nanocapsules (Damgé et. al., 1988; Damgé et. al., 1997) which have also prolonged the hypoglycemic effect of insulin in both diabetic and normal rats.

The mechanism suggested by Damgé et. al., 2007 for the intestinal uptake of insulin loaded microspheres or nanoparticles or insulin released from nanoparticles is the lymphatic uptake via the M cells of the Peyer's patches which are mostly abundant in the ileum (Damgé et. al., 2007). This explains for the substantial activity being observed with the 90 IU/kg concentration of insulin loaded microspheres and almost no activity being observed with the 30 IU/kg concentration of insulin loaded microspheres. This may be explained as perhaps a certain percentage of the encapsulated insulin may be have been released in the gastro intestinal tract and with peptides and proteins sensitive to gastrointestinal enzymatic degradation, may have led to a loss or a reduction of biological activity. Also, it can be presumed that the entire amount of encapsulated insulin is not totally absorbed from the microspheres. In other words, a certain amount of insulin may remain within the microspheres and not be released. This non-complete release from insulin loaded nanoparticles has also been observed by Attivi et. al. (Attivi et. al., 2005). When higher doses of insulin-loaded microspheres (60 and 90 IU/kg) were administered both the above

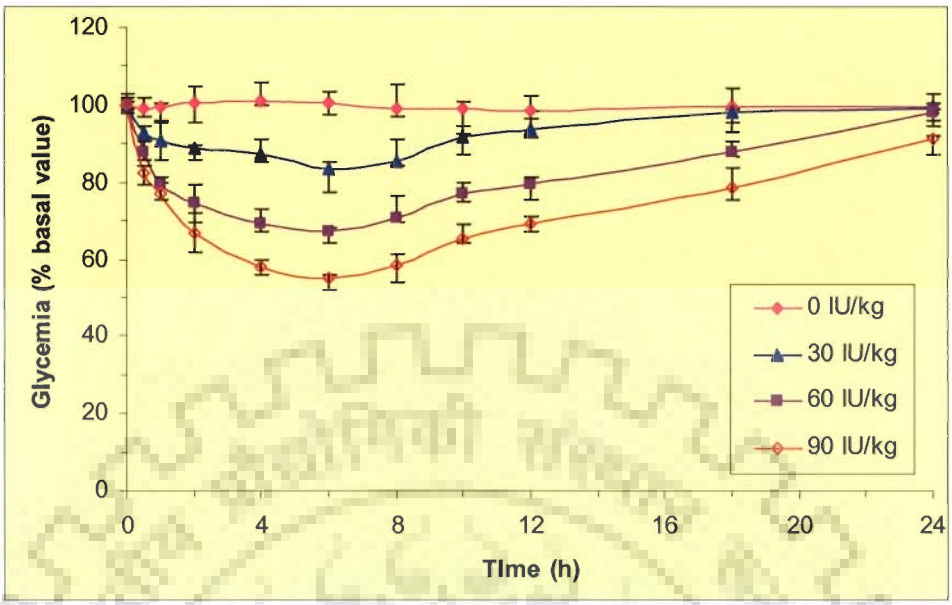

Figure 5.5: Effect of different concentrations of insulin loaded poly-ε-caprolactone microspheres after oral administration in fasted-diabetic *Sprague-Dawley* rats, ($p<0.05$); Results are expressed as means ± S.D. (6 animals per group)

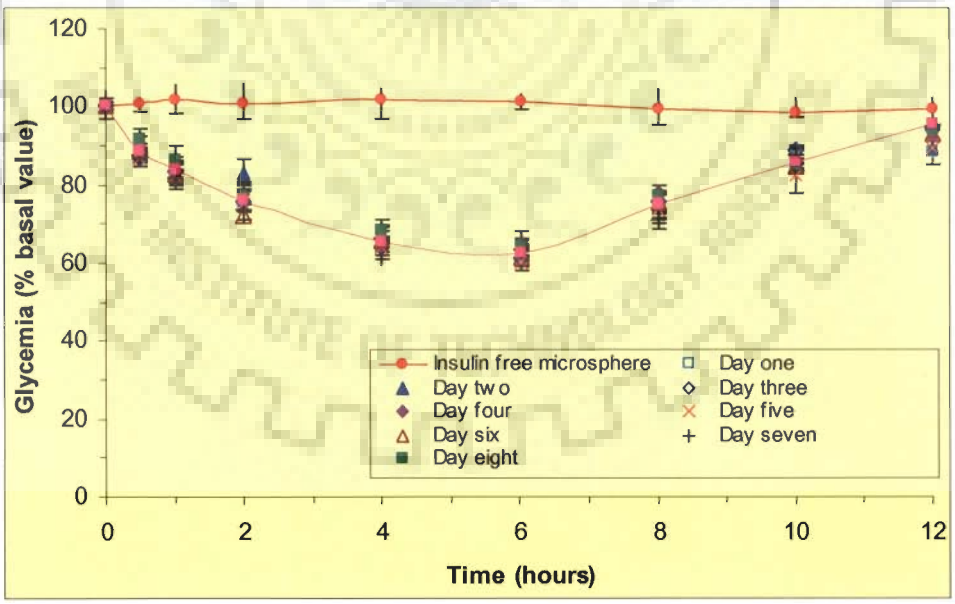

Figure 5.6: Effect of oral insulin loaded poly-ε-caprolactone microspheres on fed-diabetic *Sprague-Dawley* rats, ($p=0.001$); Results are expressed as means ± S.D. (6 animals per group)

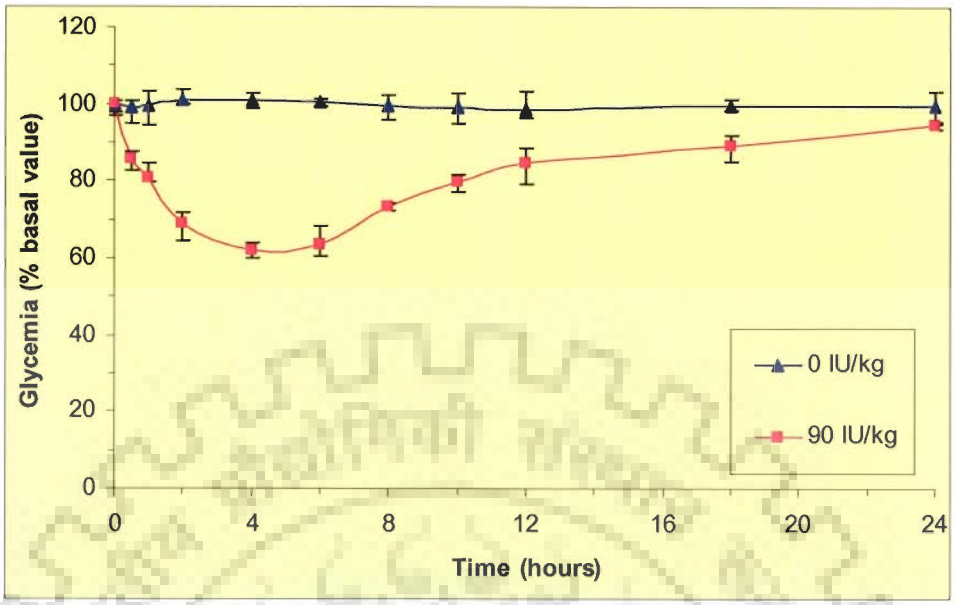

Figure 5.7: Effect of oral insulin loaded poly-ε-caprolactone microspheres on fasted-diabetic male New Zealand rabbits, (p=<0.001); Results are expressed as means ± S.D. (4 animals per group)

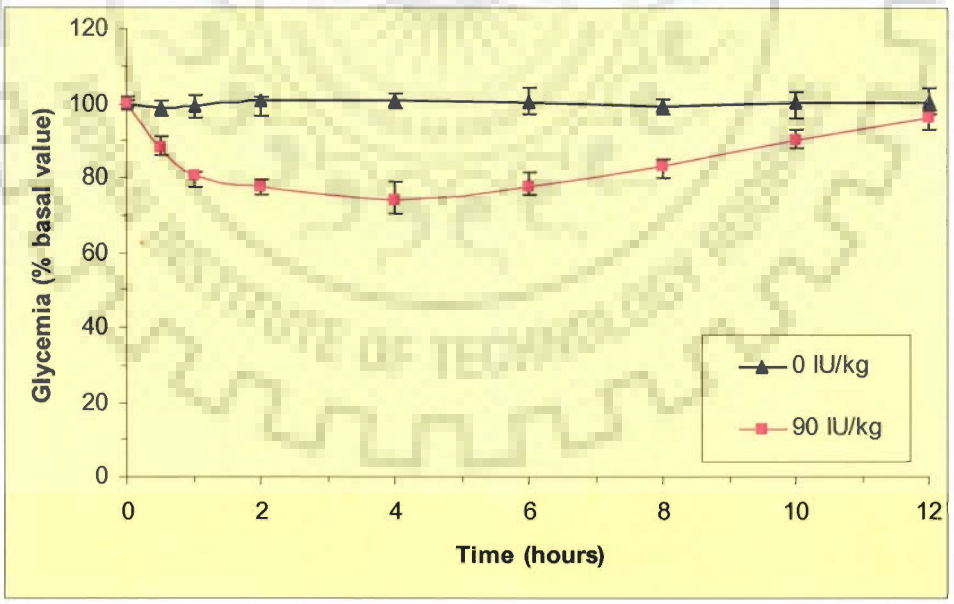

Figure 5.8: Effect of oral insulin loaded poly-ε-caprolactone microspheres on fed-diabetic male New Zealand rabbits, (p=0.001); Results are expressed as means ± S.D. (4 animals per group)

factors affected the release and absorption characteristics but they were counterbalanced by their higher doses and consequently higher concentration gradient of insulin. But in case of low dose (30 IU/kg), the remaining amount of insulin available for absorption is perhaps too low to support an efficient insulin gradient. Therefore, both partial release in gastrointestinal tract and incomplete release of insulin from the polymer microspheres could explain the lack of biological activity of 30 IU/kg insulin loaded microspheres after oral administration.

5.4.4 Effect of oral insulin loaded microspheres in fed diabetic *Sprague-Dawley* rats

Oral administration of 90 IU/kg insulin loaded PCL microspheres to fed diabetic rats reduced the initial blood glucose level by 33% ($p=0.001$) after 4 hours and 36% ($p=0.001$) after 6 hours in comparison to 42% and 45% reduction in case of fasted animals. Control animals fed with PCL microspheres without insulin showed almost no change in the blood glucose level during the experiments. Unpaired t-test followed by the Mann-Whitney Rank Sum test was carried out and results showed $p=0.001$, thus implying that there was statistically significant difference amongst the groups analyzed. In case of diabetic fed rats, lower reduction of blood glucose level than fasted diabetic rats may be explained due to the continuous absorption of glucose from the gastrointestinal tract. It was also observed that 90 IU/kg of insulin loaded microspheres administered twice daily were sufficient to maintain the desired blood glucose level. Studies were continued till 8 days and the results obtained for all the days are shown in Figure 5.6. In these experiments also, the hypoglycemia lasted for at least 6-8 hours after oral administration of PCL microspheres, which confirmed the prolonged release of active insulin from the polymeric microspheres.

5.4.5 Effect of oral insulin loaded microspheres in fasted-diabetic male New Zealand rabbits

The effect of oral administration of 90 IU/kg of insulin loaded PCL microspheres in overnight fasted diabetic rabbits is shown in Figure 5.7. Control animals fed with PCL

microspheres without insulin showed almost no change in the blood glucose level during the experiments. Insulin loaded microspheres (90IU/kg) reduced the blood glucose level by 38% (p=<0.001) after 4 to 6 hours of the oral administration. Glycemia then increased progressively after 10 hours and approached the control value. The effect of insulin-loaded microspheres lasted for at least 8-10 hours after the oral administration. Unpaired t-test followed by the Mann-Whitney Rank Sum test was carried out and results showed p=<0.001, thus implying that there was statistically significant difference amongst the groups analyzed.

5.4.6 Effect of oral insulin loaded microspheres in fed-diabetic male New Zealand rabbits

Figure 5.8 shows the effect of oral administration of 90 IU/kg of insulin loaded PCL microspheres in fed diabetic rabbits. In this case too, control animals fed with PCL microspheres without insulin loading, showed almost no change in the blood glucose level during the experiments. Insulin loaded microspheres (90 IU/kg) reduced the blood glucose level by 26% (p=0.001) after 4 hours of the administration and the hypoglycemic effect lasted for at least 8 hours after the oral administration as compared to 38% decrease observed in fasted animals where the effect lasted for almost 10 hours. Unpaired t-test followed by the Mann-Whitney Rank Sum test was done and results showed p=<0.001, thus implying that there was statistically significant difference amongst the groups analyzed. Here also, lower reduction of blood glucose level in fed diabetic rabbits than fasted diabetic animals may be explained due to the continuous absorption of glucose from the gastrointestinal tract. In this case too, 90 IU/kg of insulin loaded microspheres administered twice daily were sufficient to maintain the desired blood glucose level. Therefore, the encapsulation of insulin into microspheres prepared with PCL, allowed the preservation of the biological activity of insulin along with its prolongation of action after oral administration in fasted as well as fed diabetic animal models.

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Chapter 6

PHARMACOSCINTIGRAPHIC AND TOXICITY STUDIES

6.1 PREAMBLE

Nuclear medicine is primarily a diagnostic specialilty based on the principle of imaging of short-lived radionuclides, for example ^{99m}Tc. Besides being an aid in establishing diagnosis, the role of nuclear medicine is also to assist in planning of treatment, delivering the therapy, evaluating the results of the treatment and in detecting the recurrence of the disease. Essentially, phrmacoscintigraphy can be regarded as the study of the biological distribution of radioactive medicinal products, and incorporates radiopharmacokinetics, transport mechanisms, interaction of binding sites and the metabolic fate. The profound uses of radiopharmaceuticals in the areas of biomedicine and research are based on the fact that the living system does not differentiate between the isotopes of a given element, although this differentiation is possible through physical means (Rescigno and Seigre, 1966; Birkett, 2000). The use of stable and radioactive isotopes for the study of reaction in biological system is derived from the fact that we can follow the metabolic path of a molecule, as well as its distribution in the living organism, its participation in the various reaction, determine the turnover of its components, the rate of biochemical reactions, the existence of a precursor-successor relationship between two compartments of the system and its computation (Rescigno and Segre, 1966). Biodistribution of a radiopharmaceutical may be explained in terms of a single mechanism as a result of interaction between many different mechanisms involving initial dilution, passive and active membrane transport, protein binding, possible metabolic incorporation, elimination and excretion. Radiopharmaceuticals exhibit a huge range of physical and chemical properties. Mechanisms of biodistribution may depend on the physical form, as in the uptake of particulate material by phagocytosis, or may be brought up by the similarity of the radiopharmaceutical agent to a substrate or a

metabolite. Radiopharmaceuticals find use for nuclear diagnosis and treatment of various diseases of thyroid, lungs, liver, kidneys and in many cancers. (Sarkar et. al., 2005; Singh et. al., 2002; Bhattacharya et. al., 2001; Gestin et. al., 2000; Mittal et. al., 2005; Singh et. al., 2001)

6.1.1 Radiopharmaceuticals and radiolabeling

Radiopharmaceutical, as the name indicates, is a drug containing radionuclide. It may be defined simply as chemical substance that contains radioactive atom within its structure and is suitable for administration to humans for diagnosis or treatment of disease. These are formulated in various chemical or physical forms to deliver their radioactivity to particular parts of the body (Chopra and Mishra, 2005; Benoist et. al., 1998a; Benoist et. al., 1998b; Morandeau et. al., 2006). Furthermore, the γ-radiations emitted from these radiopharmaceuticals readily penetrate the tissuees and escape from the body allowing its external detection and measurement. In this way, the functional and morphological characteristics of various organs in the body can be evaluated (Mishra et al., 2003; Chuttani et al., 2003; Mishra et al., 2005).

Most elements have a mixture of stable and unstable isotopes. Nuclides (isotopes) containing an unstable arrangement of protons and neutrons will, at some stage, transform to either a completely stable or a more stable combination of nucleons. Such nuclides (isotopes) are said to be radioactive and are termed radionuclides (radioisotopes). Transformation, or radioactive decay, occurs with either the emission of a charged particle from the nucleus or the capture of an electron from one of the cells surrounding the nucleus. Radioactive decay thus results in a change in the balance between the protons and the neutrons, and change in the element. If the product nucleus is also in an excited state, it will de-excite to the ground state with the emission of one more γ- rays. The initial unstable nucleus is referred as the parent nuclide and product nucleus as the daughter nuclide.

For an ideal radiopharmaceutical, the radionuclide should be easily available, decay by electron capture or isomeric transition; the radionuclide should not decay by particle emission. Rather, the nuclide decaying by γ-emissions is desirable. The energy of the decay should be within the range of 30 KeV- 300 KeV. This is due to the fact that, energy below 30 KeV is absorbed by the tissue and the energy above 300 KeV is not marked by the lead or denser material.

Radionuclides with short half-lives are preferred because larger amounts of radioactivity may be administered. This improves the information obtained because more protons are available for imaging. The radiation may also be reduced because the radioactivity decays quickly with short half-life radionuclides and imaging can be performed with minimum exposure to the other body parts. Nearly 80% of the currently used radiopharmaceuticals are labeled with Technetium-99m (^{99m}Tc) (Kumari et. al., 2004; Sarkar et. al., 2005; Singh et. al., 2002 Bhattacharya et. al., 2001).

6.1.2 Advantages of technetium-99m (^{99m}Tc)

The reason for such a pre-eminent position of ^{99m}Tc in clinical use is its extremely favourable physical and radiation characteristics. The six hour physical half-life and being a pure γ-emitter (the absence of β radiations) permits the administration of mCi amounts of ^{99m}Tc radioactivity without a significant radiation dose to the patient. In other words, technetium's shorter physical half-life permits the use of a higher administered dose which translates to a higher count rate which will shorten imaging time and provide clear and better resolution images. The gamma energy of ^{99m}Tc (140 KeV) is optimal for the detector crystal used in the gamma camera and will undergo less attenuation and scatter. ^{99m}Tc is also readily available and may be produced daily from molybdenum generator in most nuclear medicine centers. Moreover, this generator eluted radioisotope is easy to obtain at very low cost. The combination of ready availability, excellent imaging properties, favourable dosimetry and high specific activity make ^{99m}Tc a logical choice for labeling.

6.1.3 Chemistry of technetium

As element 43 in the periodic table, Technetium is placed in group VIII A, in the second row of the transition metals. Therefore, its chemistry is dominated by the metal-donor complexes; compounds formed by binding of the electron deficient metal and atoms or functional groups which are capable of donating electron pairs. With only minor exceptions, all Tc-radiopharmaceuticals are metal donor complexes. Compounds which form complexes with metals (termed ligands) may have only one donor group (a monodentate ligand), or two (bidentate) or more (polydentate) donor groups.

A feature of transition metals is that each may have multiple oxidation states. For Tc, all oxidation numbers from 0 to +7 have been reported (Deutsch et. al., 1983), and a possible oxidation state of -1 has also been suggested (Huggins and Kaesz, 1964). The Mo-99/Tc-99m generator provides Tc in its highest oxidation state of +7. While complexes of Tc in this oxidation state are known, the oxidation states which dominate the chemistry of Tc are +1, +3 and +5. Nature of complex formed is dependent upon the co-ordination number (the number of ligating atoms bound to Tc), and the geometry of those donor groups around the metal co-ordination numbers from 4 to 9 are recorded for Tc (Deutsch et. al., 1983], with several co-ordination numbers and/or geometries known within each oxidation state.

6.1.4 Reduction of Tc for labeling

The chemical form of Tc available from the Mo generator is sodium pertechnetate (^{99m}Tc NaO_4). Chemically, ^{99m}Tc O_4^- is rather a non-reactive species and does not label any compound by direct addition. In Technetium-99m labeling of many compounds, prior reduction of Technetium-99m from the +7 state to a lower valence state (+3, +4 or +5) is required. Various reducing systems that have been used are stannous chloride, ascorbic acid and ferric chloride, concentrated HCl, sodium borohydride and ferrous sulphate. Among

these, stannous chloride ($SnCl_2$. $2H_2O$) is the most commonly used reducing agent in acidic medium in most preparations of ^{99m}Tc labeled compounds.

The chemical reaction that occurs in the reduction of technetium by stannous chloride in acidic medium can be stated as follows (Saha, 1998):

Step I: $3\ Sn^{2+} \longrightarrow 3\ Sn^{4+} + 6\ e^-$

Step II: $2\ ^{99m}TcO_4^- + 16\ H^+ + 6\ e^- \longrightarrow 2\ ^{99m}Tc^{4++} + 8\ H_2O$

Overall Reaction: $2\ ^{99m}TcO_4^- + 16\ H^+ + 3\ Sn^{2+} \longrightarrow 2\ ^{99m}Tc^{4++} + 8\ H_2O + 3\ Sn^{4+}$

The above equation indicates that Technetium-99m has been reduced to $^{99m}Tc\ O_4^+$. Other reduced states such as $^{99m}Tc^{3+}$ and $^{99m}Tc^{5+}$ may be formed under different physiochemical condition.

The reduced ^{99m}Tc is highly reactive and combines with a wide variety chelating agents. The chelating agents usually donate the lone pair of electrons to form co-ordinate covalent bonds with Technetium-99m. The chemical groups such as –COO, $-NH_2$ and –SH are the electron donors in compounds such as DTPA, glucoheptonate and various proteins. (Gouin et. al., 2005).

6.1.5 Complex stability

The stability of complexes can be considered in one of the two ways; thermodynamic or kinetic. The former is the equilibrium between free and complexed metal ion in a solution atleast a molar equivalent of the ligand. An equilibrium constant, K, provides a measure of thermodynamic stability (Perrin, 1964). The rate at which complexes decompose is a measure of their kinetic stability. There is no direct relationship between the two parameters. Complex decomposition rate will be dependent upon the environment. *In-vivo* stability (or the lack of it) may not automatically be inferred from the *in-vitro* data. Technetium complex

stability can influence radiopharmaceutical performance in a number of ways. For most radiopharmaceuticals, it is essential that no dissociation occurs *in-vivo* for the period during which image accusation is required. Increased background activity is usual consequence of such dissociation.

When the pharmacokinetic study is done by the radiolabeling the drug, then simple analysis of the biological sample for the presence of the radiolabeled compound easily provides the quantitative estimate of the drug concentration without the interference of the endogenous substances. Thus, the use of radiolabeling has directly shortened the study procedure and increased the reliability and the precision of the collected data.

To ascertain the absorption and distribution mechanisms of the insulin loaded PCL microspheres, radiolabeled insulin encapsulated into these microspheres was studied by gamma imaging of the animal models as shown in Figure 6.1. This technology, being non-invasive has been proven to be of great value in the assessment of a wide range of pharmaceutical formulations and new drug delivery systems. Toxicity was also determined with histopathological evaluation in healthy rats.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Insulin Human Injection USP, 100 IHU/ml, (Eli Lily and Company, USA), Poly-ε-caprolactone (M_n 42,500; Sigma- Aldrich Chemical Company, USA), Dichloromethane (Merck, Mumbai, India), Hydroxypropylmethylcellulose (M_w 10,000; Sigma-Aldrich Chemical Company, USA), Stannous chloride (S.D. Fine-Chem Ltd., India), Hydrochloric acid (S.D. Fine-Chem Ltd., India), Acetone (S.D. Fine-Chem Ltd., India) Alloxan (Sigma-Aldrich Chemical Company, USA), ITLC-SG strips (Gelman, USA). 99mTechnitium was provided by radiation safety division of Institute of Nuclear Medicine and Allied Sciences (INMAS), Defence Research and Development Organization (DRDO), New Delhi.

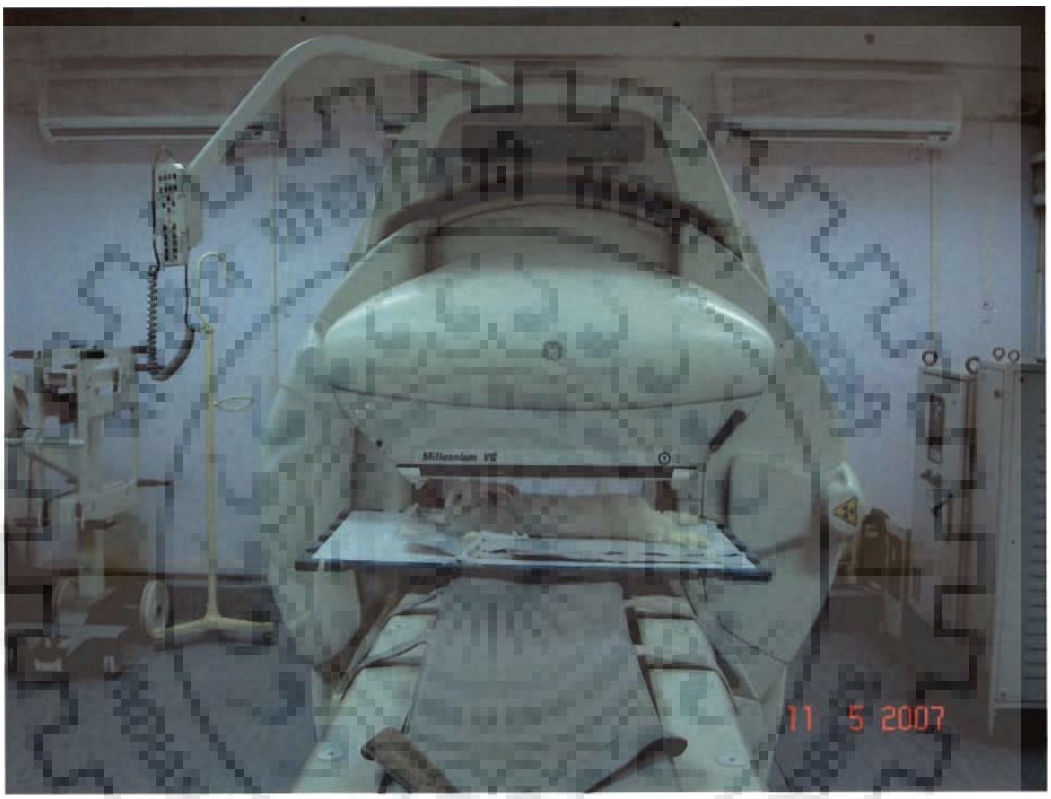

Figure 6.1: GE gamma camera (GE XR/T 4000 SPECT Gamma Camera, Germany)

Figure 6.2: Scintillation counter

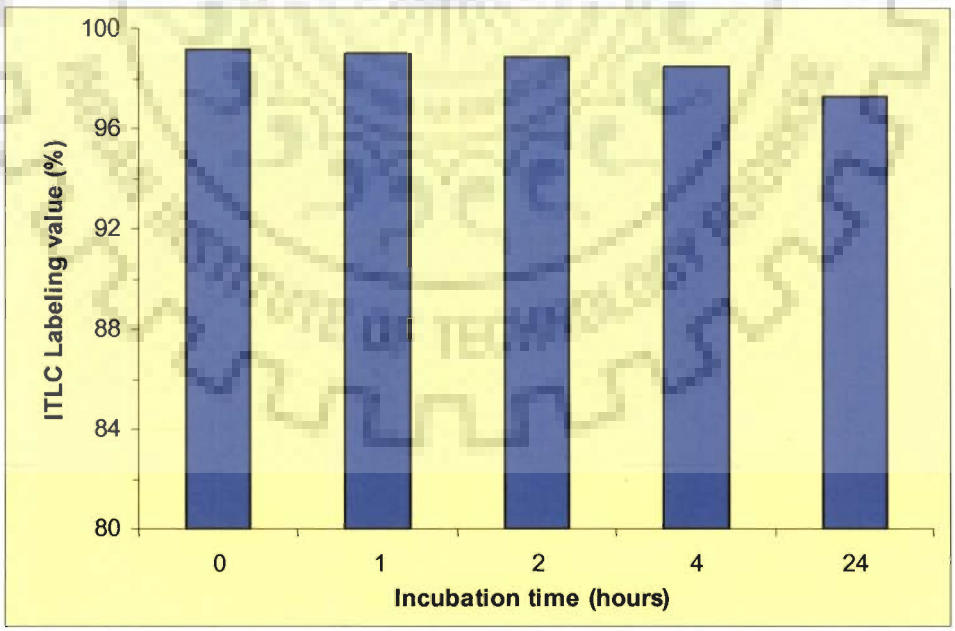

Figure 6.3: *In-vitro* stability of ^{99}Tc labeled insulin in saline

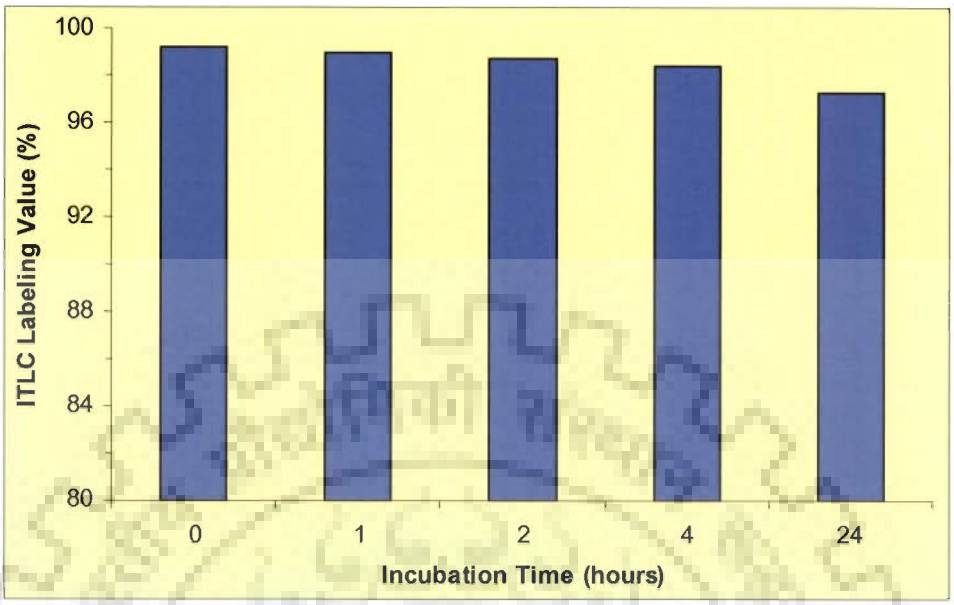

Figure 6.4: *In-vitro* stability of ^{99}Tc labeled insulin in serum

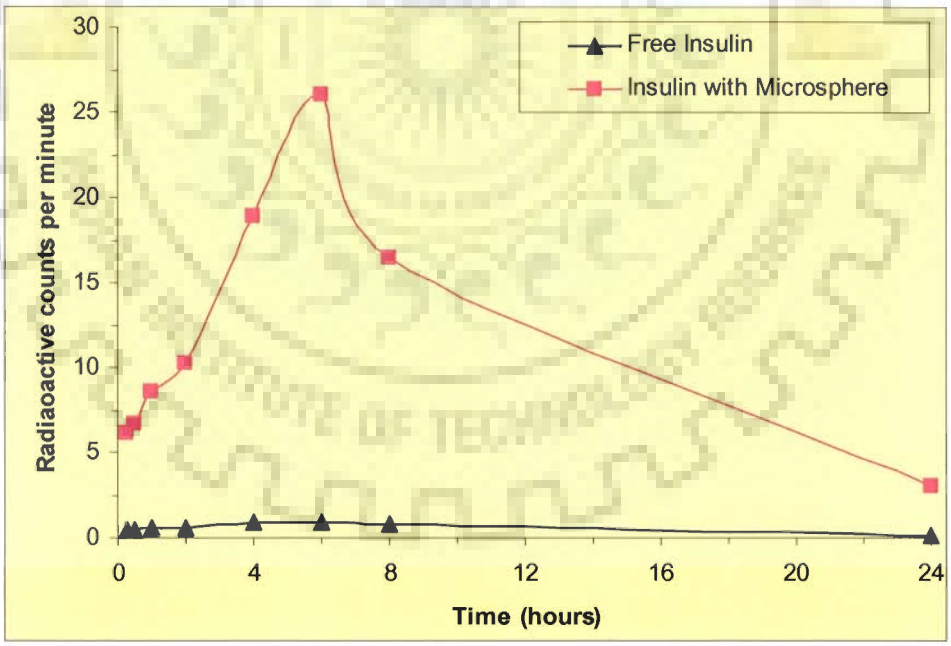

Figure 6.5: Pharmacokinetic profiles of ^{99m}Tc-insulin loaded PCL microspheres and free ^{99m}Tc-insulin after oral administration

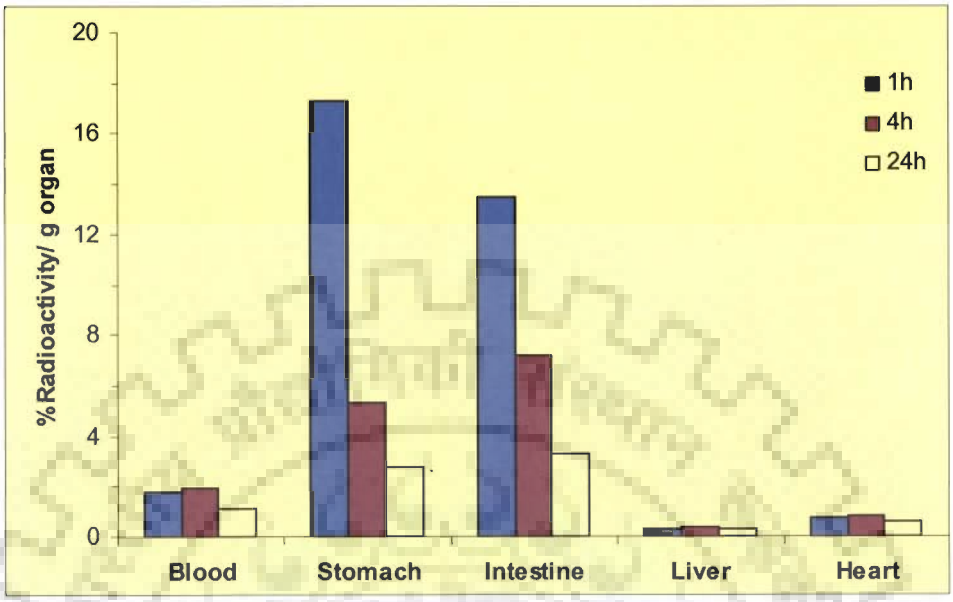

Figure 6.6: Biodistribution of free ^{99m}Tc labeled insulin per gram organ of animal

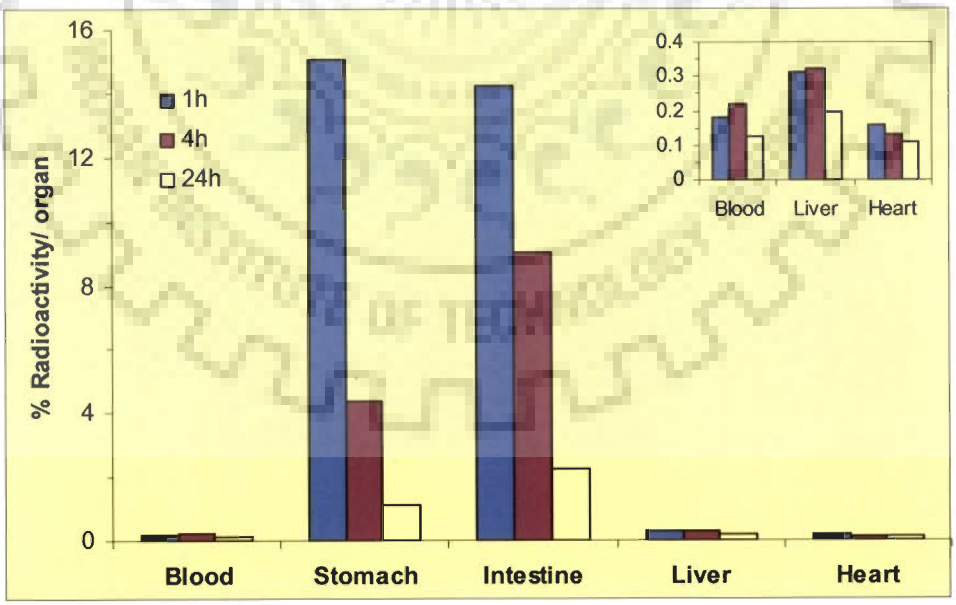

Figure 6.7: Biodistribution of free ^{99m}Tc labeled insulin per organ of animal

Experimental Animals: Sprague-Dawley rats weighing 280-330 gm (15-16 weeks old) and Male New Zealand rabbits weighing 2.5-3.0 kg were provided by experimental animal facility of INMAS, Delhi. The study protocol was reviewed and approved by Institutional Animal Ethics Committee.

6.2.2 Pharmacoscintigraphic studies

6.2.2.1 Radio labeling of insulin by ^{99m}Tc-pertechnetate as radionuclide

Insulin (100 IHU/ml) was radiolabeled with technetium-99m (^{99m}Tc), which is a suitable radiotracer for analytical procedures, because of its favorable physical properties. For the radiolabeling of insulin with technetium-99m, 10 ml of insulin (100 IU/ml) was mixed with 1 ml of stannous chloride (5mg/ml concentration solution in 0.1N HCl). The pH of the solution was kept between 5 and 6. Fifty mille baqurel (MBq) of $Na^{99m}TcO_4$ was added to it and the mixture was incubated for 15 minutes.

6.2.2.2 Evaluation of radiolabeling efficiency

The radiolabeling efficiency of the ^{99m}Tc-insulin radiocomplex was evaluated by Instant Thin Layer Chromatography (ITLC) using instant thin layer chromatography-silica gel (ITLC-SG) impregnated strips as stationary phase and 100% acetone as the mobile phase.

The ITLC-SG strips were cut into narrow strips of 1cm width and 10cm length. 2 µl sample (^{99m}Tc-insulin radiocomplex) was applied on one end of the strip 1 cm above the base. The strip was then allowed to run in the chamber containing 100% acetone, in ascending mode. When the solvent migrated to about 2/3rd of the strip's length, the experiment was discontinued and the strip was removed and air dried. This was followed by cutting the strip in two halves and each half was counted for the radioactivity retained within it. The free technetium moves with the solvent front and the reduced or hydrolyzed Tc, along with the conjugated one stays at the base (Saha, 1998). The radioactivity count left with the

lower half of the strip with respect to the total count provides the estimate of the fraction of insulin that was radiolabeled.

$$\%\ Radiolabeling = \frac{Radioactivity\ retained\ in\ the\ lower\ half\ of\ ITLC - SG\ strip}{Total\ radioactivity\ associated\ with\ the\ ITLC - SG\ strip} \times 100$$

6.2.2.3 In-vitro stability studies of insulin-^{99m}Tc radiocomplex

The ITLC procedure was used for the determination of the *in-vitro* stability of the ^{99m}Tc-insulin radiocomplex in human serum at different time intervals. About 50 µl of the radiocomplex was incubated with 450 µl of human serum at 37 °C. Aliquots at different time periods were applied on ITLC-SG strip and allowed to run in 100% acetone to check any dissociation or degradation of the labeled complex. The dissociation was estimated as the percent radiolabeled complex remaining after the incubation of 0 hour, 1 hour, 2 hours, 4 hours and 24 hours. The above process was repeated in saline.

$$\%\ Radiolabeled\ Complex\ Stability = \frac{Radioactive\ counts\ in\ the\ lower\ half\ of\ ITLC - SG\ strip\ at\ specified\ time}{Corresponding\ total\ counts\ of\ the\ ITLC - SG\ strip\ at\ that\ time} \times 100$$

6.2.2.4 ^{99m}Tc-insulin loading of poly-ε-caprolactone microspheres

Radiolabeled insulin loaded PCL microspheres were prepared by using w/o/w double emulsion solvent evaporation technique. An aqueous ^{99m}Tc labeled insulin solution (100 IHU/ml, 3 ml) was emulsified with 1% solution of PCL in dichloromethane by stirring at 6000 rpm for 2 minutes, using a mechanical stirrer (RQT-124A, Remi Motors, India). The water-in-oil emulsion, thus formed, was thereafter added to 30 ml of external aqueous phase (1% solution of HPMC) and again stirred at 4500 rpm for 150 minutes, resulting in the formation of the final w/o/w emulsion. To facilitate solvent evaporation, the final emulsion was magnetically stirred for about five hours. The microspheres, thus formed, were isolated

by centrifugation at 3000 rpm for 5-10 minutes. The microspheres were then washed with deionized distilled water and dried at room temperature.

6.2.2.5 Pharmacokinetics of ^{99m}Tc labeled insulin loaded microspheres

Diabetes was induced in the experimental rabbits by injecting a single dose of alloxan (150 mg/kg body weight) dissolved in sterilized water intravenously and their glucose levels were checked for diabetic conditions after 48 hrs (Srinivasan and Ramarao, 2007). Animals with glucose levels above ≥300mg/dl were only used in subsequent studies (Bendayan et. al., 1994).

Rabbits were chosen randomly and their body weights were measured. Free ^{99m}Tc-insulin (90 IU/kg) without loading in microspheres was orally administered to one group of animals and ^{99m}Tc-insulin loaded microspheres (90 IU/kg) were orally administered to the other group of animals. Blood samples (0.5 ml) were withdrawn from the ear vein of the animals at different time intervals of 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hours after oral administration. The samples were weighed accurately and radioactivity was measured using a scintillation counter, shown in Figure 6.2, which was earlier calibrated for ^{99m}Tc energy. Seven percent of the body weight of the animal is considered as the whole weight of the blood in the body and the data is expressed as radioactive counts per minute present in the whole blood of the body.

6.2.2.6 Biodistribution of ^{99m}Tc-insulin loaded microspheres

Biodistribution of ^{99m}Tc-insulin loaded microspheres and free ^{99m}Tc-insulin was carried out in healthy male *Sprague-Dawley* rats weighing about 220 ± 20 grams. To one group of animals, radiolabeled insulin loaded microspheres suspended in 1 ml of distilled water was administered orally. To the second group, free radiolabeled insulin was administered orally. Rats were then dissected at 1 hour, 4 hours and 24 hours post administration. Different

organs like the stomach, liver, small and large intestines, heart and blood was removed. Organs were made free of adhering tissues, washed in saline, dried, weighed and finally their radioactivity was measured in a scintillation counter. Data was expressed both as percent radioactivity per whole organ and percent radioactivity per gram organ.

6.2.2.7 Gamma camera imaging of ^{99m}Tc-insulin loaded microspheres in diabetic male New Zealand rabbits

Diabetes was induced in the experimental rabbits by injecting a single dose of alloxan (150 mg/kg body weight) dissolved in sterilized water intravenously and their glucose levels were checked for diabetic conditions after 48 hrs (Srinivasan and Ramarao, 2007). Animals with glucose levels above ≥300mg/dl were only used in subsequent studies (Bendayan et. al., 1994). Diabetic rabbits were selected randomly and divided in three groups. To the first animal group, ^{99m}Tc-insulin loaded microspheres were administered orally by force feeding. To the second group of animals, free ^{99m}Tc-insulin without loading in microspheres was administered similarly. To the third animal group, ^{99m}Tc-insulin loaded microspheres were orally administered alongwith 10 ml of ethanol solution as an enhancer. Gamma camera images of all the animals were taken using GE gamma camera (GE XR/T 4000 SPECT Gamma Camera, Germany) at different time intervals of 0.5 hour, 2 hours and 4 hours. Imaging was also done at 24 hours to determine the presence of any activity after 24 hours of administration.

6.2.2.8 Gamma camera imaging of ^{99m}Tc-insulin loaded microspheres in diabetic Sprague-Dawley rats

Diabetes was induced in the experimental animals by injecting a single dose of alloxan (140 mg/kg body weight) dissolved in saline water (0.9% NaCl solution in distilled water) intraperitoneally and their blood glucose levels were checked for diabetic conditions after 48

hrs (Rerup, 1970; Srinivasan and Ramarao, 2007). Animals with blood glucose level ≥300 mg/dl were only used for further studies. Diabetic rats were selected randomly and divided in three groups. To the first animal group, ^{99m}Tc-insulin loaded microspheres were administered orally by force-feeding. To the second group of animals, free ^{99m}Tc-insulin with out loading in microspheres was orally administered. To the third animal group, ^{99m}Tc-insulin loaded microspheres were orally administered alongwith 10 ml of ethanol solution as an enhancer. Gamma camera images of all the animals were taken using GE gamma camera (GE XR/T 4000 SPECT Gamma Camera, Germany) at different time intervals of 0.5 hour, 2 hours and 4 hours.

6.2.3 Toxicity studies

6.2.3.1 Conventional acute toxicity (LD50) test

Healthy male *Sprague-Dawley* rats were selected randomly and divided into three groups. Animals of first group were administered orally with 500mg/kg single dose of PCL microspheres suspended in 1 ml distilled water. Animals of second group were administered orally with 1gm/kg single dose of PCL microspheres suspended in 1 ml distilled water. Testing a higher dose than 1gm/kg body weight was also tried but it was very difficult to feed the animals. Animals of third group were taken as control and not fed with any polymeric microspheres. Toxicity testing was then carried out.

6.2.3.2 Repetitive dose toxicity

Healthy *Sprague-Dawley* rats (200±30gm body weight) were selected randomly and divided in three groups for repetitive dose toxicity studies. Animals of first group were fed orally with 500mg/kg dose of PCL microspheres without insulin twice daily for one month. Second group of animals were fed orally with 500mg/kg dose of PCL microspheres without insulin

twice daily for two months. Third group of animals were taken as control and not fed with any polymeric microspheres.

Animals with one month of continuous feeding and half of the animals from the control group were sacrificed and a small piece (5x10mm) of their liver, stomach, small intestine and large intestine were saved for histopathological studies. Similarly, animals with two months of continuous feeding and the remaining animals from control group were sacrificed and a small piece of liver, stomach, small intestine and large intestine of the animals were saved for histopathological studies.

6.2.3.3 Histopathological studies

6.2.3.3.1 Preparation of tissue samples for microtomy

All the samples of tissue were kept in separate vials containing 10 ml of formalin solution (HCHO 10% (w/v)) for 24 hours for the fixation of tissue. After fixation, dehydration of tissue were carried out for which samples were washed in tap water for 2 hours and were then subjected to increasing strength of alcohol i.e., 50%, 70%, 90% and 95% for 2 hours in each and finally left overnight in absolute alcohol. The samples were removed from absolute alcohol and dipped in xylene. After 2 hours, when alcohol was completely replaced by xylene, the tissue samples acquire a translucent appearance. Wax was impregnated in the tissue samples by subjecting the samples into mixture of xylene and wax in the ratio 3:1, then to another mixture of ratio 1:1 and finally to wax for 2 hours in each at temperature not exceeding 45^0C. This prevented tissue shrinkage and allowed good impregnation. For embedding of tissue samples, wax was melted completely (below 45^0C) and poured in between two Leukhart's "L" pieces. Tissue samples fully impregnated with wax were placed carefully in the molten wax inside "L" pieces. These were then placed in a refrigerator for solidification of the blocks. After solidification paraffin wax-embedded tissue blocks were carefully trimmed and labeled.

6.2.3.3.2 Microtomy and staining of tissue samples

The trimmed paraffin wax-embedded tissue blocks were mounted on moulds with the help of molten wax. These moulds were fitted on a rotary rocking microtome attached with a microtome knife having a very sharp edge. Thickness adjuster was set at 5μm. Ribbons of tissue sections were carefully collected and transferred to the clean slides containing albumin as adhesive. These slides were dried over a hot plate at a temperature not exceeding 45^0C.

The sections were rehydrated by passing the slides through xylene, decreasing strength of alcohol (absolute, 90%, 70%, 50%, 30%) and finally in water for 5 minutes in each. The sections were kept in haematoxylin and after 2 minutes, the sections were given three dips in acid alcohol (1%HCl and 70% ethanol) and then washed in running water to remove extra stain. The sections were neutralized by giving five dips in ammonia water and washing under running tap water. These sections were given three dips in eosin and then washed again to remove extra stain. The sections were again dehydrated by passing serially through 70% alcohol (3-4 dips), 90% alcohol (6-7dips), absolute alcohol (5 minutes) and finally xylene (15 minutes). The stained slides were observed under microscope fitted with an inverted camera.

6.3 RESULTS AND DISCUSSION

6.3.1 Radiolabeling efficiency and *in-vitro* stability studies

The radiolabeling efficiency of the insulin-^{99m}Tc radiocomplex was evaluated by ITLC using instant thin layer chromatography-silica gel impregnated strips. The efficiency of radiolabeling of insulin with ^{99m}Tc was found to be 99.2%.When the radiolabeled complex was incubated in saline at room temperature, there was no significant degradation of the radiocomplex.. Even after 24 hours, the complex was found to be very stable (97.3%) as shown in Figure 6.3. The insulin-^{99m}Tc radiocomplex was also incubated with serum up till 24 hours at 37°C and the stability of the complex was tested. As evident from Figure 6.4,

after 24 hours, the radiocomplex was found to be equally stable (97.25%). The above figures clearly demonstrate the high stability of the insulin-^{99m}Tc radiocomplex in both saline and serum. Therefore, it was established that serum did not produce any adverse effects on the stability of the compound.

6.3.2 Pharmacokinetics of ^{99m}Tc labeled insulin loaded microspheres

The pharmacokinetics of ^{99m}Tc labeled insulin, after oral administration of ^{99m}Tc-insulin loaded into the PCL microspheres to rabbits was studied. It was found that the clearance of radiolabeled insulin from blood was found to exhibit biphasic path as seen in Figure 6.5.

6.3.3 Biodistribution of ^{99m}Tc-insulin loaded microspheres

The biodistribution of free ^{99m}Tc-insulin and ^{99m}Tc-insulin loaded PCL microspheres in heart, blood, liver, stomach and intestine in *Sprague-Dawley* rats at various time intervals after its oral administration are shown in Figures 6.6 to 6.9. It was observed that among all the organs studied, stomach exhibited highest radioactivity per gram organ for both ^{99m}Tc-insulin loaded PCL microspheres and free ^{99m}Tc-insulin. On the contrary, intestine and stomach showed highest radioactivity per whole organ for ^{99m}Tc-insulin loaded microspheres and free ^{99m}Tc-insulin respectively. Radiolabeled insulin microspheres showed maximum per whole organ distribution in the intestine but free radiolabeled insulin concentrated mainly in the stomach. This can be explained as insulin, being a protein, undergoes very fast degradation in harsh condition of stomach when not protected by some formulation.

6.3.4 Gamma camera imaging of ^{99m}Tc-insulin loaded microspheres in diabetic Male New Zealand rabbits and *Sprague-Dawley* rats

Gamma camera imaging of diabetic male New Zealand rabbits administered with ^{99m}Tc-insulin loaded microspheres, free ^{99m}Tc-insulin and ^{99m}Tc-insulin loaded microspheres

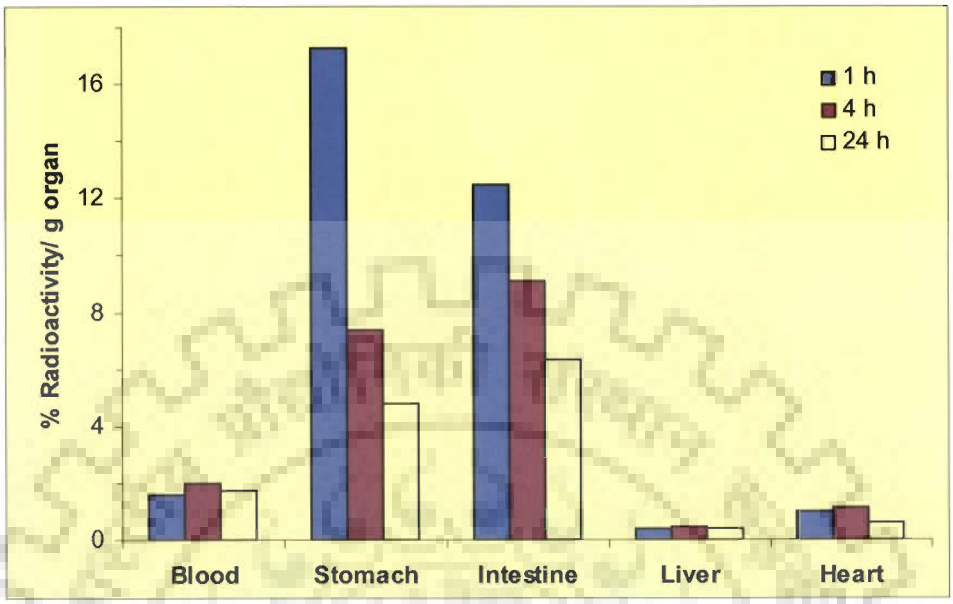

Figure 6.8: Biodistribution of ^{99m}Tc-insulin loaded PCL microspheres per gram organ of animal

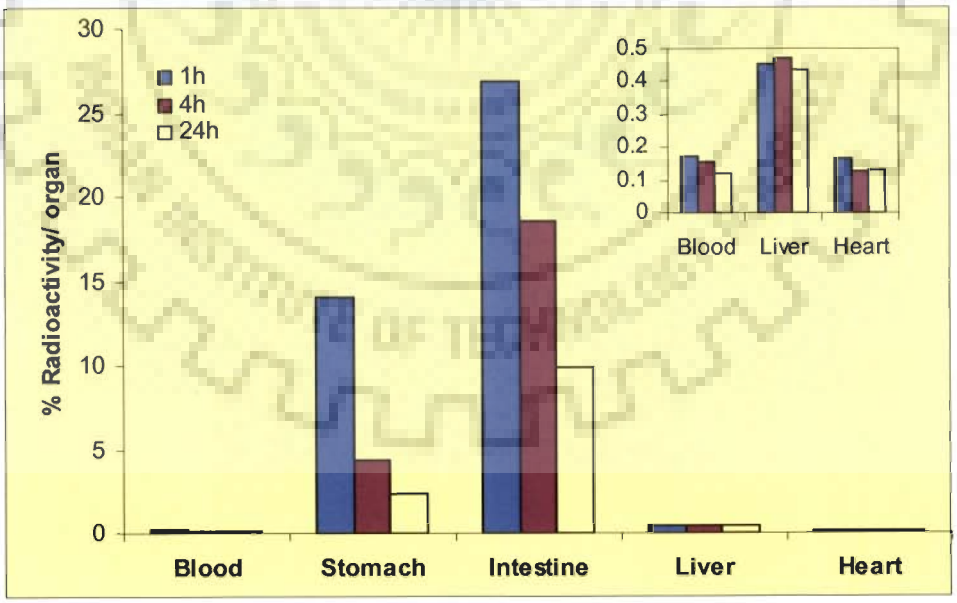

Figure 6.9: Biodistribution of ^{99m}Tc-insulin loaded PCL microspheres per organ of animal

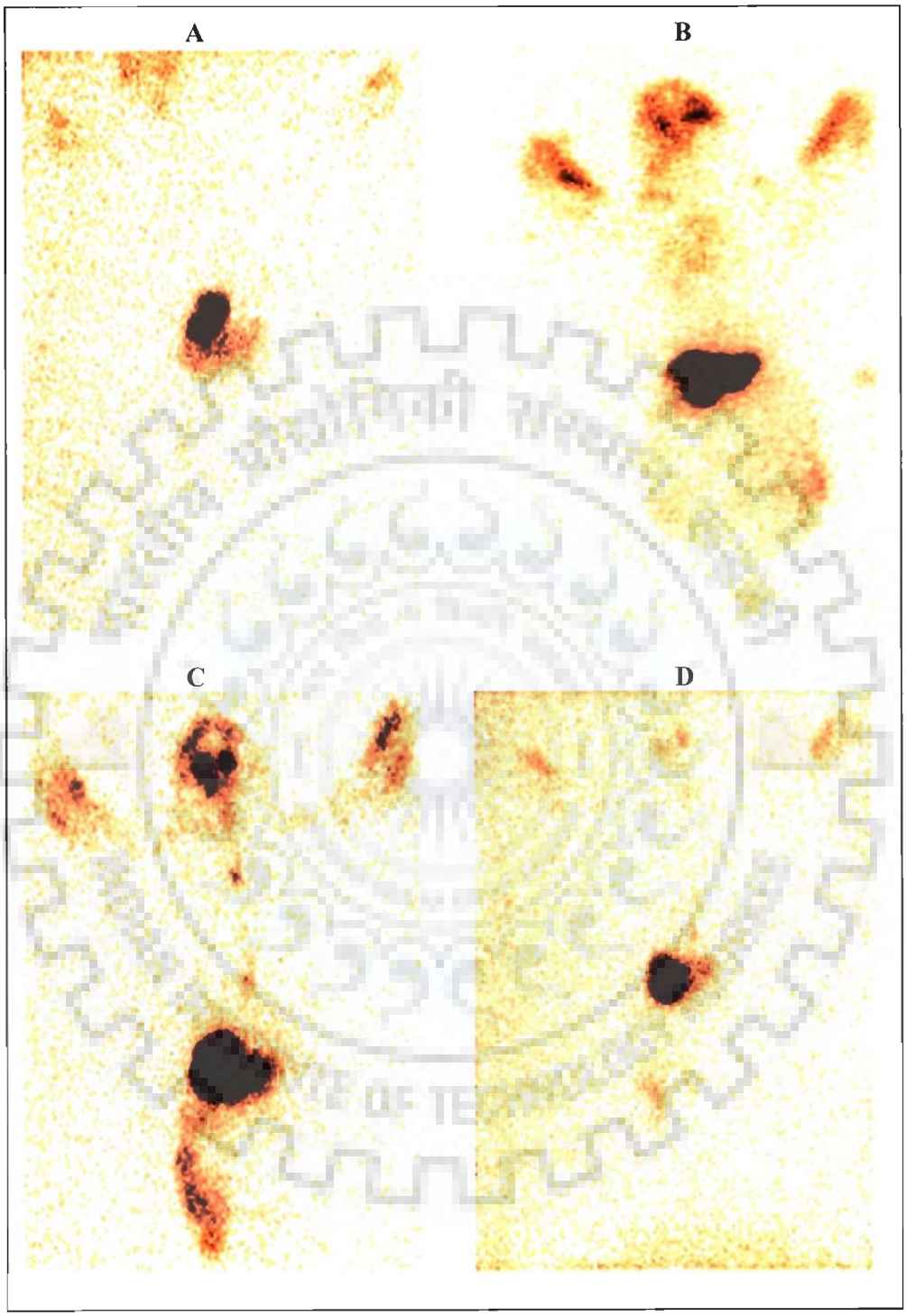

Figure 6.10: Gamma images of Male New Zealand rabbits administered with ^{99m}Tc-insulin loaded PCL microspheres at different time intervals; A: 0.5hour; B: 2 hours; C: 4 hours and D: 24hours

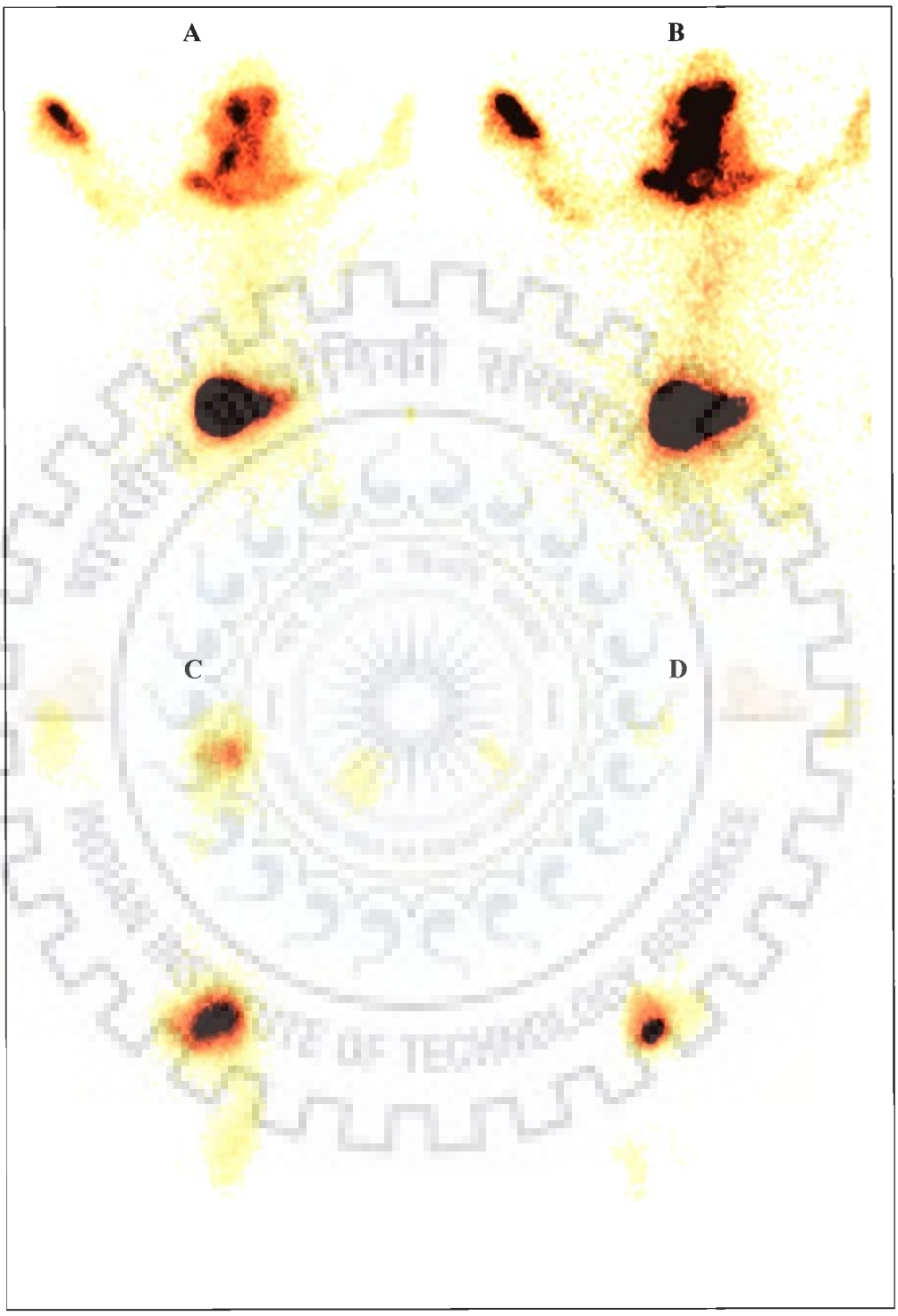

Figure 6.11: Gamma images of Male New Zealand rabbits administered with free ^{99m}Tc-insulin at different time intervals; A: 0.5hour; B: 2 hours; C: 4 hours and D: 24hours

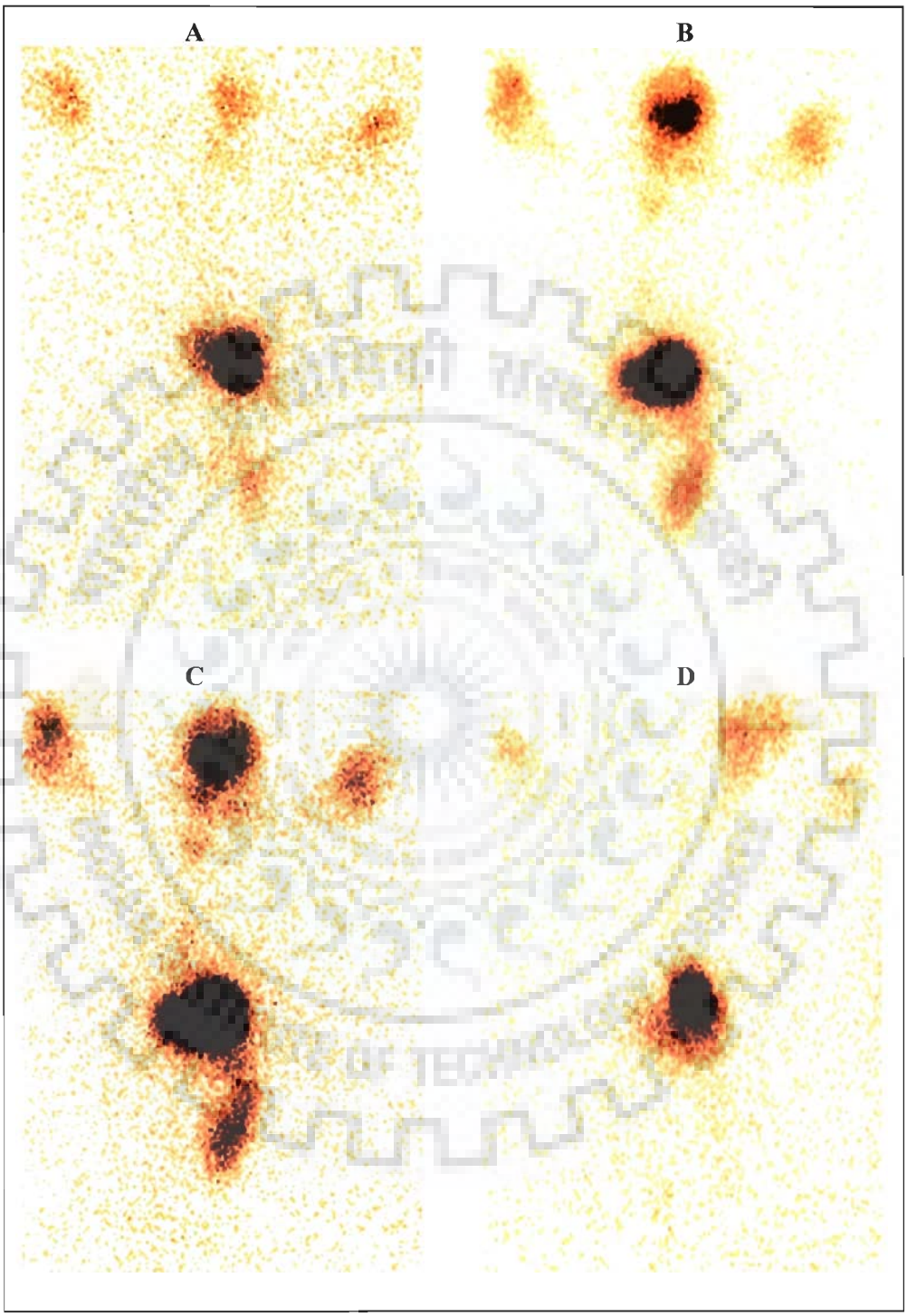

Figure 6.12: Gamma images of Male New Zealand rabbits administered with ^{99m}Tc-insulin loaded PCL microspheres along with enhancer at different time intervals; A: 0.5hour; B: 2 hours; C: 4 hours and D: 24hours

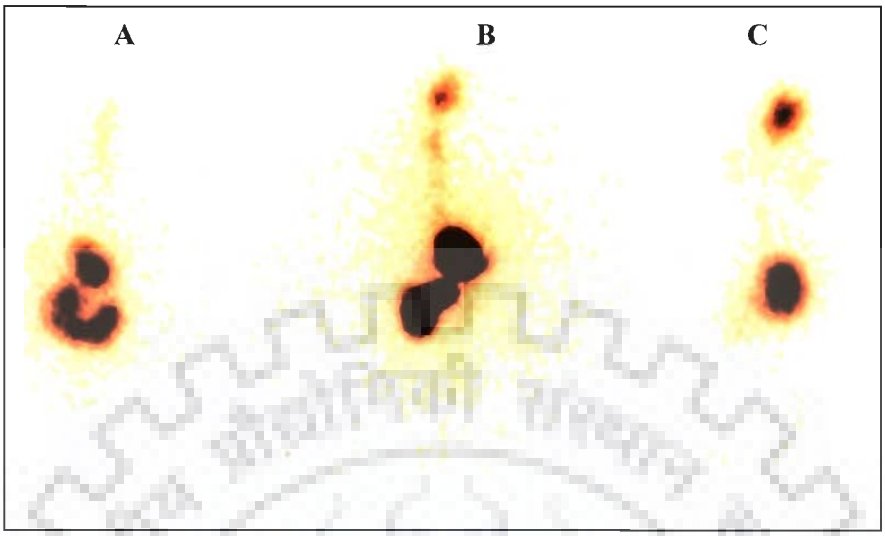

Figure 6.13: Gamma images of *Sprague-Dawley* rats administered with A: free ^{99m}Tc insulin, B: ^{99m}Tc-insulin loaded PCL microspheres with enhancer and C: ^{99m}Tc-insulin loaded PCL microspheres at 0.5 hour

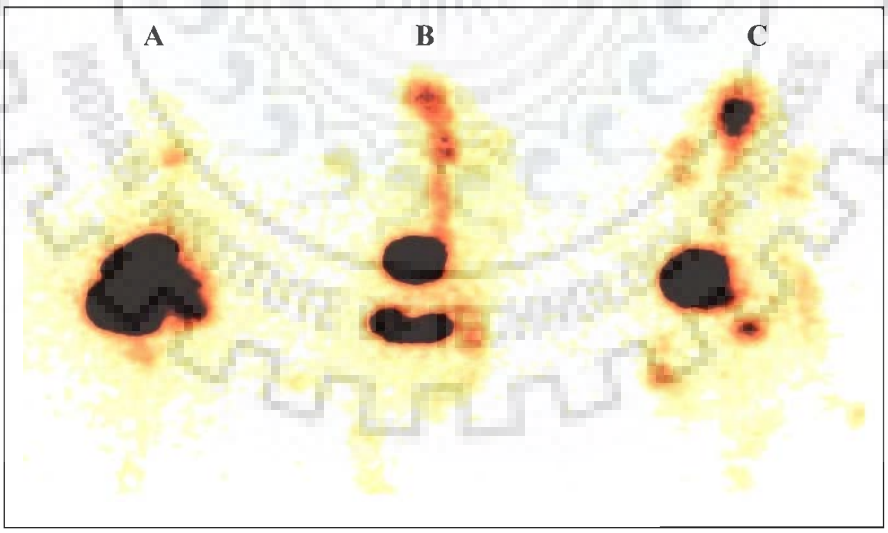

Figure 6.14: Gamma images of *Sprague-Dawley* rats administered with A: free ^{99m}Tc-insulin, B: ^{99m}Tc-insulin loaded PCL microspheres with enhancer and C: ^{99m}Tc-insulin loaded PCL microspheres at 2 hours

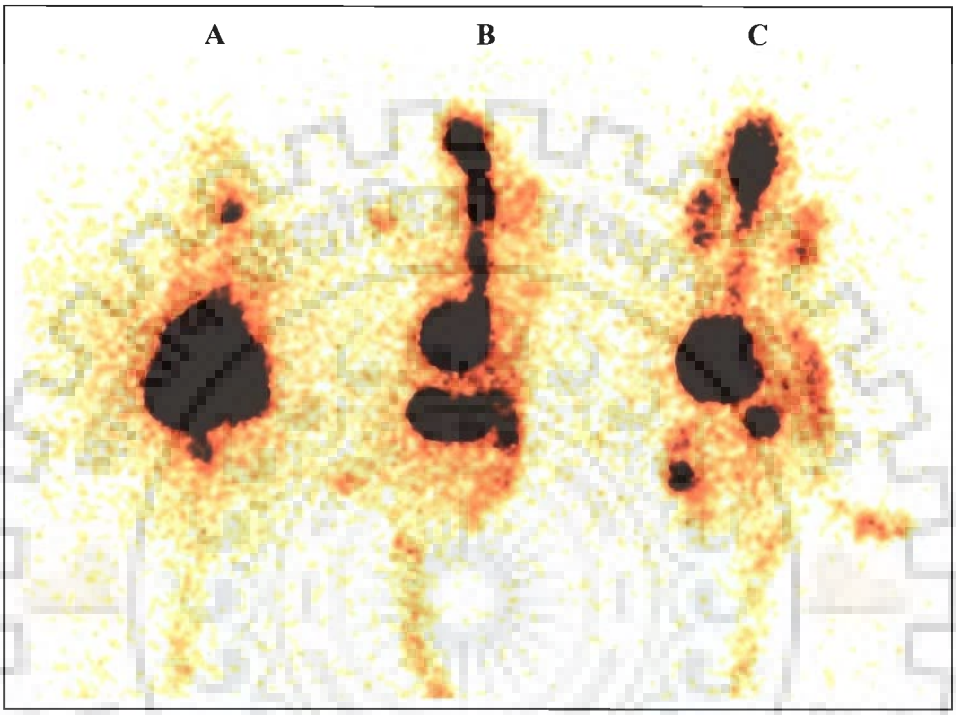

Figure 6.15: Gamma images of *Sprague-Dawley* rats administered with A: free ^{99m}Tc-insulin, B: ^{99m}Tc-insulin loaded PCL microspheres with enhancer and C: ^{99m}Tc-insulin loaded PCL microspheres at 4 hours

administered alongwith ethanol as enhancer was carried out. The images depict the variations in absorption and distribution characteristics.

Gamma camera imaging of all animals were carried out at different time intervals (0.5 hour, 2 hours, 4 hours and 24hours) and are shown in Figure 6.10-6.12(a to d). The animals fed with ^{99m}Tc-insulin loaded PCL microspheres showed the concentration of radioactivity in the stomach after 0.5 hour of oral administration, while negligible activity was observed into the surrounding tissues (Figure 6.10a). After 2 hours of administration of polymeric microspheres, good diffusion of the activity into the surrounding tissue and paws alongwith the stomach was observed, as seen in Figure 6.10b. After 4 hours of oral administration, this group of animals showed significantly higher diffusion into the intestine besides other tissues like paws and visceral organs, while the activity was also observed in stomach as depicted in Figure 6.10c. Gamma imaging of the animals was also done after 24 hours to ascertain remaining activity and it was found that small amount of radioactivity was detected in stomach even after 24 hours of administration of polymeric particles (Figure 6.10d). Decrease of radioactivity may be explained due to the short half life of ^{99m}Tc (6.02 hours).

The second group of animals were fed with free ^{99m}Tc-insulin without loading into PCL microspheres. The major concentration of activity was observed in buccal cavity and stomach at 0.5 hour as seen in Figure 6.11a. These animals showed more amount of diffusion of radioactivity into the body tissues after 2 hours of oral administration but both buccal cavity and stomach showed maximum radioactive concentration as depicted in Figure 6.11b. After 4 hours of oral administration, the animal showed some diffusion of radioactivity into the surrounding tissue alongwith the intestine but was concentrated mainly in the stomach region of the animal (Figure 6.11c). There Figure 6.11d shows negligible amount of activity at the end of 24 hours in the animal. The findings are in accordance with literature as insulin, being a protein molecule, undergoes very fast degradation in harsh condition of stomach if not protected by any formulation.

The third group of animals fed with the ^{99m}Tc-insulin loaded into the PCL microspheres alongwith 10 ml of ethanol as enhancer also showed the concentration of radioactivity into the stomach after 0.5 hour of oral administration, while negligible activity was observed into the surrounding tissues (Figure 6.12a). The animals after 2 hours of administration showed good diffusion of activity in the surrounding tissues, in the intestine as well as in the paws besides the stomach (Figure 6.12b), due to movement of ^{99m}Tc-insulin loaded PCL microspheres into the intestine. After 4 hours of oral administration, the animals showed significantly higher diffusion into the intestine, paws and other visceral organs of animal body, while the activity was also observed in stomach as seen in Figure 6.12c. There was appreciable amount of activity even at the end of 24 hours (Figure 6.12d), in this set of animals.

The movement of ^{99m}Tc-insulin loaded microspheres from stomach to small intestine was very clearly observed in this group of animals. Similar experiments were also carried out in *Sprague-Dawley* rats in a similar fashion and gamma images were taken at time intervals of 0.5 hour, 2 hours and 4 hours. The images were found to be comparable to the ones obtained from those of male New Zealand rabbits as shown in Figures, 6.13 to 6.15 (a to c).

6.3.5 Toxicity studies

6.3.5.1 Conventional acute toxicity (LD50) test

Animal of all three groups survived for two months till the end of the experiment. The body weight and behavior of the experimental animals were observed and found to be the same as that of control animals. LD50 for rats for PCL microspheres was found to be above 1gm/kg body weight.

6.3.5.2 Repetitive dose toxicity

There were no changes observed in the bodyweight and behavior of the animals undergoing repetitive dose toxicity studies till two months. All animals showed similar behaviour including the control animals. The histopathological slides of various organs of the gastrointestinal tract were studied and are reported below.

5.3 Histopathological studies

Histopathological studies of liver, stomach, small intestine and large intestine were carried out for establishing any repetitive dose toxicity associated with the PCL microspheres. Slides were prepared from the stomach, small intestine, large intestine and the liver (5x10mm) and stained and are as shown in Figures 6.16 to 6.31.

Stomach: The sections from the stomach (Figures 6.16 to 6.19) showed normal folds present in the transverse mucosa of stomach, normal surface epithelium and normal gastric glands. The *lamina propria* was composed of loose connective tissue interspersed with smooth muscle and lymphoid cells. Separating the mucosa from the underlying sub-mucosa was the layer of smooth muscle, the *muscularis mucosae*. All the cells (mucus, oxyantic and zymogen cells) appeared to be normal in shape and embedded in mucosa. The gastric epithelium and the gastric glands were within the normal histological limits.

Small Intestine: The sections of the small intestine (Figures 6.20 to 6.23) were circular, consisting of mucosa and sub-mucosa. The intestinal villi were well preserved projecting into the lumen. Between the villi, there were simple tubular glands which were normal. The smooth muscle layers did not show any histological changes.

Large Intestine: The sections from the large intestine (Figures 6.24 to 6.27) showed long intestinal glands, which showed the goblet and absorptive cells. The lining epithelium was columnar and had short irregular microvilli. The crypts of lieberkuhn were well preserved. The *muscularis mucosae* and the muscle layer were within normal limits.

Liver: The sections from the liver showed (Figures 6.28 to 6.31) normal hepatic architecture. The hepatocytes were arranged in cords and there was no evidence of toxicity in hepatic cells. The central vein showed congestion and was undialated. The kuffer cells were normal and there was no increase in the number of kupffer cells. The endothelial cells of sinusoids were also seen as normal. The branch of hepatic artery, biliary duct and portal vein were within normal histological limits. It is clearly evident from the histopathological studies of stomach, liver, small intestine and large intestine that even after two month of repetitive dose administration of the PCL microspheres to experimental animals, no toxic effect was seen. Thus, it may be concluded that two month of repetitive dose of microspheres to experimental animals showed no adverse effect on histopathology of the tissues studied.

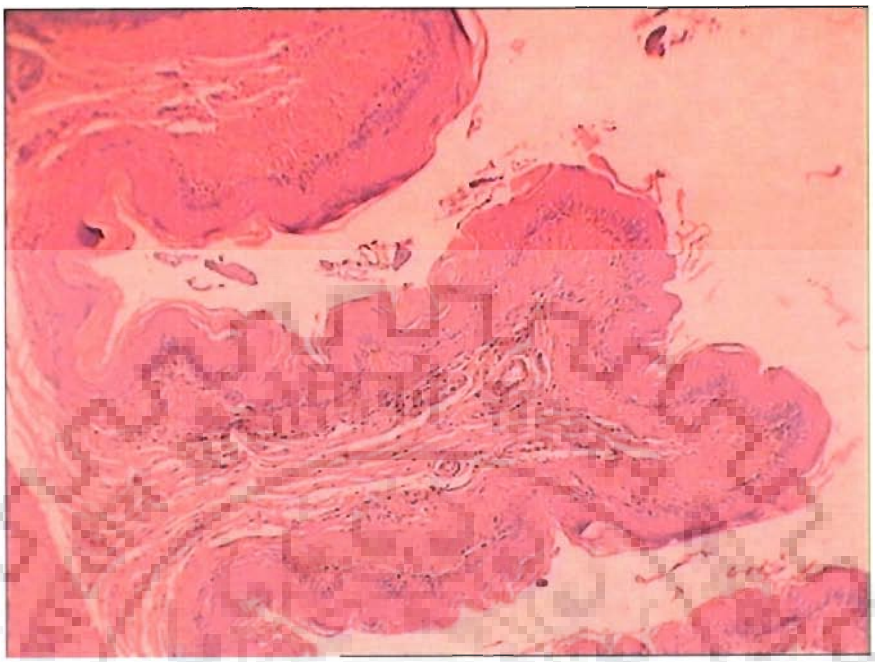

Figure 6.16: Histopathological slide showing a section of stomach of control animal (10X)

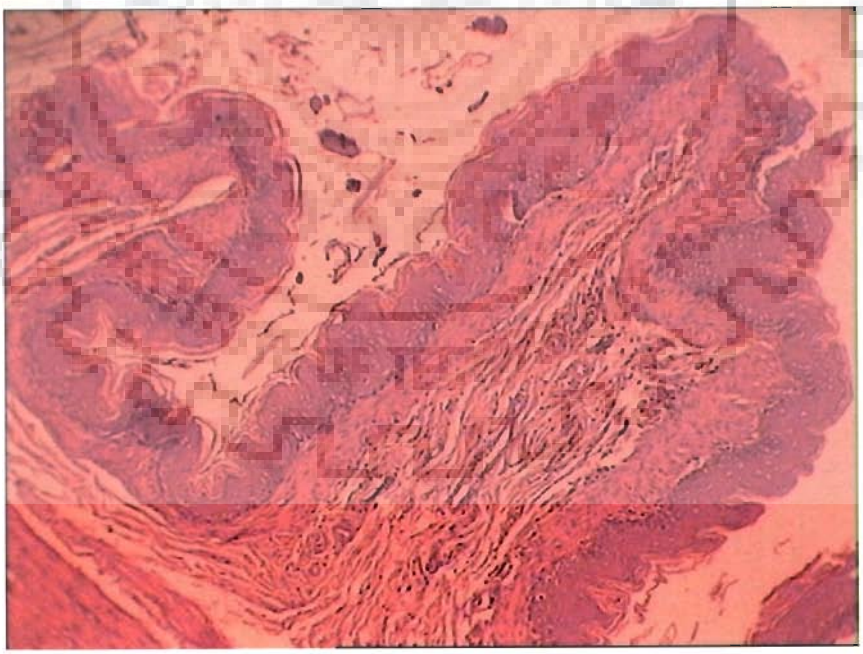

Figure 6.17: Histopathological slide showing a section of stomach of animal fed with PCL microspheres for 1 month (10X)

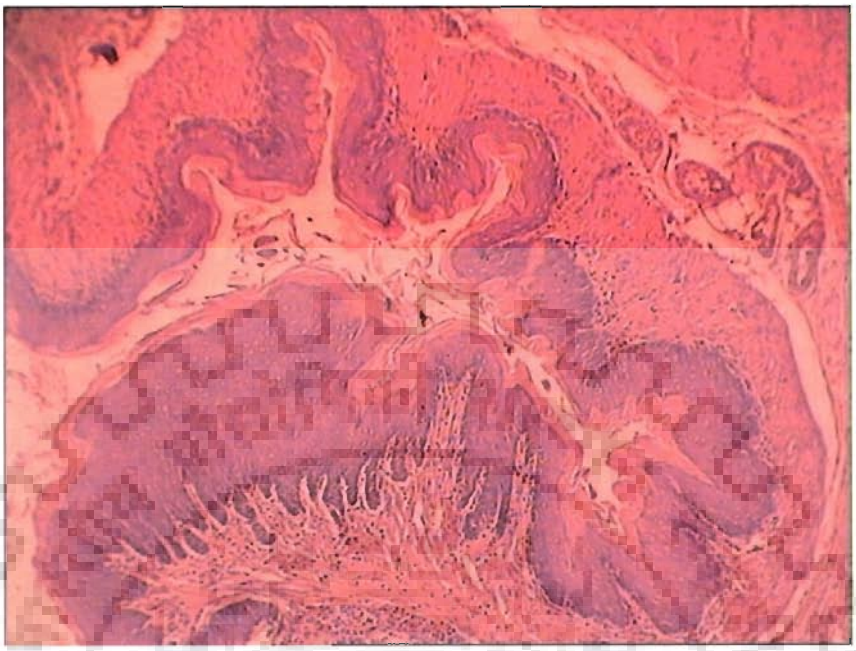

Figure 6.18: Histopathological slide showing a section of stomach of animal fed with PCL microspheres for 2 months (10X)

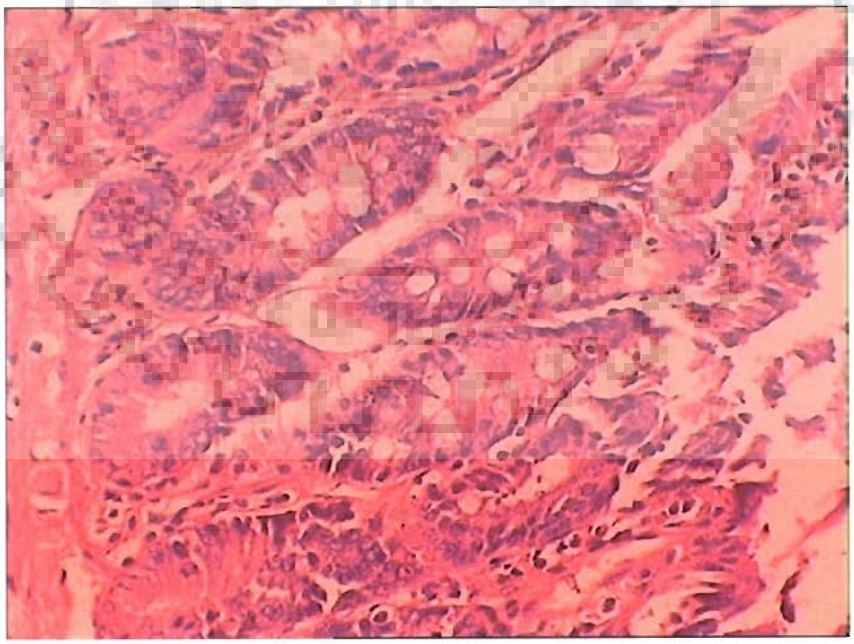

Figure 6.19: Histopathological slide showing a section of stomach of animal fed with PCL microspheres for 2 months (40X)

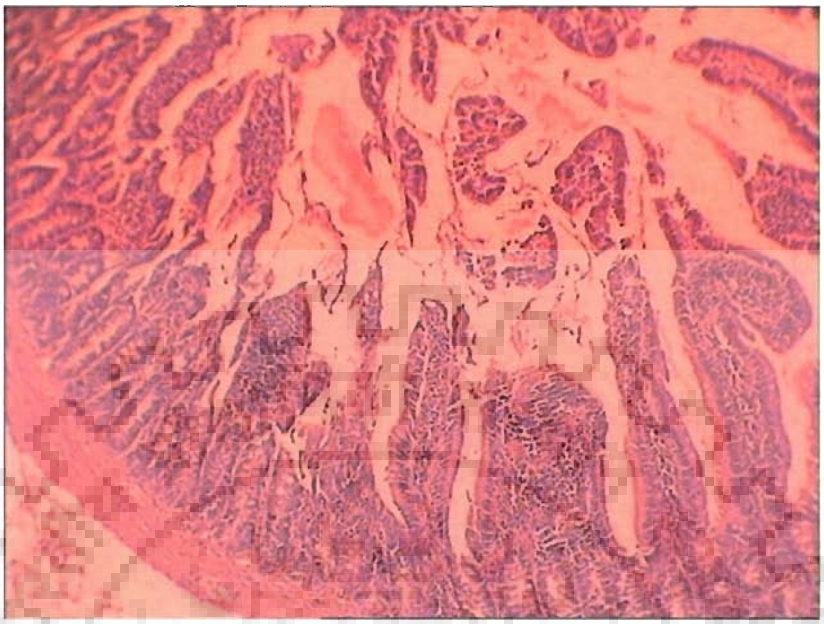

Figure 6.20: Histopathological slide showing a section of small intestine of control animal (10X)

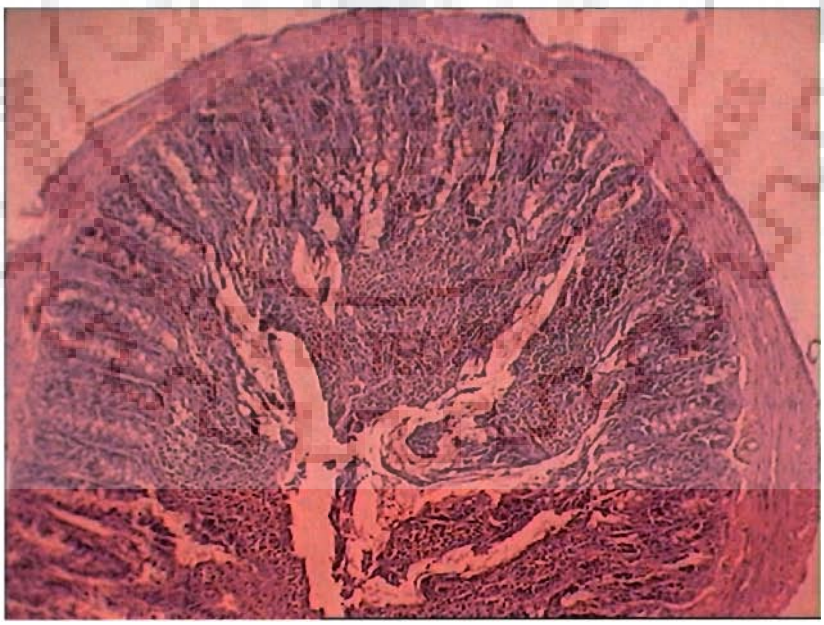

Figure 6.21: Histopathological slide showing a section of small intestine of animal fed with PCL microspheres for 1 month (10X)

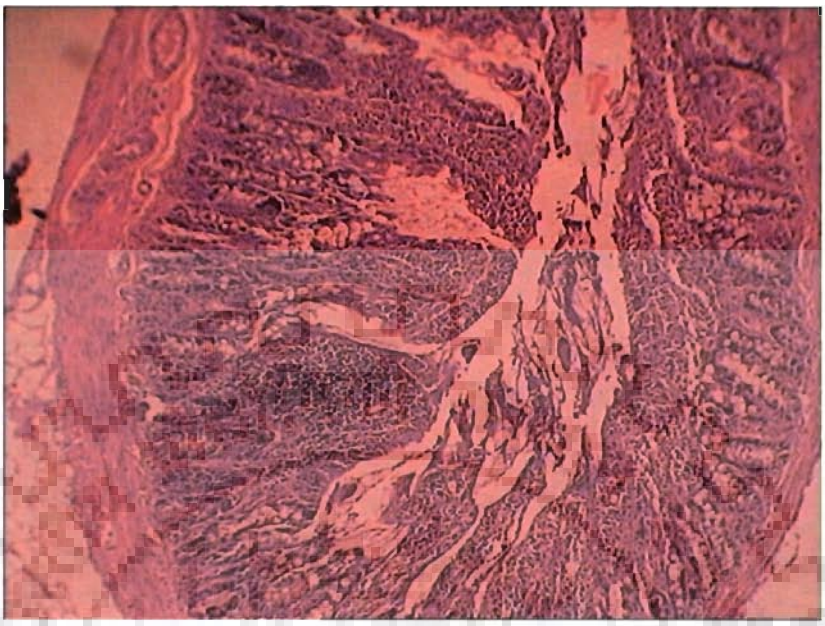

Figure 6.22: Histopathological slide showing a section of small intestine of animal fed with PCL microspheres for 2 months (10X)

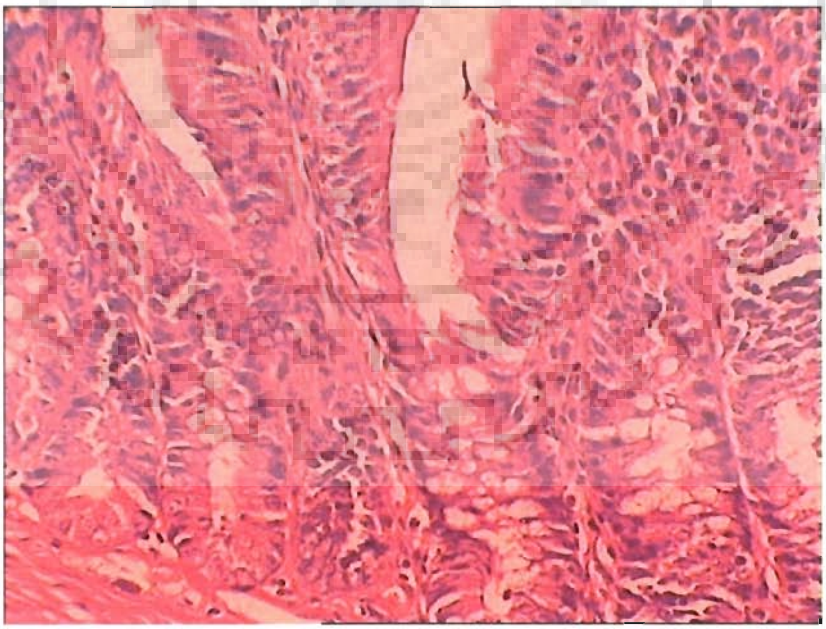

Figure 6.23: Histopathological slide showing a section of small intestine of animal fed with PCL microspheres for 2 month (40X)

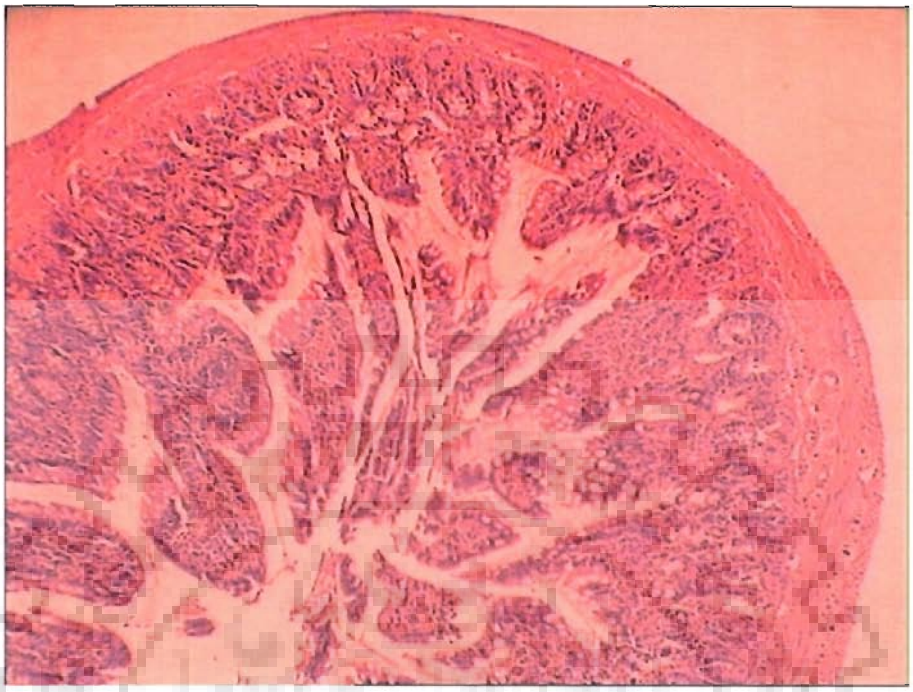

Figure 6.24: Histopathological slide showing a section of large intestine of control animal (10X)

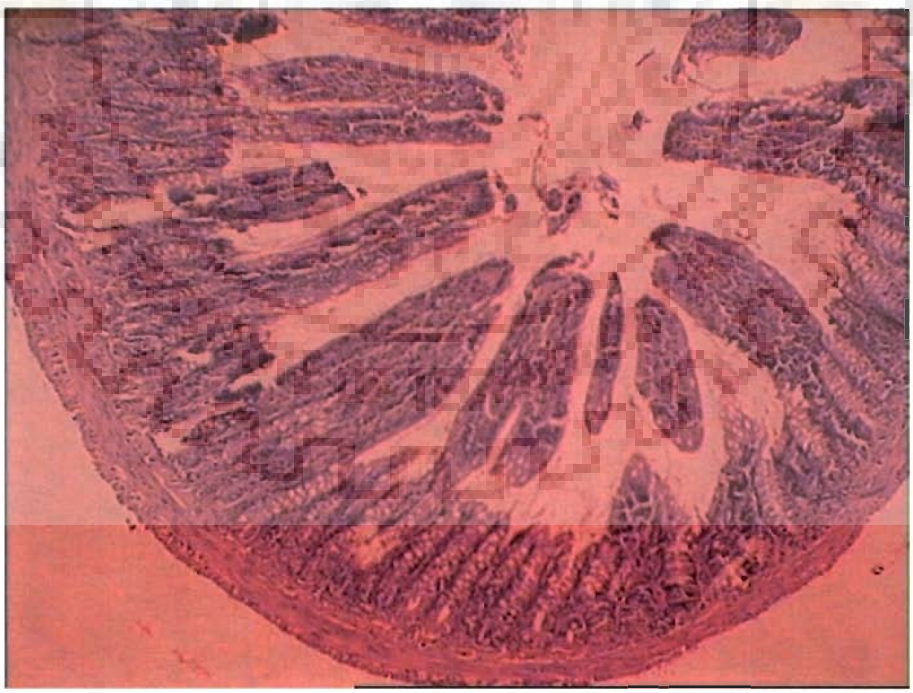

Figure 6.25: Histopathological slide showing a section of large intestine of animal fed with PCL microspheres for 1 month (10X)

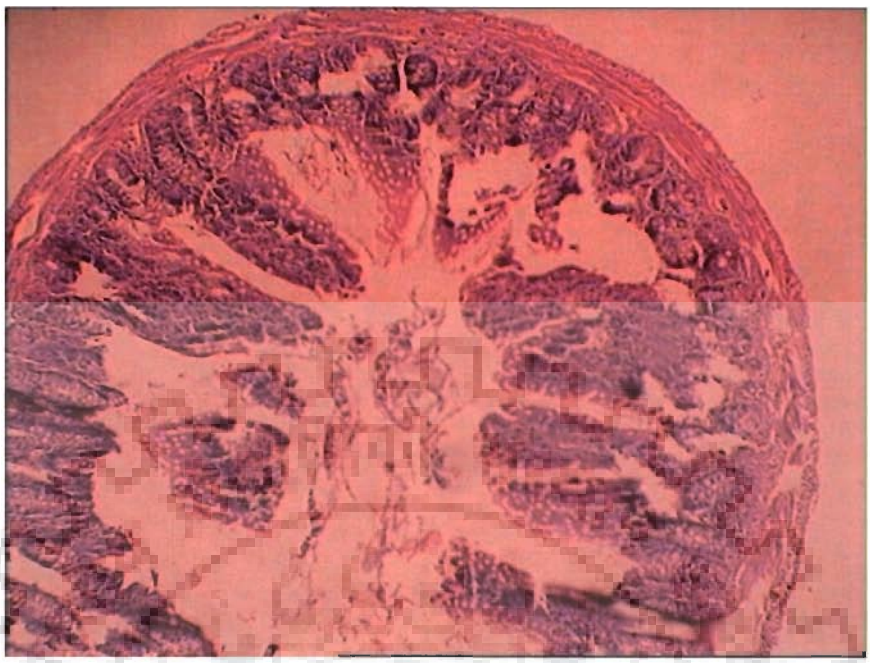

Figure 6.26: Histopathological slide showing a section of large intestine of animal fed with PCL microspheres for 2 months (10X)

Figure 6.27: Histopathological slide showing a section of large intestine of animal fed with PCL microspheres for 2 months (40X)

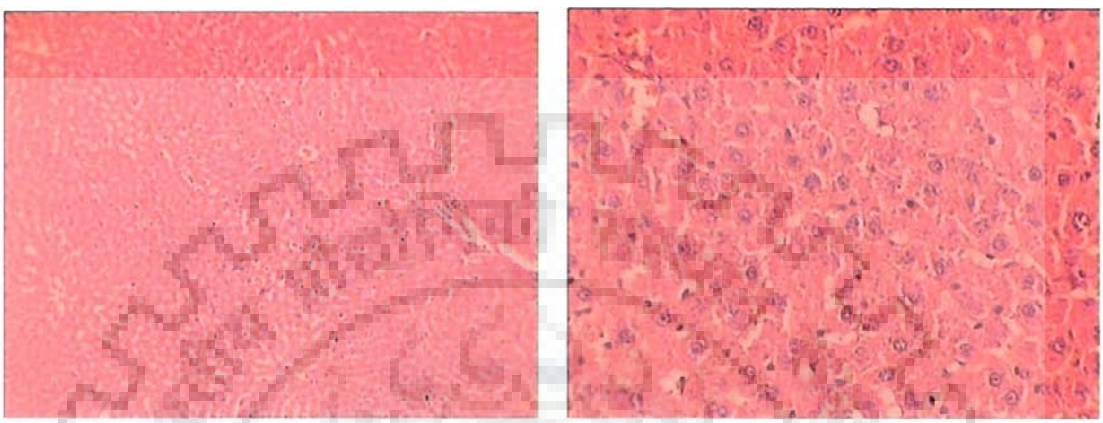

Figure 6.28: Histopathological slides showing a section of liver of control animal (10X and 40X)

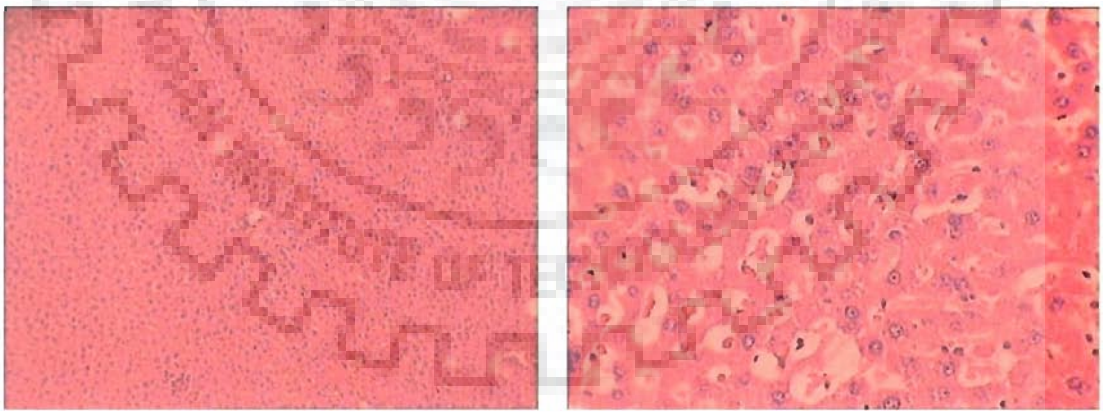

Figure 6.29: Histopathological slides showing a section of liver of animal fed with PCL microspheres for 1 month (10X and 40X)

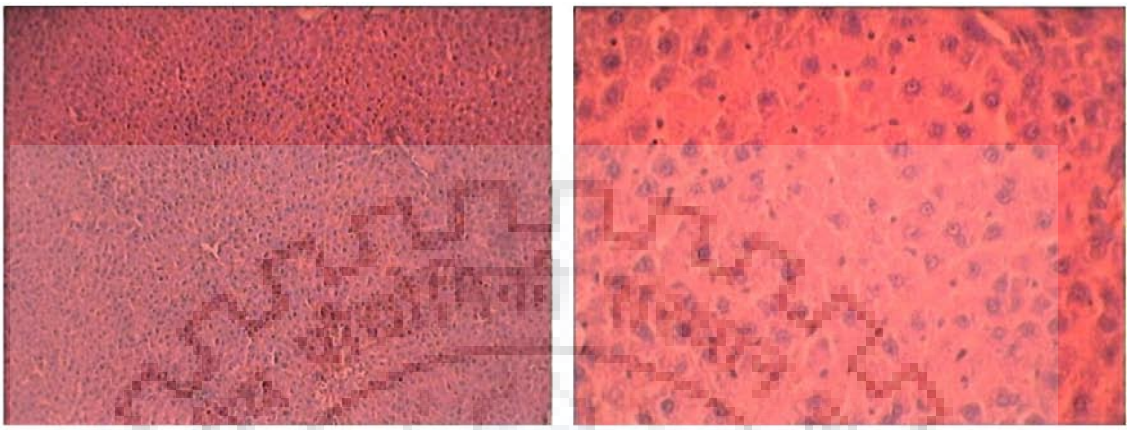

Figure 6.30: Histopathological slides showing a section of liver of animal fed with PCL microspheres for 2 months (10X and 40X)

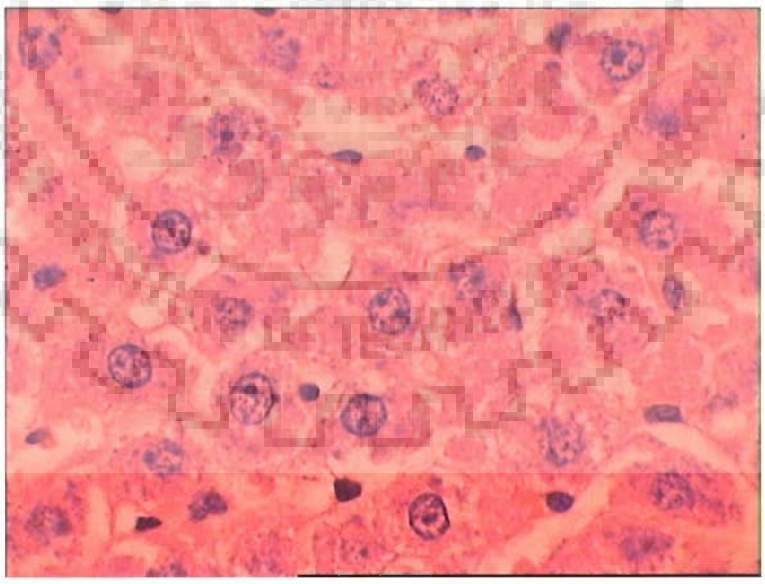

Figure 6.31: Histopathological slide showing a section of liver of animal fed with PCL microspheres for 2 months (100X)

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Diabetes is a widespread disease affecting almost 300 million people in the world. The parenteral route of administration is the only available route of insulin delivery and the treatment regimen for chronic diabetics includes upto three subcutaneous injections in a day. But parenteral administration is painful and therefore, not very patient compliant. Also there is an increased chance of infection at the site of administration. In order to develop a painless delivery systems to replace injectable dosage forms, alternative routes for administration of insulin like the nasal, transdermal and oral, have been extensively investigated. But each of these routes is associated with some limitations. The nasal route of administration is difficult to manipulate as the physiological conditions existing at the nasal epithelium may alter during various disease conditions. The prolonged use of the formulations containing proteins and peptides may totally disrupt the nasal mucosa and cilia, making this route highly unsuitable for chronic administration of drugs. Skin being an immunocompetent organ, transdermal delivery of macromolecular protein and peptide drugs causes irritancy and allergy. Also hydrophilic drugs are very difficult to deliver by this route as they donot easily permeate the skin. Oral route of delivery donot have these limitations but there are certain other challenges associated with it. They include the harsh acidic environment of the stomach, protein and peptide degradation by the digestive enzymes and the epithelial barriers of the gastrointestinal tract. Despite these challenges, oral route is a preferred route of administration if it can assure reproducible and predictable bioavailibilities. Many different strategies have been attempted to develop a biologically active oral insulin formulation by researchers world over.

The present study was aimed at the formulation of polymer based microspheres encapsulating insulin for oral delivery.

Our study began with the preformulation studies by establishing a valid analytical procedure using the micro BCA assay for the estimation of insulin. Based on the literature, poly-ε-caprolactone (PCL) was chosen as the biodegradable polymer for the formulation of the microspheres. The w/o/w double emulsion technique was selected among other incorporation methods as better protein stability and higher microencapsulation of proteins drugs within microspheres have been reported with this technique. Formulation of the PCL microspheres by using the w/o/w double emulsion solvent evaporation technique was successful.

Characterization of the PCL microspheres obtained by double emulsion solvent evaporation technique was carried out. The effect of various formulational parameters such as solvents and stabilizers, polymer concentration, stabilizer concentration and drug concentration was studied. It was concluded that dichloromethane was the best suited solvent for the organic phase. The stabilizers were found to affect the surface morphology of the microspheres considerably. Smooth and spherical microspheres were formed with HPMC as the stabilizer whereas using PVA resulted in pitted microspheres. Therefore, HPMC was chosen over PVA as the stabilizer. The microspheres were characterized for their percent yield and percent encapsulation efficiency. Surface morphology was determined by scanning electron microscopy and particles size analysis was determined by laser diffractometry. The batch of microspheres prepared with 1% PCL, 100 IHU/ml drug concentration and 1% HPMC concentration was found to give optimum results in terms of percent yield, percent encapsulation efficiency, surface morphology and particle size. The percent yield and percent encapsulation efficiency was found to be 92.2 ± 0.26% and 65.72 ± 0.13% respectively. The SEM scans revealed smooth and spherical microspheres. The particle size of the microspheres was found to be 2.33μm. Therefore, the optimum concentration of the polymer and the stabilizer (HPMC) was determined at 1% and the drug concentration chosen was 100 IHU/ml for the formulation of the microspheres.

To study the drug-distribution within the microspheres, drug–polymer interaction, surface topography and the amount of residual solvent present advanced characterization techniques like the confocal laser scanning microscopy, differential scanning calorimetry, atomic force microscopy and gas chromatography was used. The CLSM results clearly revealed the insulin distribution within the microspheres. The DSC results showed that that there was no interaction between insulin and PCL. The polymer, PCL, with a T_m of 66 °C, is tough and flexible with high permeability to low molecular species at body temperature. Also, the amount of residual solvent, dichloromethane, was well within the acceptable pharmacopoeial limit indicating safe use of microspheres for oral administration.

The PCL microspheres were evaluated for their *in-vitro* insulin release and *in-vivo* hypoglycemic effects. The microspheres were characterized by a more significant initial release (amounting to 10%), followed by a slower release (about 60% in 30 days). The initial burst release may be due to the drug desorption from the particle surface. This was then followed by the gradual release phase, resulting from the diffusion of the drug dispersed into the polymer matrix. Since PCL is a slow degrading polymer, the predominant mechanism of insulin release from these microspheres was diffusion. The effect of polymer, stabilizer and drug encapsulation concentration on the *in-vitro* release profiles was studied. It was found that microspheres with higher polymer concentration (1.5% and 2%) showed the least drug release. Microspheres fabricated with a low stabilizer concentration in the external water phase have a more rapid insulin release. A correlation was also established between the drug concentration, encapsulation efficiency and the release rate. A higher insulin concentration resulted in higher insulin encapsulation within the microsphere which further led to a more rapid release rate. This is because high insulin encapsulation resulted in a larger concentration gradient between the microspheres and the *in-vitro* release medium.

The biological efficacy alongwith the hypoglycemic action of the insulin-loaded microspheres was determined after oral administration in diabetic *Sprague Dawley* rats and

male New Zealand rabbits. In order to determine and compare the hypoglycemic effect of orally administered insulin-loaded PCL microspheres with free insulin administered subcutaneously, insulin-loaded polymeric microspheres were administered orally and free insulin was administered subcutaneously to overnight fasted diabetic rats. Blood glucose concentrations were measured by AccuChek® blood glucometer (Roche, Germany) and the changes in blood glucose level versus time profiles were observed. In case of the subcutaneous administration of free insulin, the glycemia decreased significantly after 30 minutes, the maximal decrease being reached after 1 hour. When insulin entrapped microspheres were administered orally, the glycemia decreased to a lesser extent and its effect was observed between 2 to 12 hours post administration, with the maximal decrease noted from 4 to 8 hours. In diabetic *Sprague Dawley* rats, 90 IU/kg insulin-loaded microspheres appreciably reduced glycemia by about 45% ($p<0.05$) after 6 hours of the administration. In fasted diabetic male New Zealand rabbits, insulin-loaded microspheres reduced the blood glucose level by 38% ($p=<0.001$) after 4 to 6 hours of the treatment. In case of fed diabetic animals, lower reduction of blood glucose level was observed. This may be due to the continuous absorption of glucose from the gastrointestinal tract. In these experiments also, the hypoglycemia lasted for at least 6-8 hours after oral administration of PCL microspheres, which confirmed the prolonged release of active insulin from the polymeric microspheres. The results showed that the encapsulation of insulin into microspheres prepared with PCL allowed the preservation of its biological activity after oral administration alongwith its prolongation of action.

Pharmacoscintigraphic studies were carried out to further evidence our *in-vivo* results. Radiometric detection of orally administered drugs labeled with a suitable radiotracer is believed to be the best technique to trace the exact path and morphology of the drug and the efficiency of formulation to deliver the drug at desired place in the gastrointestinal tract. Insulin was radiolabeled with a suitable radiotracer, ^{99m}Tc. The radiolabeling efficiency was

evaluated by ITLC and was found to be 99.2%. The radio-complex stability in saline and serum was determined and found to be 97%. The ^{99m}Tc-insulin was formulated into the PCL microspheres and orally fed to the experimental animals. Gamma camera imaging of diabetic Male New Zealand rabbits administered with ^{99m}Tc-Insulin loaded microspheres, free ^{99m}Tc-Insulin and ^{99m}Tc-Insulin loaded microspheres administered alongwith ethanol as enhancer was carried out. Images showed that after 4 hours of oral administration, animals fed with ^{99m}Tc-Insulin loaded into the PCL microspheres showed significantly higher diffusion into the stomach and intestine besides some diffusion in other tissues like paws and visceral organs as compared to those fed with free ^{99m}Tc-insulin without loading into PCL microspheres which showed major concentration of activity in buccal cavity and stomach till 2 hours after oral administration only. Images also showed that animals fed with ^{99m}Tc-insulin loaded into the PCL microspheres with ethanol showed similar but enhanced results than those seen with the administration of the microspheres alone. Biodistribution studies of free ^{99m}Tc-insulin and ^{99m}Tc-insulin loaded PCL microspheres was carried out in organs like the stomach, liver, intestine, heart and blood in *Sprague-Dawley* rats at various time intervals after its oral administration. It was observed that among all the organs studied, stomach exhibited highest radioactivity per gram organ for both ^{99m}Tc-insulin loaded PCL microspheres and free ^{99m}Tc-Insulin. On the contrary, intestine and stomach showed highest radioactivity per whole organ for ^{99m}Tc-insulin loaded microspheres and free ^{99m}Tc-insulin respectively. Therefore, absorption and distribution studies of the radiolabeled insulin loaded PCL microspheres were successfully ascertained by pharamcoscintigraphy.

To determine any toxic effects of the insulin-loaded PCL microspheres, toxicity studies were carried out in *Sprague-Dawley* rats. Histopathological studies of liver, stomach, small intestine and large intestine were carried out. It is evident from the histopathological studies that even after two month of repetitive dose administration of the microspheres to experimental animals, no toxic effect was seen.

All these studies and results led to the following conclusions:

- *Formulation of the poly-ε-caprolactone microspheres by using the w/o/w double emulsion solvent evaporation technique was successful.*
- *The poly-ε-caprolactone microspheres prepared were well characterized in terms of percent yield, percent encapsulation efficiency, surface morphology and particle size.*
- *The poly-ε-caprolactone microspheres showed moderate encapsulation and distribution of insulin within the microspheres with no insulin-PCL interaction and an acceptable amount of residual solvent indicating safe use of microspheres for administration.*
- *The in-vitro release profile of insulin from the poly-ε-caprolactone microspheres showed an initial release, followed by a slower release of insulin due to diffusion from the microspheres.*
- *The bioactivity or the hypoglycemic effect of insulin encapsulated in the poly-ε-caprolactone microspheres was retained as demonstrated by the in-vivo studies.*
- *The absorption and distribution studies of the radiolabeled insulin loaded poly-ε-caprolactone microspheres were successfully ascertained by pharamcoscintigraphy.*
- *Histopathological studies showed that poly ε-caprolactone microspheres were non-toxic.*

Therefore, poly ε-caprolactone microspheres are capable of delivering insulin over prolonged periods after oral administration, making them a potential candidate for oral insulin delivery.

Future Scope of Work:

- Increasing the encapsulation efficiency of insulin within the microspheres and using a combination of strategies like use of safe penetration enhancer, muco-addhesive properties, use of protease inhibitor in order to reduce the dose required to control the blood glucose level and elicit better therapeutical response may be further researched.

- The hypoglycemic effect of insulin loaded poly-ε-caprolactone microspheres was established only in rats and rabbits. Further research to determine its hypoglycemic effect in large animals like monkeys and finally human clinical trial may be envisaged.

- Evaluation of the long term toxicity and immunological response of the insulin loaded poly-ε-caprolactone microspheres in animals is necessary before carrying out human clinical trials.

APPENDIX-I

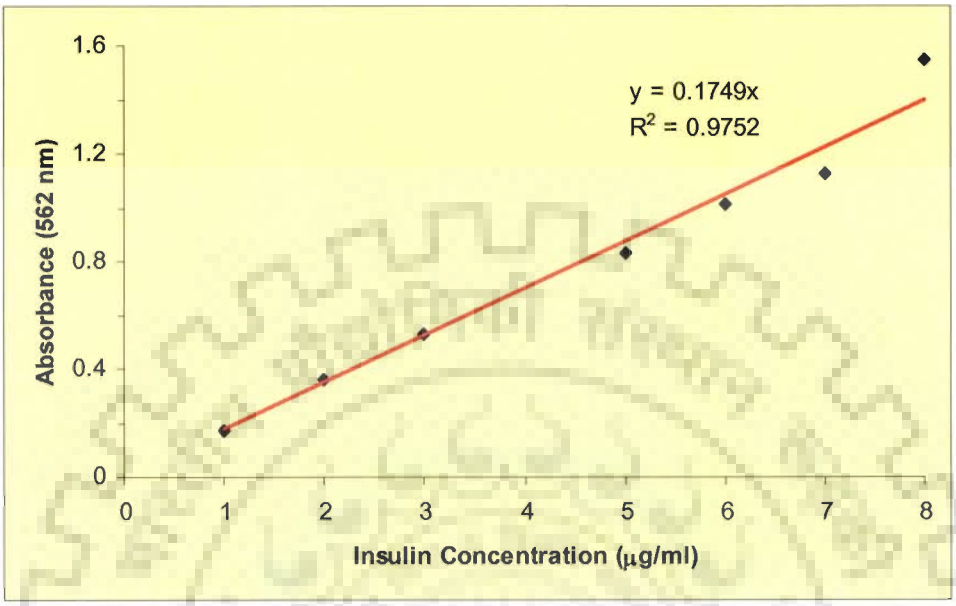

Figure IA: Standard plot of insulin in water

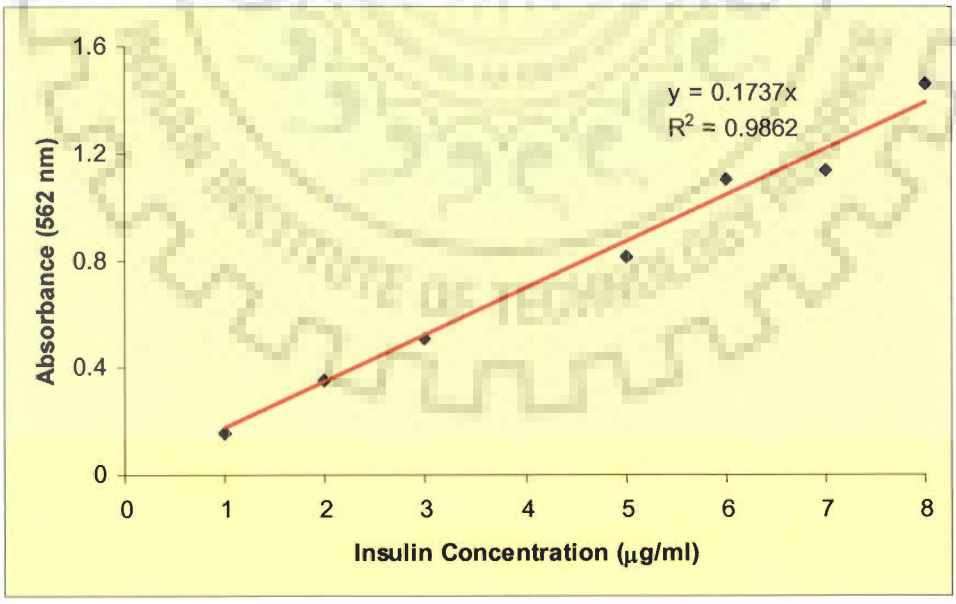

Figure IB: Standard plot of insulin in buffer, PBS (pH-7.4)

CURRICULUM VITAE

ANINDITA MUKERJEE

EDUCATION

July 2003 onwards	**Degree pursuing: Ph.D.** **Department:** Biotechnology **Institute:** Indian Institute of Technology Roorkee, Roorkee, India **Thesis:** Polymer mediated delivery of antidiabetic drug **Supervisor:** Dr. V. Pruthi, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India **Supervisor:** Dr. V. R. Sinha, Professor, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India
July 2002	**Degree awarded: B. Pharmacy.** First rank in University **Department:** University Institute of Pharmaceutical Sciences **Institute:** Panjab University, Chandigarh, India.

RESEARCH EXPERIENCE

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE, ROORKEE, INDIA
Doctoral Research Fellow (July 2003- February 2008)

- Formulation of polymeric microspheres/nanospheres for drug delivery.
- Characterization of formulated microspheres/nanospheres - using different techniques like Scanning Electron Microscopy, Laser Particle Size Analyzer, Atomic Force Microscopy, Confocal Microscopy, Differential Scanning Calorimetry.
- *In- vitro* and *in-vivo* drug-release studies of the formulated polymeric microspheres using drug estimations and pharmacoscintigraphy
- Toxicity studies of the polymeric microspheres by histopathological evaluation

PANJAB UNIVERSITY, CHANDIGARH, INDIA
Undergraduate Research Project

- Carried out extensive literature survey of all medicinal properties of *Hibiscus-rosa-sinensis*
- Submitted a project report on 'The Medicinal Properties of *Hibiscus-rosa-sinensis'*

PUBLICATIONS

REFEREED JOURNALS

1. Mukerjee A. and Pruthi V. (2006) Effect of solvents and stabilizers on the preparation and morphology of insulin-loaded poly-ε-caprolactone microspheres, *Research Journal of Biotechnology*, vol. 1 (2), pp-12-15

2. Mukerjee A. and Pruthi V. (2007) Oral insulin delivery by polymeric nanospheres, *Journal of Biomedical Nanotechnology*, vol. 3, pp-68-74

3. Mukerjee A., Sinha V.R. and Pruthi V. (2007) Preparation and characterization of poly-ε-caprolactone carrier particles for controlled insulin delivery, *International Journal of Biomedical and Pharmaceutical Engineering,* vol. 1(1), pp-40-44

4. Mukerjee A. and Pruthi V. (2007), Drug delivery: techniques for polymeric microsphere preparation, *Research Journal of Biotechnology,* vol. 2 (4), pp-58-63

5. Mukerjee A., Zhu R., Hinterdorfer P., Sinha, V.R and Pruthi V. (communicated) Structural characterization and release studies of insulin entrapped biodegradable poly-ε-caprolactone nanospheres for drug delivery, *Drug Delivery*

6. Mukerjee A., Mishra A. K. and Pruthi V. (communicated) Pharmacoscintigraphic evaluation of orally administered insulin-loaded poly-ε-caprolactone microspheres in diabetic animal models, *Journal of Controlled Release*

7. Mukerjee A., Ranjan A.P. and Pruthi V. (communicated) Studies on microsphere morphology and *in-vitro* release of poly-ε-caprolactone microspheres, *Research Journal of Biotechnology*

8. Mukerjee A., Mishra A. K. Sinha, V.R. and Pruthi V. (communicated) *In-vivo* release and toxicity evaluation of insulin entrapped poly-ε-caprolactone microspheres after oral administration, *International Journal of Pharmaceutics*

9. Mukerjee A., Sinha V.R. and Pruthi V. (communicated) Poly-ε-caprolactone microspheres as carriers for drug delivery, *European Journal of Pharmaceutics and Biopharmaceutics*

CONFERENCE PROCEEDINGS

A. International conferences held outside India

1. Mukerjee A. and Pruthi V (2005) Challenges in protein delivery: a role of biodegradable polymeric microspheres and nanospheres. Proceedings, International conference on environmental, industrial and applied microbiology, Biomicroworld, Badajoz, Spain, pp 279

2. Mukerjee A. and Pruthi V. (2006) Biodegradable polymeric nanospheres as potential carriers for oral drug delivery. Proceedings, International conference on Bio-nanotechnology- future prospects in Emirates, Dubai, pp-47-50

3. Mukerjee A. and Pruthi V. (2006) Preparation and characterization of poly-ε-caprolactone carrier particles for controlled insulin delivery. IEEE proceedings,

International conference on biomedical and pharmaceutical engineering 2006 (ICBPE 2006), Singapore, 5 pages, cd-rom

4. Mukerjee A. and Pruthi V. (2007) Structural characterization of polymer nanoparticles as microcarriers for antidiabetic drugs. Proceedings, The IX Annual Linz winter workshop, Linz, Austria, pp-132-137

5. Mukerjee A. and Pruthi V (2007) Development of insulin-loaded poly-ε-caprolactone nanospheres as a depot system for parenteral delivery, Proceedings, Advanced Materials & Nanotechnology, Wellington, New Zealand, cd-rom

6. Mukerjee A. and Pruthi V. (2007) Microencapsulation and characterization techniques for poly-ε-caprolactone microparticles entrapping insulin. Proceedings, 1st international conference on industrial processes for nano and micro products, London, pp-211-216

B. International conferences held in India

1. Mukerjee A. and Pruthi V. (2004) Poly-ε-caprolactone in controlled drug delivery. Proceedings, International conference on chemistry biology interface -2004, Delhi, pp23-26

2. Mukerjee A., Ranjan A. P. and Pruthi V. (2006) Biodegradable polymeric nanospheres for sustained insulin delivery. Proceedings, International Nano-bioscience conference, Pune, pp-44

3. Mukerjee A. and Pruthi V. (2006) Insulin delivery by polymeric nanospheres. Proceedings, Indo-US symposium on advanced drug delivery, Chandigarh, pp-42

4. Mukerjee A. and Pruthi V. (2006) Formulation and optimization of insulin-loaded polymeric nanospheres using response surface methodology. Proceedings, 59th annual Indo-Canadian session of Indian institute of chemical engineers, Chemcon 2006, Gujarat, 343-cd-rom

5. Mukerjee A. and Pruthi V (2007) Chitosan microparticles as carriers for antidiabetic drugs. Proceedings, 9th Indo-Korean symposium on biochemical engineering and biotechnology, Delhi, pp-68

6. Mukerjee A., Chuttani K. and Pruthi V (2007) Evaluation of insulin loaded polymeric microspheres by gamma imaging for oral drug delivery. Proceedings, International conference on new horizons in biotechnology, Trivandrum, pp-294

C. National conferences and seminars

1. Mukerjee A. and Pruthi V. (2004) Novel protein delivery systems, IIT Roorkee Technical Festival (Cognizance), Roorkee

2. Mukerjee A., Rao C. and Pruthi V. (2005) Novel techniques for biosurfactant enhanced remediation of heavy metal contamination. Proceedings, 7th National symposium on biochemical engineering and biotechnology, Delhi

3. Mukerjee A. and Pruthi V. (2005) Characterization of insulin encapsulated biodegradable polymeric microspheres. Proceedings, ACTON 2005, Roorkee

4. Mukerjee A. and Pruthi V. (2005) Biodegradable polymers for controlled drug delivery. Proceedings, 58th annual session of Indian institute of chemical engineers, Chemcon 2005, Delhi

5. Mukerjee A. and Pruthi V. (2006) Biodegradable microspheres for drug delivery. Proceedings, National conference on environmental technology: biological perspectives issues and challenges, Mumbai

6. Mukerjee A. and Pruthi V. (2006) Nanoparticles in drug delivery. Presented in Therapeutics and diagnostic products for reproductive health: recent trends and future prospects, Roorkee

7. Mukerjee A. and Pruthi V. (2006) Role of synthetic biodegradable polymers in drug delivery. Presented in Biomaterials, tissue engineering and medical diagnostics, Delhi

8. Mukerjee A. and Pruthi V. (2006) Biodegradable polymers for drug delivery. Presented in National biotechnology conference-2006-current trends and future perspectives, Roorkee

9. Mukerjee A. and Pruthi V. (2007) Chitosan microparticles for protein drug delivery. Proceedings, International symposium on new frontiers in marine natural product research (NFMNPR), Goa

10. Mukerjee A. and Pruthi V. (2007) *In-vivo* studies of insulin encapsulated poly-ε-caprolactone nanoparticles for oral delivery. Proceedings, 60th annual session of Indian institute of chemical engineers, Chemcon 2007, Kolkata

AWARDS AND HONORS

- University Medal in B. Pharmacy-for First rank in University in this course (August 1998- July 2002)

- Gian Harkishan Singh Gold Medal- for being the outstanding student in B. Pharmacy course (August 1998- July 2002)

- Awarded merit scholarships for all the four years of the B. Pharmacy course (1998-2002)

- Meritorious in academics throughout school, always been in the top 1 percent.

- Recipient of National Doctoral Fellowship from All India Council of Technical Education, Government of India, for the period: January 2004 – January 2008.

- Recipient of 'Outstanding Paper Award' for the paper 'Preparation and characterization of poly-ε-caprolactone carrier particles for controlled insulin delivery' at the International conference on biomedical and pharmaceutical engineering 2006 (ICBPE) at Singapore in December 2006

- Recipient of 'Best Poster Award' for the paper 'Formulation and optimization of insulin-loaded polymeric nanospheres using response surface methodology' at the 59th annual Indo-Canadian session of Indian Institute of Chemical Engineers, Chemcon 2006, at Bharuch, Gujarat in December 2006

GRANTS

- Recipient of Foreign Travel Grant for Young Scientists from Indian National Science Academy (INSA), Government of India, in 2006 for attending a conference in Singapore

- Recipient of Foreign Travel Grant for Young Scientists from Department of Biotechnology (DBT), Government of India, in 2006 for attending a conference in Singapore

- Recipient of Foreign Travel Grant for Young Scientists from Department of Science and Technology (DST), Government of India, in 2007 for attending a workshop and conference in Linz, Austria

- Recipient of Foreign Travel Grant for Young Scientists from Council for Scientific and Industrial Research (CSIR), Government of India, in 2007 for attending a workshop and conference in Linz, Austria

WORKSHOPS

- Attended workshop on 'Bioinformatics, Tools and its Application' at Indian Institute of Technology Delhi, Delhi, March 30th -31st , 2004

- Attended Winter School at the Johannes Kepler University of Linz, Linz, Austria, January 30th – February 1st, 2007

RELEVANT COURSES TAKEN

Graduate Level

- Biochemistry
- Applied Microbiology
- Molecular Diagnostics and Therapeutics Biotechnology
- Delivery of Protein Pharmaceuticals using Biodegradable Microspheres- term paper submitted

Undergraduate Level

- Pharmaceutics
- Pharmacology
- Pharmaceutical & Medicinal Chemistry
- Pharmacognosy

COMPUTER SKILLS

- Environment: Windows
- Software used: MS-Office- MS-Word, MS-Excel, MS- Power Point

OTHER ACTIVITES

- Participated in organizing technical sessions for the 'XXIV Annual Symposium of Reproductive Biology and Comparative Endocrinology', a national conference, organized by the Department of Biotechnology, IIT Roorkee, Roorkee, India in 2006

- Officiated as a Judge for the event 'Ad-o-mania' at the IIT Roorkee Technical Festival (Cognizance) in 2006

PERSONAL INFORMATION

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