STUDIES ON BOVINE CASEIN AS A CARRIER MATRIX FOR CONTROLLED DRUG DELIVERY

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by

M. S. LATHA

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DECLARATION

I, M. S. Latha hereby certify that I had personally carried out the work depicted in the thesis entitled "**STUDIES ON BOVINE CASEIN AS A CARRIER MATRIX FOR CONTROLLED DRUG DELIVERY**" except where external help sought and acknowledged.

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CERTIFICATE

This is to certify that Smt. M. S. LATHA in the division of Polymer Chemistry of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph. D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled "**STUDIES ON BOVINE CASEIN AS A CARRIER MATRIX FOR CONTROLLED DRUG DELIVERY**" was carried out under my direct supervision.

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Dr. A. Jayakrishnan
(Guide)

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. . . To my husband

Acknowledgement

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SYNOPSIS

SYNOPSIS

Controlled drug delivery aims at increasing the therapeutic efficacy of the drug while minimizing its toxicity. Successful application of many clinically established drugs in conventional dosage forms is limited in scope because of a multitude of problems. Controlled drug delivery products give new life to old pharmaceuticals. It provides the possibility of protection and masking, reduced dissolution rate, facilitation of handling and targeting of the active agent. Several issues must be resolved in devising a drug delivery system. Choice of the method for achieving controlled release in a particular application depends on the physico-chemical properties of the carrier, potency and properties of the drug and the location and access to the target site.

Drug carriers play a significant role in controlled release technology. Drug release profile and *in vivo* performance depend largely on the properties of the carrier matrix. The versatile nature of polymers allows them to be uniquely suited as carriers in drug delivery. The trend in controlled release technology has been towards biodegradable carriers which degrade in the body to non-toxic degradation products. Biodegradable drug carriers obviate the

need for follow-up surgical removal of the delivery device after the drug is depleted and therefore are more suited for parenteral application. Natural and synthetic polymers have been investigated as biodegradable drug carriers. Natural polymers remain attractive because they normally pose less toxicity of their own.

The study reported in the thesis was aimed at evaluating the potential of bovine milk protein casein as a carrier for controlled drug delivery. Although serum proteins such as albumin have good *in vivo* tolerance, the possibility of contamination of blood products with viruses such as HIV and hepatitis B makes carriers such as albumin less attractive as a drug carrier today. Therefore, although considerable work has already been done on albumin as a carrier, there is a need for more safe and efficient protein-based drug carriers having properties comparable to that of albumin. It was demonstrated that dug release properties of albumin and casein bear remarkable resemblance with drugs such as methotrexate. Although milk protein casein is abundant and inexpensive this has been investigated only to a limited extent.

The thesis consists of four chapters. The first chapter provides a general background, objectives and relevance of the proposed investigation. The second chapter gives an outline of the materials used and the experimental techniques employed in the study. Placebo and drug-loaded casein microspheres were prepared by glutaraldehyde cross-linking using a steric stabilization method. Outlined are the methods of preparation and characterization of casein microspheres loaded with drugs such as theophylline, 5-fluorouracil,

mitoxantrone and progesterone. Estimation of the amount of drugs incorporated and the method for following up the release profiles of incorporated drugs in simulated biological fluids *in vitro* are presented in this chapter. Appropriate *in vivo* experiments in laboratory animals to evaluate the therapeutic efficacy of microsphere/drug formulations of theophylline, 5-fluorouracil, mitoxantrone and progesterone are also presented.

In chapter 3, the results of the experiments are presented and discussed. The first part of this chapter deals with preparation and characterization of placebo casein microspheres prepared by glutaraldehyde cross-linking. Microspheres of wide size range were prepared and implantation studies were carried out in animal models in order to examine the tissue response and toxicity of microspheres. Microspheres were found to be well tolerated by the living tissue without any adverse tissue reactions. The study demonstrated the feasibility of using casein microspheres for sustained drug delivery.

The second part of chapter 3 describes the use of casein microspheres for sustained oral delivery. Using theophylline as the model drug, it was shown that a sustained release could be obtained and therapeutically desirable serum concentration could be maintained over prolonged periods. Glutaraldehyde cross-linked casein microspheres were demonstrated to be resistant to proteolytic enzymes at concentrations prevailing in the gastrointestinal tract *in vitro*. In a bioavailability study in rabbits, it was shown that cross-linked milk protein microspheres could sustain theophylline concentration for prolonged periods *in vivo*.

In the third part, the use of casein microspheres for the delivery of anti-neoplastic agents is presented. Casein microspheres loaded with 5-fluorouracil, a casein-5-fluorouracil conjugate and mitoxantrone-loaded casein microspheres were prepared and evaluated *in vitro* as well as *in vivo*. Sustained release lasting several days was seen for all the three formulations *in vitro*. In vivo evaluation against Ehrlich ascites carcinoma in mice showed that 5-fluorouracil-loaded casein microspheres and casein-5-fluorouracil conjugate were not very effective against this carcinoma, but mitoxantrone-loaded microspheres showed significant antitumour effect in comparison with the free drug.

The fourth part discusses the possibility of using casein microspheres loaded with an antifertility steroid such as progesterone. Using progesterone-loaded microspheres, it was demonstrated in a rabbit model that sustained delivery of this steroid was possible for prolonged periods without peaking and troughing of plasma concentration during the entire period of the study. While the release from the steroid administered as a powder intramuscularly did not last for more than 4 days *in vivo*, the microencapsulated steroid showed sustained release lasting over five months. From this study, it appeared that casein microspheres crosslinked with glutaraldehyde matrix would be a potential carriers for long term delivery of antifertility steroids.

The last part of this chapter deals with the preparation of calcium cross-linked casein in the form of beads and granules. Although calcium crosslinked starch and alginate have been investigated as potential drug carriers, calcium crosslinked casein has not so far been investigated as a drug delivery

matrix although calcium crosslinking of casein is a well known reaction. The potential of calcium caseinate as an oral delivery system was therefore examined. Theophylline-loaded calcium caseinate beads were prepared and evaluated for *in vitro* drug release profiles. Data obtained suggested that drugs which are only absorbed within the confines of the small intestine would be more suitable candidates for sustained delivery using this matrix. Calcium cross-linked granules were also prepared and evaluated for sustained oral delivery of the model drug theophylline.

Chapter 4 gives an overview of the major conclusions of the study and an outline for possible future research in this area.

CHAPTER 1
INTRODUCTION

INTRODUCTION

1.1 Background

Drug delivery using controlled release technology began in the 1950's and has continued to expand so rapidly that there are many products both on the market and in development. Controlled drug delivery aims at increasing the therapeutic efficacy of the drug while minimizing its toxicity. Substantial decline in the rate of appearance of new drugs due to increased safety requirements associated with drug registration in all major countries and the growth in the science of pharmacokinetics due to advances in precise analytical techniques have led to significant advances in drug delivery technology. Controlled release technology offers promise to a variety of problems in medicine (Robinson 1978; Lewis 1981; Juliano 1987; Johnson and Lloyd-Jones 1987; Chien 1990; Judah 1990; Rao and Jeyanthi, 1991; Agis 1992; Cohen *et al.*, 1994).

Successful application of many clinically established drugs in conventional dosage forms is limited in scope because of a multitude of problems. Controlled drug delivery products give new life to old pharmaceuticals. It provides the

possibility of protection and masking, reduced dissolution rate, facilitation of handling and targeting of the active agent (Juliano 1991; Koch 1991; Robinson and Manger 1991; Florence and Jani 1994). Optimal therapy requires the persistence of drug concentration at a constant level within the therapeutic range as long as the desired effect is accomplished. Persistance of the agent is often reduced by different mechanisms like degradation, elimination etc. Conventional application of rapidly cleared drugs in a multiple dose regime often results in peaks and valleys of drug concentration in the systemic circulation. This may lead to overdosing and underdosing in the case of drugs with narrow therapeutic range. Drug concentration profile in blood from administration of multiple doses of a conventional dosage form and a single dose of a long acting controlled release dosage form is shown in Figure 1.1.

Controlled release dosage forms can maintain the concentration of the drug within the therapeutic range for a long time . Hence, frequency of dosing can be reduced to avoid toxicity due to excess concentration in blood (Juliano 1980; Chien 1982). Controlled release technology is expanding to include drugs that can be targeted to specific organs as well as drugs that can be released in response to specific biological processes (Kwon *et al.*, 1990; Kim et al., 1994; Yuk *et al.*, 1995).

Several issues must be resolved in devising a drug delivery system. A thorough understanding of the drug carrier, the drug and the target site are necessary for designing a controlled drug delivery system. Choice of the method for achieving controlled release in a particular application depends on

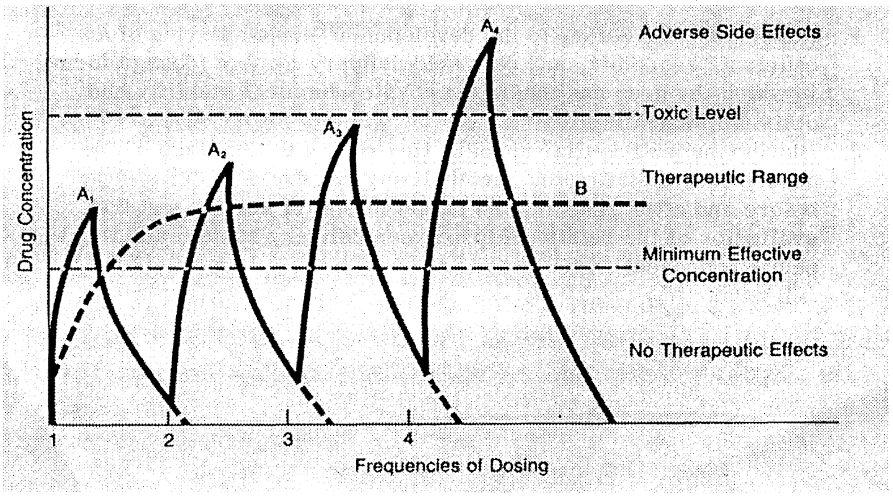

Figure 1.1 Drug concentration profile in blood from administration of multiple doses of a conventional dosage form (A_1, A_2, A_3, A_4) and a single dose of a controlled release dosage form (B).

the physico-chemical properties of the drug carrier, potency and properties of the drug and the location and access to the target site. Drug release from a carrier matrix is governed by the property of the carrier, mode of dispersion of the drug, interaction of the drug with the carrier matrix and the morphology of the entire system (Carraher and Gebelein 1982; Kissel *et al.*, 1991). Rate of release has to be optimised to obtain the desired effect. Cost is also important. Cost of material and the processing cost determine the commercial feasibility of the controlled delivery device.

Drug carriers play a significant role in controlled release technology. Drug release profile and in vivo performance depend largely on the properties of the carrier matrix. Polymers have received considerable attention in this area (Carraher and Gebelein 1982; Migliaresi *et al.*, 1988; El-Nokaly *et al.*, 1993;

Khare and Peppas 1993; Dumitru 1994). Polymers in the form of slabs, cylinders, membranes, microspheres, drug conjugates, microcapsules etc., have been used in drug delivery. Polymeric microspheres have been investigated to a great extent as drug carriers (Davis *et al.*, 1984; Monica *et al.*, 1990; Khare *et al.*, 1991; Carli 1993; Couvreur and Puisieux 1993). As opposed to other physical forms of drug carriers such as rods, cylinders and slabs, the main advantage of microspheres is that they can be injected into the body in a suitable vehicle using a hypodermic needle. Microsphere-based systems can be used for oral and parenteral delivery. Microspheres can be prepared from both natural and synthetic polymers.

1.2 Polymers as Drug Carriers

For wide applications in controlled drug delivery, it is imperative that a range of rates and durations of drug release be achievable. The versatile nature of polymers allows it to be uniquely suited as carriers in drug delivery. A broad spectrum of performance characteristics can be obtained by a judicious manipulation of polymer properties. A variety of polymeric carriers has been described (Gebelein *et al.*, 1990; Linhardt *et al.*, 1990; Masaharu *et al.*, 1990; Robert 1990; Couvreur and Vauthier 1991; Chan *et al.*, 1991; Okano and Yoshida 1993) and many are in commercial application. Ocusert^{R} , Progestasert^{R} , Transderm^{R} , Norplant^{R} , etc. are some of the commercially available polymeric controlled drug delivery devices (Brown *et al.*, 1976; Chien 1982; Dumitru 1994). Polymers have the advantage that they are available with a wide variety

of mechanical and physical properties, are readily formed into desired shapes, and can be inert towards the host tissue. They are also available at reasonable cost (Chien 1982).

1.3 Criteria for Use of Polymers in Controlled Release Applications

Polymers should satisfy certain criteria for drug carrier applications. The polymeric carrier should be biocompatible and should be free from elutable impurities and additives. There are many polymers that meet this criteria. Folkman and Long were the first to establish the concept of drug delivery using polymeric implants (Folkman and Long 1964; Long and Folkman 1966). They studied the feasibility of using silicone, a nondegradable polymer and demonstrated that by confining a depot of therapeutic agent within the polymeric capsule, sustained release rate can be achieved within therapeutic range for a prolonged period of time. Many synthetic non-biodegradable polymers have been investigated as drug carriers (Table 1.1) (Baker 1987). Silicones were found to be excellent materials because of their biocompatibility, ease of fabrication and high permeability to many drugs. However, they have been found to cause autoimmune diseases in recent reports (Granchi et al., 1995; Netscher *et al.*, 1995). Non-degradable polymers used for the long term delivery of drugs remain in the body unless they are removed. Although such systems have many merits, surgical implantation and removal of the drug depleted device have a negative impact on the acceptability of such devices.

Table 1.1
Non-biodegradable polymers used in drug delivery

Silicones
Polyethylene
Polyisobutylene
Polyurethane
Poly(ethylene vinyl acetate)
Poly(methyl methacrylate)
Poly(ethyl methacrylate)
Poly(butyl methacrylate)
poly(2-hydroxyethyl methacrylate)

The trend in controlled release technology has been towards biodegradable carriers which degrade in the body to non-toxic degradation products (Pitt and Schindler 1980; Feijen 1986; Linhardt 1989; Hutchinson and Furr 1990; Xin and De 1990; Gref *et al.*, 1994). It obviates the need for follow-up surgical removal after the drug is depleted and is more suited for parenteral application. An important requirement of such polymers is that the degradation products should be non- toxic because they eventually enter systemic circulation or result in tissue deposition. Long term toxicological evaluation of the degradation products therefore is important in determining the clinical suitability of such carriers. Natural and synthetic polymers have been investigated as biodegradable drug carriers. Table 1.2 shows some biodegradable natural and synthetic polymers used in drug delivery applications (Dunn and Ottenbrite 1991). Natural polymers such as proteins and polysaccharides undergo enzymatic degradation in the body. Most synthetic polymers contain hydrolyzable linkages like amide, ester, urea, and urethane.

The physiological environment in which the system is placed in the body affects the rate of degradation.

Table 1.2
Biodegradable polymers used in drug delivery

Natural polymers	Synthetic polymers
Albumin	Poly(lactic acid)
Gelatin	Poly(glycolic acid)
Casein	Poly(caprolactone)
Starch	Poly(hydroxy butyrate)
Collagen	Poly(hydroxy valerate)
Chitin	Polyanhydrides
	Polyorthoesters
	Polyamino acids
	Phosphazenes
	Poly(ethylene glycol)

Drug delivery using natural biodegradable polymers continue to be an area of active research despite the emergence of synthetic biodegradable polymers (Kost and Shefer 1990; Simon 1990; Roos *et al.*, 1991; Carter *et al.*, 1992; Ohya *et al.*, 1993; Rubino *et al.*, 1993; Lou *et al.*, 1994). Natural polymers remain attractive because they normally pose less toxicity of their own. Most of the natural polymers are biodegradable and are generally biocompatible. One significant advantage of natural polymers over synthetic polymers is their excellent homogenicity in terms of molecular weight and monomer (amino acid or sugar) sequences.

1.4 Mode of Attachment of Drug to the Carrier

The binding force that holds the drug to the carrier matrix can either be physical or chemical. In addition to this, hydrophobic and electrostatic interaction may also occur. Depending on the mode of attachment, drug release from the matrix also varies.

1.4.1 Physical Binding

Drug release is expected to be faster if physical entrapment alone is involved. Here, the drug exists in its native form and its properties remain the same after being released from the matrix. Release in this case can be diffusion controlled, or degradation controlled depending on the nature of the carrier matrix.

Drug release from a non-biodegradable matrix or from a matrix which degrades after drug is completely released is modulated by a diffusion controlled mechanism. Rate of release in such cases vary with permeability of the polymer, surface area of the device, size of the drug molecule etc. In polymers with ionizable functional groups, drug release is affected by solvent pH. Solvent controlled release is seen in poly(malic acid halfester). Drug is released in alkaline environment of the intestine but not in gastric fluid where pH is 1-2 (Dunn and Ottenbrite 1991). When diffusion is slow compared to degradation, release depends on degradation of the matrix (Baker 1987).

1.4.2 Chemical Binding

Slow release can be achieved by covalent attachment of the drug to the carrier matrix. The polymer should have reactive functional groups to which drug can be bound through a functionality available on the drug. Drugs with an NH_2 group can be attached to the matrix containing either -COOH, -OH or -CHO groups. There are various methods for attaching drugs to the matrix (Hurwitz *et al.*, 1975; Shen and Ryser 1979; Kim and Oh 1988; Thau *et al.*, 1991). Attachment of drug to the matrix can be permanent in which case drug can exert its action in the bound form, or can be attached via a labile bond which cleaves in the body environment releasing the free drug for action. In permanent attachment, drug can be a component of the backbone or as pendant from it by a covalent nonhydrolysable bond (Donaruma *et al.*, 1980; Dumitru 1994). In some cases, activity of the drug is compromised. For example, daunomycin attached to N-hydroxypropyl methacrylamide was found to be less effective than free drug and the puromycin conjugate was virtually ineffective against L1210 leukaemia (Duncan *et al* 1987). Poly(glutamic acid)-adriamycin conjugate was also totally ineffective against the same tumour. Using spacers, it has been demonstrated that such drawbacks could be overcome. It was shown that poly(glutamic acid) conjugated to adriamycin through lysosomally cleavable spacer such as Gly-Gly-Gly-Leu-Adr and Gly-Gly-Adr enhances drug activity as evidenced by the mean survival time of mice with L1210 leukaemia cells implanted intraperitoneally (Hoes *et al* 1993).

1.5 Route of Administration

Drugs can be administered into the body via different routes. Route of administration is important in devising a drug delivery system.

1.5.1 Oral Delivery

History of sustained oral delivery began with the introduction of Spansules[R] in 1952 (Dumitru 1994). Oral delivery is the simplest and most convenient way of drug administration. However, the design of such dosage forms are limited because of the gastro-intestinal (GI) physiology and varying transit time. In oral drug delivery, the system has to pass through frequently changing environments in the GI tract. There is also patient to patient variation in GI content, stomach emptying time and peristaltic activity (Robinson 1978). Although limitations are many, it offers less potential danger than the parenteral route. Full understanding of the biological parameters is necessary for the development of an effective drug delivery system. Recently, there has been much progress in the study of in vivo performance of oral dosage forms (Johnson and Lloyd-Jones 1987).

If the drug is a protein or a polypeptide, oral route fails due to the inactivation of the protein or peptide by gastric acid and intestinal enzymes, poor bioavailability and interference from other bacteria and viruses in the GI tract. Recently, Eldridge *et al.* (1990) studied the possibility of oral vaccination using biodegradable microspheres of size less than 10 μm which protect vaccine antigens from degradation and target delivery to the Peyer's

patches. Sustained vaccine release which induced a strong IgA response in diverse mucosal tissue was demonstrated. Bioavailability of drugs with limited solubility in the stomach or intestine and small absorption rate constant can be increased by increasing the retention time in the stomach. Use of polymers with bioadhesive properties can prolong the residence time of the dosage form in the stomach. Methylcellulose, poly(ethylene glycol), poly(vinyl pyrrolidone) etc. are some of the polymers showing bioadhesive properties and were investigated for oral delivery (Johnson and Lloyd-Jones 1987). Longer et al (1985) studied chlorothiazide release from albumin microspheres by oral administration by mixing it with polycarbophil, a bioadhesive polymer. Improved drug delivery was observed in this case. Vyas and Jain (1992) prepared bioadhesive polymer grafted starch microspheres bearing isosorbide dinitrate for buccal administration. They compared the drug release and *in vivo* performance with conventional oral treatment and was found to be promising.

1.5.2 Parenteral Delivery

Parenteral administration provides easy passage for the drug into the systemic circulation. Drug absorption and elimination are rapid in this case resulting in a relatively short period of drug action. Most of the microsphere-based controlled delivery systems are developed with the aim of using them for parenteral administration. Microspheres used for parenteral delivery should be sterile and should be dispersible in a suitable vehicle for injection. Hydrophilic microspheres have the potential advantage of aqueous

dispersability as opposed to hydrophobic microspheres for reconstituting them for injection. Surfactants in small concentrations are often necessary for reconstituting hydrophobic particles for injection in aqueous vehicles which are reported to cause adverse tissue reactions and affect the release of the incorporated drug (Kramer 1974). Retention of microspheres in circulation after intravenous application was reported to be improved by optimising the size of the microspheres, their surface properties etc. It was reported that coating microspheres with a hydrophilic polymer such as poloxamer, poly(ethylene glycol) etc. increases the longevity of the system in circulation (Klibanov *et al.*, 1990; Mori *et al.*, 1991; Torchilin *et al.*, 1992). However, long circulating microspheres are still a far cry since they are prone to be phagocytosed by the floating macrophages of the reticuloendothelial system.

1.6 Drug Targeting

Interaction of the drug molecule with the site of action results in pharmacological effect. For optimal therapy, it is desirable to maintain the drug concentration in the target tissue within therapeutically effective dose range as long as the treatment requires. Very often, excess drug has to be administered to have desirable therapeutic concentration because of the distribution of drug throughout the body (Chien 1982). This may lead to toxicity in non-target site. Rationale behind the concept of targeting is to bring drug molecule into intimate contact with the target site by localisation of the drug to a particular area in the body, to a particular group of cells within the body or even to intracellular

structures and preclude the distribution of drug throughout the body. This reduces serious side effects due to interaction of drug with non- target cells and opens up the possibility of using highly toxic substances especially in cancer chemotherapy. Targeting also reduces the concentration of drug necessary for therapeutic action because of the reduced loss of drug due to distribution in the body (Chien 1982; Tomlinson 1991).

There are various approaches in this area (Buri and Gumma 1985; Goldberg *et al.*, 1991). Microspheres have been investigated for targeted delivery by manipulating their natural distribution (Illum *et al.*, 1982; Porter *et al.*, 1992). Depending on their size and surface charge, microspheres will distribute itself to different organs (Davis and Illum 1983). Mechanical entrapment of particles greater than 7 μm in the capillary beds of lungs is exploited for the targeted delivery of drugs to the lungs via venous supply. Microspheres of size 5-10 μm have been found to remain in Peyer's patches after oral administration (Eldridge *et al* 1990).

Magnetic drug targeting is another means of delivering drugs to single or multiple regional targets. Magnetic microspheres have been used for localising the system at specific *in vivo* targets by placing a magnet externally over the target area (Sugibayashi *et al.,* 1982; Seigel and Langer 1984; Haffeli *et al.*, 1994). Albumin microspheres containing Fe_2O_3 have been used for the delivery of anti-inflammatory agents directly to the knee joint and for the delivery of adriamycin to the tail of rat (Widder *et al* 1983). Gupta and Hung (1990) studied the efficacy of magnetic albumin microspheres for the delivery of

doxorubicin in rats. It was found that compared to the free drug, a one third dose of microsphere entrapped drug resulted in almost eight times higher drug exposure at the target site. In addition, drug delivery to all non-target tissues was substantially reduced. Magnetic targeting is an efficient but expensive method of drug targeting. Therapeutic response can be achieved at one tenth of free drug dose using this method. It affords drug release for 30 min to 30 h as desired. The main draw back of this method is that a portion of the magnet entrapped to magnetise the spheres is deposited permanently in target tissue (Ranney and Huffaker 1987).

Attaching specific ligands to the drug can also direct it to the specific cells (Arnon and Hurwitz 1983). Human albumin attached to succinoyl-Alan-Alan-Pro-Val-CH_2Cl an active site-directed inhibitor of human leukocyte elastase was successfully used to direct the inhibitor to the lung of rats. Specificity conferred by antibody molecules is now being exploited for drug targeting. Use of monoclonal antibodies for drug targeting is progressing towards clinical utility (Johnson and Lloyd-Jones 1987; Seymour *et al.*, 1991). Davis and Illum (1983) found that monoclonal antibody attached to poly(cyanoacrylate) particles showing strong interaction with the antigenic tumour cells *in vitro*. Such approaches hold considerable promise in cancer chemotherapy.

Intracellular targeting can be achieved by using some proteins and polysaccharides. Intracellular targeting obviate the problem of poor efficacy of many chemotherapeutic agents due to their poor intracellular influx. Microspheres

have been described to act as a phagocytic stimulus (Fernandez-Repollet and Schwartz 1988). Hoffman *et al* (1984) demonstrated that streptomycin-loaded albumin microspheres were avidly taken up by the macrophages and the drug was released inside the phagocytic cells after ingestion and intracellular degradation of microspheres. Ponpipom *et al* (1981) found an increase in uptake of glycoprotein with mannose residues by macrophages. Flourescein labelled casein and albumin microspheres of less than 5 μm were avidly taken up by the mouse myleomonocyte leukemia cells, a macrophage like cell line (Knepp *et al* 1993). Tumour cells display high uptake of protein microspheres and after internalisation drug is released on digestion of the matrix by enzymes (Oppenheim and Stewart 1979).

1.7 Protein Microspheres in Drug Delivery

Protein microspheres have attracted considerable attention because of their biodegradability and nontoxicity. Moreover, the large number of functional groups present in the proteins offer sites for attachment of the drug. Of the various proteins investigated as drug carriers, albumin and gelatin have received much attention.

1.7.1 Albumin Microspheres

Considerable work has been done on human serum albumin (HSA) as a drug carrier. Albumin is the major plasma protein constituent and is the natural circulating carrier. The use of HSA microspheres has been first suggested by

Kramer (1974) and thereafter a large number of patents and publications have emerged on this topic (Egbaria and Friedman 1990; Newman 1990; Dilova and Shishkove 1993; Cummings *et al.*, 1994; Novotn and Zinek 1994). The use of albumin microspheres in drug delivery has been extensively reviewed by Morimotto and Fujimotto (1983); Gupta and Hung (1989a; 1989b) and by Arshady (1990). Major application of albumin microspheres is in the delivery of chemotherapeutic agents as it offers high local drug concentration while minimizing systemic toxicity over a specific area for a longer period of time. Fujimoto *et al* (1985), Burger and McVie (1985), and Noteborn *et al* (1988) evaluated the targeted delivery of chemotherapeutic agents using albumin microspheres. Microspheres loaded with mitomycin C was found to be more effective than the free drug when given via the hepatic artery to treat metastatic cancer. Burger and McVie (1985) showed that cisplatin-loaded albumin microspheres were ten times more effective in localizing the delivery of the drug in patients with hypervascular liver cancer. Preclinical and clinical studies using albumin microspheres demonstrated their biocompatibility. Apart from the slight nausea, pain and fever, no major side effects have been observed.

Albumin microspheres prepared by most techniques have been found to be hydrophobic to some extent that small quantities of surfactants were needed to disperse them in aqueous vehicles. Surface hydrophilicity is important in dispersing the particles in a non-aggregatory manner in aqueous vehicles. Also, it has been suggested by Longo *et al* (1982) that the presence of surfactants used for dispersion might provoke tissue reactions and affect the release of

drugs. Illum *et al* (1986) have found that hydrophobic particles are cleared more rapidly from circulation than hydrophilic particles. Knepp *et al* (1993) found that a high payload of water soluble drugs could be incorporated into preformed microspheres if they are hydrophilic in nature. Using a polymer solution to stabilize the albumin droplets and cross-linking the protein with glutaraldehyde via the organic phase resulted in highly hydrophilic microspheres (Longo *et al* 1982).

Drug release from albumin microspheres can be controlled by changing their cross-linking density and the drug to albumin ratio (Kim and Lee 1986). But, high cross-linking retards the rate of degradation. Drug to albumin ratio has its limiting factors such as solubility of the drug in albumin solution and the burst effect that occurs at high drug payloads. This can be overcome by chemically attaching the drug to the protein. Since there are many functionalities in the protein to which the drug can be anchored, high drug payloads can be obtained by chemically binding the drug to the matrix. Release spanning over long periods of time can be obtained by this method. Mukhopadhyay *et al* (1989) demonstrated that methotrexate coupled to maleylated bovine serum albumin was effective in delivering the drug to macrophages by using the scavenger receptor mediated endocytic pathway to achieve selective killing of intracellular parasites leishmania mexicana amazonensis amastigotes residing in the macrophages. They have proposed that similar approach may be useful for the treatment of other diseases in which the macrophages are the primary target including tuberculosis, leprosy, monocytic leukaemia and heavy metal storage diseases.

Since albumin binds many drugs strongly, this binding would retard drug release until the microspheres are degraded by proteolytic enzymes (Sjoholm and Edman 1979). Lee *et al* (1981) demonstrated that albumin microspheres loaded with progesterone produced no adverse response in rabbits and a sustained release for about 20 days was demonstrated *in vivo*. The intramuscular life of albumin microspheres cross-linked using 1% glutaraldehyde used in this study was reported to be about 2 months. The authors could not detect any remnants of injected microspheres after 2 months in the muscle.

Albumin microspheres of suitable size have been found to be useful for localised drug delivery. Rifampicin loaded albumin microspheres were found to be useful for the localised delivery of the drug to the lungs (Pande *et al* 1991). *In vivo* distribution studies showed that 62% of the drug could be localised in the lungs by controlling the particle size.

Chemically modified albumin microspheres with an anionic polypeptide such as poly(glutamic acid) afford ion-exchange. Goldberg *et al* (1984) prepared adriamycin- loaded microspheres of human serum albumin and human serum albumin containing poly(glutamic acid). Poly(glutamic acid) containing microspheres showed much lower toxicity with the elimination of necrotic reactions at the injection site. Drug release was found to be a function of ionic strength of the medium. High payloads of adriamycin (40-50%) could be incorporated into preformed microspheres from aqueous drug solutions.

Albumin microspheres have also been investigated for oral drug delivery (Longer *et al.*, 1985; Lewis *et al.*, 1992). Retention of the microspheres in the

stomach can be improved by mixing albumin microspheres with a bioadhesive polymer (Longer *et al* 1985). Albumin microspheres containing chlorothiazide and polycarbophil placed in a capsule showed reduced rate of gastric emptying and improved bioavailability. Radiolabelled albumin chelates (Mabuchi *et al.*, 1988; Nishimura *et al.*, 1989) and microparticles (Guiot and Couvreur 1986) are used clinically for imaging purposes. Albumin has the advantage that it can be easily radiolabelled. Technetium and iodine labelled albumin microparticles have been used in imaging.

1.7.2 Gelatin Microspheres

Another protein that has been investigated as a drug carrier in microsphere form is gelatin (Jeyanthi and Rao 1987; Tabata and Ikada 1987; Oner and Groves 1993; Esposito *et al.*, 1994; Rao *et al.*, 1994; Nakaoka *et al.*, 1995). It is non-toxic, biocompatible and biodegradable. Leucuta (1990) studied the possibility of using formaldehyde cross-linked gelatin and ethylcellulose coated gelatin microspheres as oral delivery system using nifedipine as the drug. First order release was obtained for gelatin microspheres and zero order release was seen from ethyl cellulose coated microspheres. *In vivo* study of cross-linked gelatin spheres in volunteers showed that a sustained release is obtained. Glutaraldehyde cross- linked hydrophilic gelatin microspheres containing methotrexate was prepared and the release was reported to be zero order in simulated gastric and intestinal fluid (Narayani and Rao 1994). Studies by Forni *et al* (1992) on the effect of loading and dynamic swelling on drug

release of aminophylline-loaded and clonidine hydrochloride-loaded gelatin microspheres showed that loading has a greater effect on drug release than polymer relaxation. Gelatin nanoparticles are reported to be taken up by some tumour cells which do not take up albumin nanoparticles. Oppenheim and Stewart (1979) studied the uptake of fluorescein-labelled albumin and gelatin nanoparticles by certain tumour cells. Tumour cells used for the study were EMT6 (mouse mammary) WEHI-3 (mouse myelomonocytic) and SP-1 (rat squmaus cell). All of them were found to take gelatin but not albumin particles. The EMT6 cells showed 80% uptake, SP-1 10% and almost all cells of WEHI-3 showed gelatin uptake.

1.7.3 Casein Microspheres

Guerin and Levy were the first to suggest the possibility of formation of milk protein microcapsules (Guerin and Levy 1983; Levy and Guerin 1987). Desoize *et al* (1986) studied for the first time the use of casein as a carrier matrix. Chen *et al* (1987) prepared doxorubicin-loaded casein microspheres by glutaraldehyde stabilization of the aqueous phase containing the protein in a water-in-oil emulsion. They compared the physical properties, drug loading characteristics and release rate from microspheres in vitro with albumin microspheres prepared by similar method. Compared to albumin, drug content was found to be lower in casein and the release rate from casein was found to be slow. They also compared the tumour inhibitory effect of doxorubicin-loaded microspheres in rats bearing tumour SP 107 by intratumoural injection.

Casein microspheres containing 11 μg doxorubicin were found to have similar inhibitory effect on tumour growth compared to 85 μg of drug incorporated in albumin microspheres. In their method of preparation, the microspheres showed marked deviation from spherical geometry. Particles obtained were porous, distorted and were hardly spherical in shape. Size distribution was also found to be broad. These authors also investigated the biodegradation rate of albumin and casein microspheres by radiolabelled techniques. Radiolabelled microspheres were administered intravenously into rats to target lungs and via hepatic artery to target liver. In both cases, the degradation rate of casein was found to be slower than albumin. In vitro degradation studies using trypsin showed a different pattern. There was found to be a latent period for albumin in which it swelled and then degraded whereas the degradation of casein was faster. The authors sugggested that this may be due to the enzyme specificity. Casein microspheres cross-linked with glutaraldehyde, having good spherical geometry were prepared for the first time using a surfactant stabilization technique with sorbitan sesquioleate as the surfactant from aqueous alkaline solution of the protein in a water-in-oil emulsion by Knepp *et al* (1993). Enzymatic degradation using protease showed that the rate of degradation is dependent on cross-linkdensity. Microspheres with lower cross-linking density degraded faster than those with higher cross-linking density. With methotrexate as a model drug, both albumin and casein showed similar loading of approximately 15 wt% (Jayakrishnan *et al* 1994). Casein microspheres were found to be smooth and spherical and more hydrophilic than albumin. *In vitro* release from

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both albumin and casein were found to be similar. Mitoxantrone incorporated into casein microspheres was found to be more effective against Lewis lung carcinoma compared to the free drug when administered intratumourally in mice. Fluorescein- labelled casein microspheres of size $<5\mu$m were also found to be avidly taken up by the mouse myelomonocyte leukemia cells in culture (Knepp *et al* 1993).

1.8 Aim and Scope of the Study

The carrier matrix is important in determining the *in vivo* performance of the controlled release dosage form. Although serum proteins such as albumin have good in vivo tolerance, the possibility of contamination of blood products with viruses (hepatitis, HIV) makes albumin less atractive as a drug carrier today (Bogdanov *et al* 1995). Therefore, although considerable work has already been done on albumin as a carrier, there is a need for more safe and efficient protein-based drug carrier having drug carrier properties comparable to that of albumin.

Milk is abundant and forms an integral part of our daily diet. Although the milk protein casein is abundant and inexpensive, this has been investigated only to a limited extent for possible controlled drug delivery applications. It has been found that clotting of blood and milk shows remarkable similarity (Jolles and Henschen 1982) and a sequence homology exists between K-casein and the gamma chain of fibrinogen. Studies on the comparative allergic strength of many bovine milk proteins such as casein, alpha-lactalbumin and

beta-lactoglobulin suggest that none of them is a prominant allergen (McKenzie 1967). Crosslinking the protein using glutaraldehyde also makes it least immunogenic in nature. The work of Jayakrishnan *et al* (1994) has already shown that casein microspheres similar in size and morphology to albumin microspheres could be prepared by similar techniques. It was also demonstrated that drug release properties of albumin and casein microspheres bear remarkable resemblance with a model drug such as methotrexate. Therefore the bovine milk protein casein appears to have considerable potential as a drug carrier comparable in scope to serum albumin. However, unlike albumin, the milk protein casein has been investigated only to limited extent as a carrier for controlled drug delivery.

The objective of this study is therefore,

1. To prepare glutaraldehyde cross-linked, smooth, spherical microspheres of casein having wide size range comparable to that of albumin microspheres using polymeric stabilising agents, and to characterize them with respect to their shape, surface morphology, internal structure and partcle size distribution.
2. To determine the in vitro degradation of such microspheres in simulated gastric fluid and intestinal fluid in the presence of proteolytic enzymes in order to evaluate their potential for sustained delivery of oral drugs.
3. To determine the biodegradability and toxicity of the microspheres intramuscularly injected into rats and to evaluate the potential of using them for sustained parenteral drug delivery.

4. To prepare microspheres containing a model oral drug such as theophylline and to determine the *in vitro* release profiles with respect to cross-link density, particle size and drug payload.
5. To examine the bioavailability of theophylline from casein microspheres *in vivo* in a rabbit model and compare it with the free drug given in powdered form.
6. To prepare progesterone-loaded microspheres and to determine the *in vitro* release profiles with respect to size, cross-link density and drug payload and to evaluate the bioavailability of this anti-fertility steroid after intramuscular injection in rabbits for possible fertility regulation.
7. To determine the uptake of cytotoxic drugs such as mitoxantrone and 5-fluorouracil (5-FU) by loading into preformed microspheres and during microsphere preparation and to study the in vitro release profiles in phosphate buffer with and without enzymes.
8. To prepare drug-protein conjugates by attaching 5-FU covalently to casein and to determine the *in vitro* release profiles in the presence and in the absence of proteolytic enzymes.
9. To evaluate the anti-tumour activity of mitoxantrone and 5-FU loaded microspheres and 5-FU-casein conjugates against Ehrlich ascites carcinoma (EAC) in mice.
10. Finally, to examine the possibility of cross-linking the protein using calcium and to evaluate the potential of calcium caseinate for controlled delivery of drugs.

CHAPTER 2
MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Materials

Bovine casein, bovine albumin, glycine, theophylline, 5FU, progesterone, protease (type VIII), glutaraldehyde (25% biological grade), trichloroacetic acid, (TCA), hexamethylene diisocyanate and pyridine were purchased from Sigma Chemical Co., St Louis, USA. Mitoxantrone manufactured by American Cyanamid company was a gift from Prof. E.P. Goldberg of the University of Florida, USA. Biomedical grade aliphatic polyurethane Tecoflex^{R} 80A employed as the suspension stabilizer was from Thermedics Inc (Woburn, MA, USA). Calcium chloride used was from S. D. Fine Chem. Ltd., Bombay, India. Progesterone Oucheck ELISA kit was from Cambridge Veternary Sciences, England. All other reagents were of highest purity commercially available locally. Distilled water was employed throughout.

2.2 Preparation of Glutaraldehyde Cross-linked Casein Microspheres

2.2.1 Preparation of Casein Solution

One gram casein was taken in a 15 mL beaker and 5 mL of 0.5 M NaOH solution was added. It was then stirred well using a glass rod until the solution became homogeneous. The solution was then kept for 10 min for the froth formed during stirring to disappear. This solution (pH ~13) was used for the preparation of the microspheres.

2.2.2 Viscosity Determination of Casein Solution

Viscosity of the solution was determined using a Brookfield Synchro-Lectric Viscometer (Model RVF-100, Brookefield Engineering Lab. Inc., USA). The spindle of the viscometer is dipped in casein solution and rotated. Reading was noted when the rotation was allowed to stop. Reading obtained was multiplied by the spindle factor to obtain the viscosity of the solution expressed as centipoise (cP).

2.2.3 Preparation of Glutaraldehyde Saturated Toluene (GST)

GST was prepared according to the method of Longo *et al* (1982). Toluene (200 mL) and aquous glutaraldehyde (25 mL) were taken in a beaker. It was then homogenized using a probe type sonicator (Soniprep, Model 150, MSE Scientific Instruments, UK) for 1 min. and the toluene layer was separated

using a seperating funnel. It was further dried over anhydrous sodium sulphate and was used for protein cross-linking.

2.2.4 Preparation of Placebo Casein Microspheres

2.2.4.1 Preparation of Microspheres by Paddle Stirring

A 1.6% (w/v) solution of polyurethane was made in dichloromethane (DCM) by dissolving the polymer in the solvent overnight by magnetic stirring. This solution, 40 mL was mixed with 25 mL of hexane in a 100 mL round-bottomed flask. The solution was stirred (500 to 1500 rev/min) using a stainless steel half-moon paddle stirrer at room temperature and 2 mL of a 20% solution of casein in 0.5M NaOH was added dropwise and the stirring continued for 5 min. GST prepared as described in section 2.2.3 was then introduced into the flask and the stirring continued for 1 h. After decanting the solvent, the microspheres were washed with a 1:1 mixture of DCM-hexane and centrifuged at 2000 rev/min. The process was repeated 5-6 times to remove the polymer. Complete removal of the polymer was ensured by adding a few drops of the washings into distilled water and checking for the presence of any polymer film. They were then washed with acetone, distilled water and sodium bisulphite solution and again with distilled water. Finally, the spheres were washed once with acetone and dried under vacum.

Microspheres of different size range were prepared by varying the concentration of polymeric stabilizer, stirring speed etc. Unless otherwise specified, microspheres designated as having high, medium and low cross-linking

densities were prepared using 15, 10 and 5 mL of GST for cross-linking the above standard recipe throughout the text.

2.2.4.2 Preparation of Microspheres by Homogenization

Microspheres of smaller size (<20 μm) were prepared by using a homogenizer (Tempest, Virtishear, USA) for dispersion instead of a paddle stirrer. Casein solution added to the organic medium containing the stabilizing agent in a round-bottomed flask was subjected to homogenization for 1 min. GST was then introduced for the cross-linking reaction. It was then stirred using the paddle stirrer for 3 h at 1000 rev/min. Microspheres were then processed as described in section 2.2.4.1.

2.2.5 Preparation of Placebo Albumin Microspheres

Microspheres of albumin were also prepared in a similar fashion except that the protein was dissolved in distilled water. As in the case of casein microsphere it was then dispersed in the polymer solution at a stirring speed of 1500 rev/min. It was then compared with casin microsphere prepared under the same conditions.

2.2.6 Quenching Aldehyde Handles in the Microspheres

Microspheres (0.5 g) were taken in a screw- capped test tube and treated with 15 mL of a 20% solution of glycine. It was then rotated for 4 h in a haemotology mixer (Fisher Scientific, Model 346, USA) filtered, washed with distilled water and dried.

2.2.7 Preparation of Theophylline-Loaded Casein Microspheres

A 20% solution of casein was prepared in 0.5M NaOH. Powdered theophylline (0.4 g, 0.6 g, and 0.8 g) was mixed with 2 mL of the casein solution and microspheres were prepared as described in section 2.2.4.1. The stirring speed employed was 1000 rev/min. Microspheres prepared were in the sodium salt form. Drug incorporation is expressed as weight in gram of the drug in 100 g of the microspheres (Wt%). Spheres in their acid form were prepared by washing them with 0.1 M HCl and drying.

2.2.8 Preparation of 5-FU-Loaded Casein Microspheres

Aqueous solutions of 5-FU at 1.5 and 3.0% concentrations were made in distilled water by heating to around 60°C. Approximately 100 mg of the spheres (cross-linked with 10 mL GST and size 10–150 μm) was equilibrated in 2 mL of the drug solution at 60°C for 6h. Spheres were centrifuged, washed once with distilled water and dried in vacuum. Glycine-quenched microspheres were also loaded with 5-FU in a similar fashion from a 3% solution.

2.2.9 Preparation of 5-FU-Casein Conjugate

The 6-(5-fluorouracil-1-yl)hexyl isocyanate was prepared according to the method of Ouchi *et al* (1989). To 0.15 g of 5-FU taken in a 50 mL round-bottomed flask was added 10 mL of dry pyridine and stirred with a magnetic pellet. After complete dissolution, 0.25 g of hexamethylene diisocyanate was added and the contents stirred for 2 h at 90°C. To this solution,

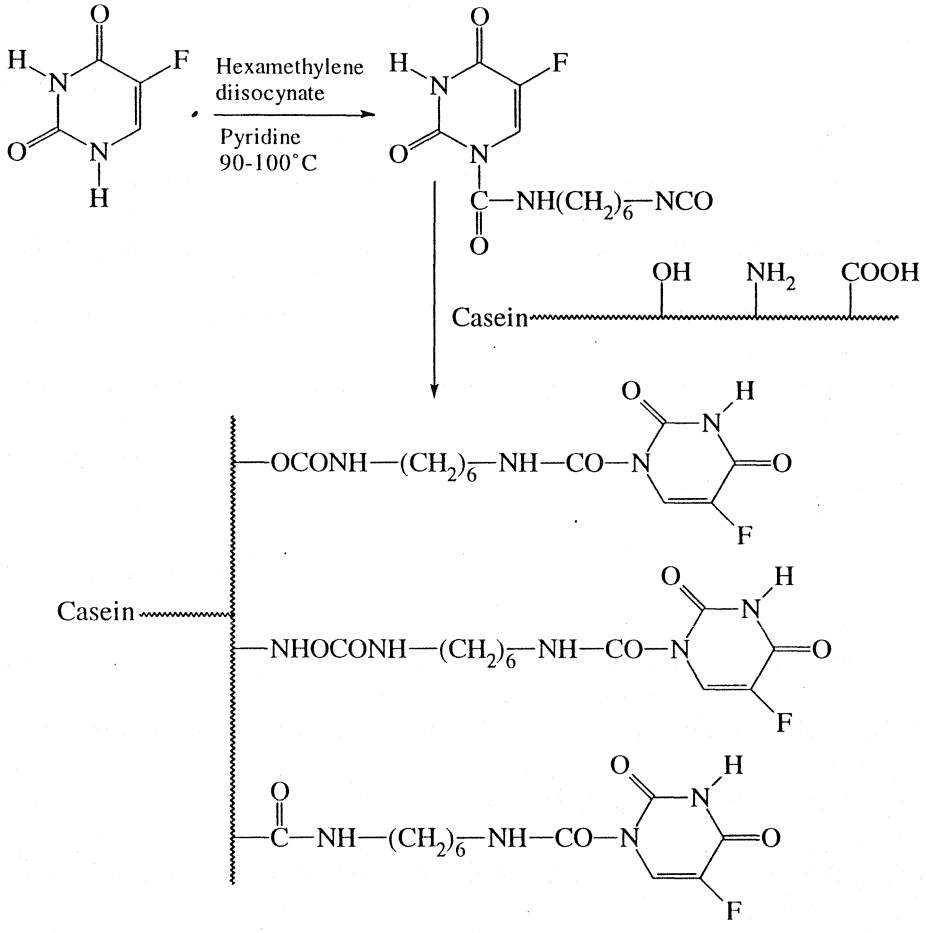

Figure 2.1 Structure of casein-5-FU conjugate

0.5 g of casein was introduced and the stirring continued for 24 h at room temperature (27°C). Precipitation of the conjugate was effected by the addition of acetone. The precipitate was washed with pyridine, followed by copious amounts of water, dried and ground in an agate mortar to partilcle size less than 200 μm. Figure 2.1 shows the possible structure of casein-5-FU conjugate.

2.2.10 Preparation of Mitoxantrone-Loaded Casein Microspheres

2.2.10.1 Loading into Preformed Spheres

One hundred mg of the microspheres (cross-linked with 10 mL GST and size 10-20 μm) was equilibrated with 2 mL of a saturated solution of the drug in water at room temperature for 6 h following which the microspheres were washed with water and centrifuged. Glycine quenched microspheres were used for drug incorporation.

2.2.10.2 *In situ* Loading

Two milliliters of 20% casein solution in 0.5 M NaOH was mixed with mitoxantrone (10 and 20% by weight of casein) and dispersed in a mixture of 40 mL DCM and 25 mL hexane containing 1% polyurethane as the suspension stabilizer in a 100 mL round-bottomed flask. The dispersion was homogenized using a homogenizer (Tempest Virtishear, USA) for 1 min, and 10 mL GST was introduced into the flask. The recipe was then stirred using a paddle stirrer at 1000 rev/min for 3 h. The microspheres formed were processed as described in section 2.2.4.2. The method generated particles having an average size of 20 $\pm$ 12 μm as determined using particle size analyzer.

2.2.11 Preparation of Progestrone-Loaded Casein Microspheres

Progesterone (10 and 20% by weight of casein) was added to 2 mL of a 20% casein solution in 0.5 M NaOH and mixed well until the solution was

homogeneous. Microspheres were prepared as described in section 2.2.4.1, at a stirring speed of 1500 rev/min.

2.3 Preparation of Calcium Caseinate

2.3.1 Preparation of Theophylline-Loaded Calcium Caseinate Beads

Five millilitres of a 20% solution of casein in 0.5 M NaOH with theophylline (50, 60 and 66% by weight of casein) dispersed homogeneously in it was forced through a 22 G needle from a syringe into 50 mL of calcium chloride solution taken in a beaker. The droplets were made to fall from a height of approximately 9 cm from the surface of the solution. Calcium chloride solutions of various concentrations (2 M, 4M and 6M) were used to prepare beads of different cross-linking density. After the beads were allowed to equilibrate in solution for 6 h, the solution was decanted, washed thrice with distilled water, frozen and lyophilized. Beads having different drug loadings were prepared by incorporating the required amount of the drug in casein solution.

2.3.2 Preparation of Theophylline-Loaded Calcium Caseinate Granules

Five millilitres of a 20% solution of casein in 0.5 M NaOH with theophylline (50, 60 and 66% by weight of casein) dispersed homogeneously in it was mixed with .02 moles of calcium chloride in 2 mL water. The gel obtained was dried in vacuum at room temperature, crushed in an agate mortar and sieved into the desired size using test sieves. The sieved particles were further washed with copious amount of water, dried and stored in a desiccator.

2.4 Estimation of Free Glutaraldehyde in GST and in the Microspheres

Glutaraldehyde in GST and in the microspheres was estimated by the ε-aminocaproic acid method (Lynn *et al* 1990). GST (1 mL) or microspheres (5 mg) was taken in a screw capped test tube. It was then mixed with 5 mL of 0.01 M phosphate buffer and rotated in a haemetology mixer (Fisher Scientific, USA) overnight to extract the glutaraldehyde to the aqueous medium. This solution was mixed with 5 mL of 0.006 mole (%) ε-aminocaproic acid in 0.01 M phosphate buffer and kept at 90°C in a water bath for 90 min. It was chilled and the absorbance was noted at 253 nm and 300 nm. Concentration and hence amount of glutaraldehyde was calculated from the calibration curve.

2.5 Internal Structure of the Microspheres

Microspheres (75-150 μm size cross-linked with 10 mL GST) were incubated in 2% osmium tetroxide for two weeks. It was then washed with distilled water and dried. Microspheres were then embedded in araldite (AR 502). Semithin sections of 1–2 μm were cut using glass knives in an ultramicrotome (Model 2088 ultratome, LKB-V, Sweden). Sections were stained with 1% toluidine blue and examined in transmission electron microscope (Hitachi Model H-600, Japan) and in optical microscope, (Nikon, binocular optiphot, Japan).

2.6 Scanning Electron Microscopic Studies

Microspheres were sprinkled onto double sided adhesive tape fixed on aluminium stubs, sputter coated with gold and examined in the microscope (Jeol JSM 35C) or (Hitachi Model S-2400, Japan) at a suitable accelerating voltage.

2.7 Thermal analysis

Differential thermal analysis (DTA) of the placebo spheres and the spheres loaded with theophylline was done using a Dupont (Model 990) thermal analyser system with standard DTA cell accessory in an atmosphere of nitrogen at a heating rate of 10°C per min. Differential scanning calorimetry (DSC) was done using a Dupont (Model 910) thermal analyser system under the same conditions.

2.8 Swelling Studies

2.8.1 Preparation of Biofluids

2.8.1.1 Preparation of Simulated Intestinal Fluid

Simulated intestinal fluid was prepared by dissolving 6.8 g monobasic potassium phosphate in 250 mL water, adding 190 mL of 0.2 M NaOH and 400 mL water, adjusting the pH of the solution to 7.5 and then making up to 1000 mL with water.

2.8.1.2 Preparation of Simulated Gastric Fluid

Simulated gastric fluid was prepared by dissolving 2 g NaCl in 7.0 mL HCl and making up to 1000 mL with water.

2.8.1.3 Preparation of Phosphate Buffer (0.1 M, pH 7.4)

Disodium hydrogen phosphate (17.97 g) and sodium hydrogen phosphate (5.73 g) were dissolved in distilled water and made up to 1000 mL.

2.8.2 Kinetics of Swelling in Biofluids

The kinetics of swelling of placebo casein spheres in biofluids was examined in the following manner. One tenth of a gram of the microspheres was taken in different test tubes and 10 mL of the biofluids was added into each tube and incubated at 37°C. Tubes were taken at different time intervals, the spheres were filtered and blotted using a Whatman 1 filter paper and transferred into small beakers and weighed. Microspheres were then washed with acetone and dried to constant weight at under vacum. From the difference in weight of the dry and swollen spheres, the fluid content was estimated using the following relation:

$$\text{Fluid content } (\%) = \frac{100 \times \text{Wt. of swollen spheres} - \text{Wt. of dry spheres}}{\text{Wt. of swollen spheres}}$$

Fluid content at steady state is taken as equilibrium fluid content (EFC)

2.9 *In vitro* Degradation of Microspheres

2.9.1 Using Turbidity Measurement

In vitro degradation of the microspheres in presence of protease was followed by measurement of turbidity of the suspension at 600 nm in a UV-visible spectrophotometer (Schimadzu, Model UV-240, Japan). To 10 mL of phosphate buffer containing various amounts of protease in a test tube was introduced 5 mg of the microspheres (75-150 μm) and incubated at 37°C. The turbidity of the dispersion was measured for various periods of time till the solution attained clarity.

2.10 Particle Size Analysis

2.10.1 Analysis Using Laser Diffraction

Microspheres were suspended in a solution of chloroform containing 30% (v/v) acetone and sonicated for 2 min in a bath type sonicator (Vibronics, Model 80 W, Bombay) to ensure complete dispersion before analysis. The chloroform/acetone mixture was found to have the optimum density for keeping the particles in suspension by mild stirring during analysis. Particle size analyses of the microspheres were carried out using a computerised laser based particle size analyser (Galai CIS-1, Israel).

2.10.2 Analysis Using Test Sieves

Around 500 mg of the microspheres were sieved through standard test sieves (Filterwel, Bombay, India) and the fractions that passed through each sieve

but retained on the other sieve were weighed in an analytical balance. The percentage weight fraction was then plotted against the particle size.

2.10.3 Analysis Using Optical Microscope

Fifty to sixty spheres were randomly selected and the fractions were subjected to particle size measurement using the travelling microscope of the gonometer (Rume'-Hort, 100-00-230, USA).

2.11 Estimation of Drug Loading

2.11.1 Estimation of Theophylline Loading

Theophylline loaded casein microspheres/granules/beads (5 mg) was powdered in an agate mortar and extracted with 5 mL of methanol in a screw capped test tube over 3 days by rotating the tubes in a haematology mixer (Fisher, Model 346, USA). After filtering through a 0.45 μm filter (Sigma, USA), a known volume was made up in a 100 mL standard flask and the absorbance was measured at 274 nm in a UV-Vis spectrophotometer (Hitachi, Model 220, Japan). No other component was found to absorb at this wavelength. The efficacy of the method was checked by powdering equal amount of theophylline and placebo casein spheres/beads/granules and extracting the drug as before and estimating the concentration of the drug in the methanol extract spectrophotometrically. The accuracy of the method was found to be within $\pm$ 2%. Similar extraction procedure has been validated by earlier workers (Thanoo *et al* 1992). All assays were carried out in triplicate.

The incorporation efficiency of theophylline in the microspheres was determined from the ratio of the weight of theophylline incorporated to the weight of theophylline initially taken. The percentage actual and theoretical loadings reported were calculated on the basis of the amount of theophylline incorporated and the amount of theophylline initially employed for 100 g of casein.

2.11.2 Estimation of Mitoxantrone Loading

Mitoxantrone loaded microspheres were digested using protease and analysed for the total drug content. Microspheres, 2 mg was added to 10 mL of phosphate buffer containing 2 mg of protease and incubated at 37°C. After complete digestion, the protein was precipitated using TCA, centrifuged and the aqueous layer was analysed for the drug using a UV-Vis spectrophotometer (Shimadzu, Model UV-240, Japan) at 610 nm according to knepp *et al* (1993). The accuracy of the method was ascertained by incubating a known amount of drug and placebo spheres in protease solution, precipitating the protein after digestion using TCA and analysing the drug as before. Protein digestion and precipitation did not interfere with the determination of the drug content.

2.11.3 Estimation of 5-FU Loading

2.11.3.1 Estimation of 5-FU in Drug Conjugate

Drug conjugate, 6 mg was refluxed with 10 mL of 3 M NaOH for 2 days in a 50 mL round-bottomed flask fitted with a water condensor. Two mL of

this solution was mixed with 2 mL of 3 M HCl and the precipitated protein was removed by centrifugation at 2000 rev/min. The clear solution was analysed for 5-FU at 266 nm spectrophotometrically according to Ouchi *et al* (1989).

2.11.3.2 Estimation of 5-FU in the Microspheres

Microspheres, 2 mg was added to 10 mL of phosphate buffer containing 2 mg of protease and incubated at 37°C. After complete digestion, the protein was precipitated using TCA, centrifuged and the aqueous layer was analysed for the drug using a UV-Vis spectrophotometer (Shimadzu, Model UV-240, Japan) at 266 nm. The accuracy of the method was ascertained by incubating a known amount of the drug and placebo spheres in protease solution, precipitating the protein after digestion using TCA and analysing the drug as before. Protein digestion and precipitation did not interfere with the determination of 5-FU.

2.11.4 Estimation of Progesterone

Progesterone content in the microspheres was analysed after digesting the matrix as described in section 2.11.2. After removing the protein content by precipitation using TCA, the supernatant was assayed for progesterone at 247 nm spectrophotometrically (Dunn 1981).

2.12 *In vitro* Release Studies

2.12.1 *In vitro* Release of Theophylline

Release studies were carried out using a paddle type dissolution apparatus (USP standard) in simulated gastric and intestinal fluids. Drug loaded microspheres were taken in the dissolution medium maintained at 37°C and stirred at a speed of 100 rev/ min using a Teflon^R paddle stirrer. Aliquots of 1 mL were removed at various time intervals after the microspheres were allowed to settle down and assayed spectrophotometrically at 274 nm. An equal volume of the dissolution medium was immediately added after withdrawal of each aliquot to maintain a constant volume.

2.12.2 *In vitro* Release of 5-FU

Drug release from microspheres and casein-5-FU- conjugate was followed in phosphate buffer. Microspheres or drug-casein conjugate (50 mg) was added to 100 mL buffer (0.1 M, pH 7.4) at 37°C and stirred at 100 rev/min using a Teflon^R paddle stirrer. Aliquotes of 1 mL were withdrawn at various time intervals after the microspheres were allowed to settle down and 5-FU content was analysed at 266 nm spectrophotometrically. An equal volume of buffer was added to the medium after withdrawal of each aliquot. Drug release was also followed in the presence of proteolytic enzyme (0.005% protease). In this case, protein in the aliquot was precipitated using TCA and centrifuged. The supernatant was filterted through a 0.45 μm filter (Sigma USA) and then analysed for the drug content.

2.12.3 *In vitro* Release of Mitoxantrone

Experimental details were similar to those described in section 2.12.2.

2.12.4 *In vitro* Release of Progesterone

Microspheres (10 mg) was added to 100 mL phosphate buffer (pH 7.4, 0.1 M) and kept at 37°C. Aliquots of 1 mL were taken at different intervals of time and analysed using UV spectrophotometer at 247 nm. Release studies in the presence of proteolytic enzyme (0.005% protease) were conducted after precipitating the proteins using TCA as described in Section 2.12.2 for 5-FU.

2.13 *In vivo* Studies

2.13.1 Biodegradation and Biocompatibility of Microspheres in Rats

The *in vivo* degradation of placebo casein spheres injected intramuscularly into rats was studied over a one year period. Highly cross-linked microspheres was only employed in this study in order to examine the maximum tissue residence time achievable by the microspheres. For this purpose, microspheres were prepared by cross-linking the standard recipe using 15 mL GST, 30 mL GST and with 30 mL GST followed by 3 mL 25% aquous glutaraldehyde. Wistar rats were employed in the study. Microspheres, (75–150 μm size) 5 mg were suspended in 1 mL physiological saline and injected into the gluteal muscle using a 21 G needle. Each animal received two injections on either side of the muscle. Animals were sacrificed at different time intervals and the tissue along with the microspheres at the site of injection was removed and fixed in 10%

buffered formalin. Sections having a thickness of 5 μm were cut, stained with haematoxylin and eosin and were examined in the microscope for the extent of biodegradation and tissue compatibility.

2.13.2 Bioavailability of Theophylline in Rabbits

Rabbits weighing 1.6 to 2.5 kg were used for the study. Animals were fasted for 24 h prior to the administration of the drug. Each rabbit ($n = 3$) was given a dose of 20 mg/kg body weight of theophylline powder or an equivalent dose in microsphere/granule/bead form with 10 mL water orally through a catheter. Water was provided ad libitum during fasting and throughout the experiment. Blood samples (1 mL each) were collected at 1, 2, 3, 4, 6, 9, 12 and 24 h from the ear vein after administration. Samples were incubated at 37°C till clotted. It was then centrifuged at 1500 rev/min for 20 min and the serum was separated. Theophylline from the serum was extracted and analysed in the following way.

2.13.2.1 Analysis of Theophylline in Serum

Various methods available for the determination of theophylline concentrations in the serum have been reviewed (Hendeles *et al* 1978). The following procedure was adopted in the present study. Into 0.5 mL of serum was added 0.4 g ammonium sulphate and theophylline was extracted using 15 mL of chloroform/hexane (7:3) mixture by stirring the contents magnetically for 40 min. The organic layer was separated and 10 mL of the same was extracted using 3 mL of 0.1 M (pH 9.0) carbonate buffer after shaking in a haematology

(Fisher scientific, Model 346, USA) mixer for 30 min. The aqueous layer was then separated and the absorbance of the same was measured at 274 nm. The accuracy of the method was ascertained by adding known amounts of theophylline into pure serum and extracting the same as before. The recovery was always more than 90%.

2.13.2.2 Pharmacokinetic Analysis

The elimination rate constant K_{el} was calculated from the semi-logarithmic plot of serum concentration versus time. K_{el} was determined from the slope of the terminal linear portion of the curve using linear regression analysis. Elimination half-life values $t_{1/2}$ were calculated by dividing 0.693 by the elimination rate constant. The area under the curve $AUC_{0-\infty}$ is calculated from

$$AUC_{0-\infty}=AUC_{0-t}+C_t/K_{el}$$

AUC_{0-t} is the area under the curve from the time 0 to t calculated using the trapezoidal rule. C_t is the concentration at time t (Maruyama *et al* 1989).

2.13.3 Antitumour Effect on Ehrlich Ascites Carcinoma (EAC)

2.13.3.1 Antitumour Effect of Mitoxantrone-Loaded Microspheres in Mice

Swiss albino mice weighing 25 to 35 g were employed in the study. Four groups of 7 mice were used. They were inoculated with 2×10^6 EAC cells in 2 mL phosphate buffered saline (PBS) intraperitoneally using a 23 G needle.

On the second day after inoculation, one group received microspheres (of size 10-20μm, cross-linked with 10 mL GST and drug pay load 10.5%) equivalent to 1 mg of mitoxantrone in 2 mL PBS containing 0.05% Tween 80, another group received 1 mg of free drug in the same vehicle, the third group received placebo spheres in the same manner and the fourth group was given PBS alone, all intraperitoneally. Animals were provided commercial rat diet and water ad libitum. Therapeutic efficacy was determined by monitoring changes in body weight and survival time of each animal for 60 days. Values were compared for their significance using the students' *t*-test.

2.13.3.2 Antitumour Effect of 5-FU Loaded Microspheres/Drug Conjugate- in Mice

Experiment was similar to 2.13.3.1 except that microspheres and drug conjugate equivalent to 2 mg 5-FU were administered. Microspheres of size 10-20 μm cross-linked with 10 mL GST and containing 7% 5FU and conjugate having 15% drug loading were employed in the study.

2.13.4 Bioavailability of Progesterone in Rabbits

Male rabbits (New Zealand White) weighing 1.7 to 1.75 kg were used for the study. Microspheres of size 75-150 μm, cross-linked with 10 mL GST and containing 10% by weight of progesterone were used for *in vivo* experiments. Each rabbit (n=3) was given a dose of 5 mg/kg body weight of progestrone powder or equivalent dose in microsphere form in 2 mL sterile saline containing

0.1% Tween 80, intramuscularly into the gluteal muscle. Blood samples (1 mL) was collected immediately before administration, and at different time intervals after drug administration upto six months. Blood sample was centrifuged at 3000 rev/min for 10 min and the plasma was seperated. Progesterone concentration in plasma was determined by enzyme-linked immunosorbent assay (ELISA) using the kit obtained from Cambridge veternary Sciences, Cambridge, England in a microplate reader (Biotek, EL 311, USA) as described below.

The 96-well ELISA plates were exposed, emptied the contents and tap dried on an absorbent paper. Test samples (10 μl) each were loaded into the wells of plate in duplicate. Conjugate (200 μL) was added to each well and kept at room temperature for 30 min. Wells were then emptied and washed thrice with cold water and tap dried on absorbent paper. Substrate reagent 200 μl were loaded into each empty wells. Wells were then covered with a sheet of paper and kept at room temperature for 30 min. Stopping solution (100 μl) was added to all wells and the absorbances were read at 405 nm. Standards given in the kit was used for the preparation of calibration curve. The standard curve was run on each plate along with the test samples every time in each assay, in order to avoid plate to plate assay variation.

CHAPTER 3
RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Preparation and Characterization of Casein Microspheres

3.1.1 Preparation of Glutaraldehyde Cross-linked Casein Microspheres Using Low Concentrations of Polymeric Dispersing Agents

Two methods are widely employed for the preparation of protein microspheres. One involves heat denaturation of the protein at temperatures ranging between 100 and 180°C and the other, chemical cross-linking of the protein using a suitable cross-linking agent such as glutaraldehyde in vegetable oil emulsions (Kramer 1974; Senyei *et al.*, 1981). Both methods have limitations. The heat denaturation technique can not be employed in the case of drugs such as adriamycin which has low thermal stability and the aldehyde cross-linking affects activity of drugs such as methotrexate, epinephrine, salbutamol etc. (Gupta and Hung 1989a). Microspheres prepared by these methods are somewhat hydrophobic in character such that surfactants are needed for their homogeneous dispersion in water (Widder *et al* 1979). Longo *et al* (1982) and Goldberg *et al* (1984) postulated that surface hydrophilicity is important because a hydrophilic surface may enhance surface physical and chemical

behaviour *in vivo*. These authors also suggested that the surfactants used in small concentrations to disperse the particles in aqueous vehicles may be undesirable as they may influence tissue reactions and *in vivo* release. They prepared hydrophilic albumin microspheres using a steric stabilization method. Highly concentrated solutions (25- 30%) of poly(methyl methacrylate) or poly(oxyethylene)- poly(oxypropylene) were employed as the dispersion medium to effect stabilization of aqueous albumin droplets. Two unique features of their method for albumin microsphere preparation as compared to other methods are the addition of the glutaraldehyde cross-linking agent in an organic medium to the aqueous albumin dispersion and the use of concentrated polymer solutions as the organic phase for preparing the dispersion. Uniform, round, hydrophilic microspheres were readily prepared by this process.

The technique reported by Goldberg *et al* (1984) is distinctly superior to other methods of albumin microsphere preparation as the method generates spheres of excellent sphericity, a very smooth surface and high hydrophilicity. However, the high concentrations of the polymer solutions employed to stabilize the droplets were often difficult to be removed completely by repeated washings and centrifugation especially from preparations of micron or submicron particles. Lower concentrations of these polymers did not give rise to good stabilization effect for the protein solution droplets. The method reported here involves the use of very low concentration of polymer solution for the preparation of casein microspheres.

Casein is a milk protein used commercially as glues, plastics, fibres and in paints. Casein is insoluble in water and can be solubilised in acid and

in alkaline medium. Because of its limited and variable solubility in acids, alkaline solutions are used for most of its applications. Dilute solutions of sodium hydroxide, potassium hydroxide and ammonium hydroxide could be employed. Concentration of the solutions could be anywhere from 0.1 M to 1.0 M. Solubility increases with increase in concentration of the alkali. Concentrations of the casein solutions in such solutions could be in the range 5.0% to 30%. Ease of microsphere preparation depends on the viscocity of the casein solution. Microspheres used in this study were produced from casein solution in sodium hydroxide solution. Viscosity of casein solutions of different concentrations prepared in 0.5 and 1.0 M sodium hydroxide solution is shown in Table 3.1.

Table 3.1
Viscosity of casein solution of different concentrations in 0.5 and 1 M NaOH solution

Concn. of casein solution in 0.5 M NaOH (%)	Viscosity of the solution (cP)	Concn. of casein solution in 1.0 M NaOH (%)	Viscosity (cP)
5.0	23.2	5.0	22.4
10.0	28.0	10.0	17.6
15.0	60.0	15.0	60.0
20.0	240.0	20.0	200.0
25.0	Not completely soluble	25.0	400.0
30.0	,,	30.0	760.0

A 20% solution of casein in 0.5 M NaOH was found to have optimum viscosity to be dispersed as good spherical droplets using a suitable stabilizing

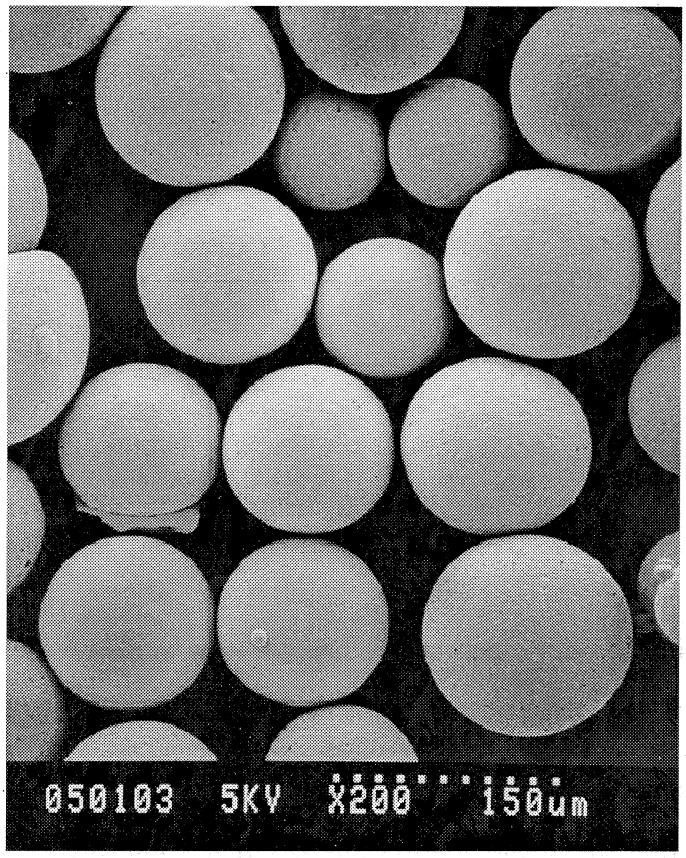

Figure 3.1. SEM of casein microspheres prepared by paddle stiring using 1% solution of polyurethane as the dispersion medium.

agent. Casein microspheres cross-linked with glutaraldehyde were prepared as described in chapter 2 (Section 2.2.4). Although low hydropilic-lipophilic balance (HLB) surfactants such as sorbitan sesquioleate were capable of producing a good dispersion of casein in non-aqueous solvents at a concentratrion of around 2% (Knepp et al., 1993; Jayakrishnan *et al.*, 1994), it was found that the aliphatic polyurethane Tecoflex^{R} 80A provided steric stabilisation of the protein solution droplets at much lower concentrations and generated spheres of excellent sphericity (Figure 3.1). The polyurethane used was medical grade,

structure of which is shown in Figure 3.2.

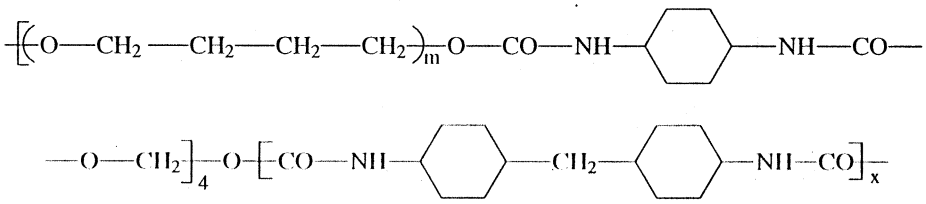

Figure 3.2 Structure of polyurethane (Telecoflex 80A).

Glutaraldehyde cross-linking was used for hardening the protein solution droplets. Compared to other aldehydes that could be used to cross-link polymers, glutaraldehyde has the advantage that it reacts relatively fast, is able to span various distances between the protein molecules and is able to react with a large number of available amino groups present in the molecule to form a more tightly cross-linked network (Fein and Filachione 1957; Fein *et al.*, 1959; Bowers and Carter 1964). Casein microspheres prepared in this study involves the addition of glutaraldehyde as a saturated solution in toluene. Addition of the cross-linking agent through an organic medium limits the cross-linking predominantly to the surface of the microspheres. This increases the swelling ability of the microspheres since the matrix is not extensively cross-linked internally. Concentration of glutaraldehyde in GST was calculated by reacting with ε-aminocaproic acid (Lynn *et al* 1990) after extracting it in phosphate buffer as described in chapter 2 (Section 2.4). Concentration of

glutaraldehyde in GST was found to be around 2%. Excess aldehyde present in the microspheres was removed by washing with sodium bisulphite solution. Residual aldehyde in the microspheres was examined by extracting in phosphate buffer and analysing using the ε-aminocaproic acid method (Chapter 2, Section 2.4). Microspheres showed no detectable residual glutaraldehyde in them. Aldehyde handles present in the microsphere were endcaped with glycine as described in Chapter 2, Section 2.2.6.

Albumin microspheres of comparable size could also be prepared by this method (Figure 3.3). This method may also be used for the preparation of other protein and polysaccharide microsphers.

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3.1.2 Evaluation of Casein Microspheres for Drug Delivery

To appreciate the feasibility of using casein in drug delivery application it is useful to study the general characteristics of the microspheres and set the stage for further study.

The distribution of microspheres introduced into the body and drug release from microsphere formulations depend largely on the particle size of the microspheres. Drug targeting could also be acheived by exploiting the natural distribution of microspheres when they are introduced into the body (Davis and Illum 1983). Microspheres of small size were reported to be taken up by macrophages. This opens up the possibility of intracellular targeting

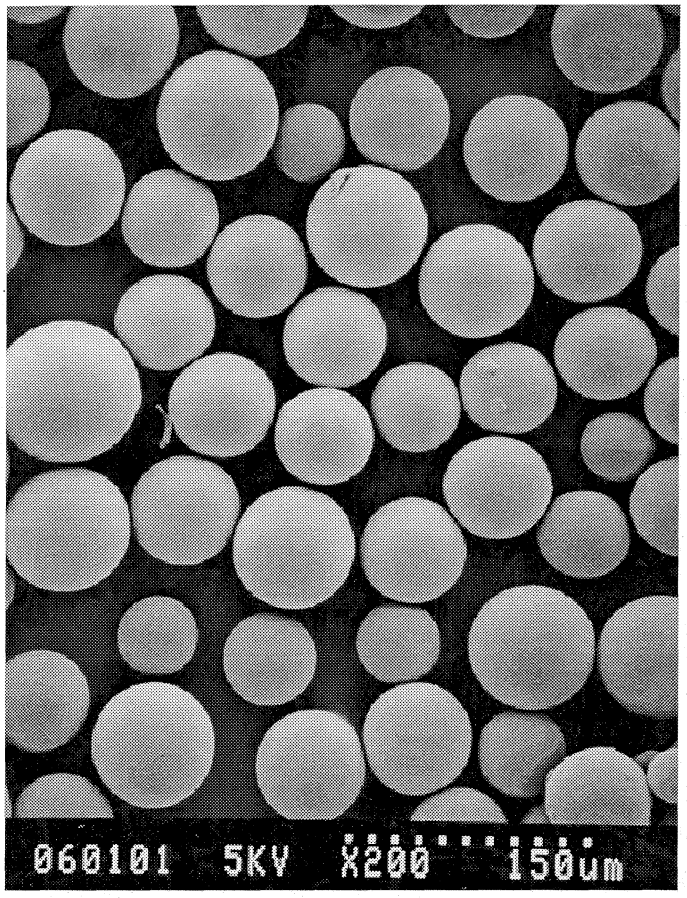


Figure 3.3. SEM of albumin microspheres prepared by paddle stirring using 1% solution of polyurethane as the dispersion medium.

in macrophage-associated diseases. The method reported here affords the preparation of microspheres of wide size range. Particle size of the microspheres was found to be largely dependent on the method of dispersion. Microspheres obtained by paddle stirring was of large size (Figure 3.1). Smaller spheres of size 2-10 μm were obtained when dispersion was carried out using a homogenizer. Figure 3.4 shows the SEM of microspheres prepared after dispersing using a homogenizer. Size distribution was also affected by the

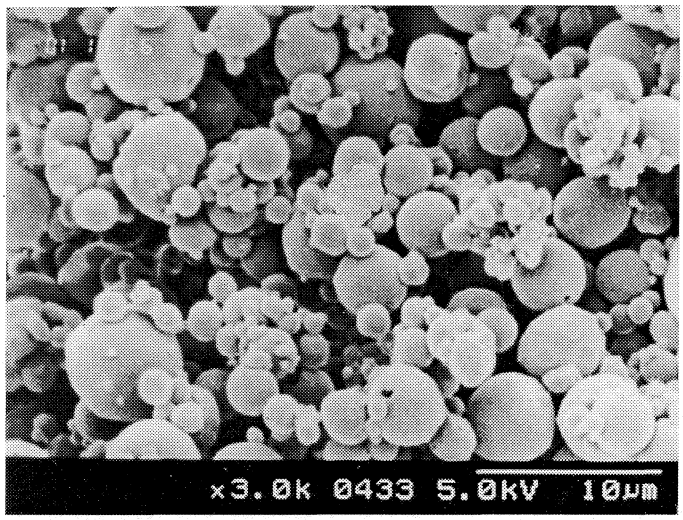


Figure 3.4. SEM of casein microspheres prepared by homogenization using 1% solution of polyurethane as the dispersion medium.

concentration of the polymeric stabilizer used and stirring speed employed as shown in Figures 3.5 and 3.6. Particle size distribution was determined by laser analysis as described in Chapter 2 (Section 2.10.1). Figure 3.5 shows the variation in distribution with stirring speed and Figure 3.6 shows the variation in distribution with the concentration of the polymeric stabilizer.

Internal structure of the microspheres was examined by employing 1-2 μm thick sections prepared as described in Chapter 2 (Section 2.5) by optical and transmission electron microscopy. Figure 3.7a shows the internal structure of placebo casein spheres by optical microscopy and Figure 3.7b shows the same under a transmission electron microscope. Microspheres were found to be highly porous which should facilitate the loading of many drugs after the microspheres are prepared and washed free of all impurities.

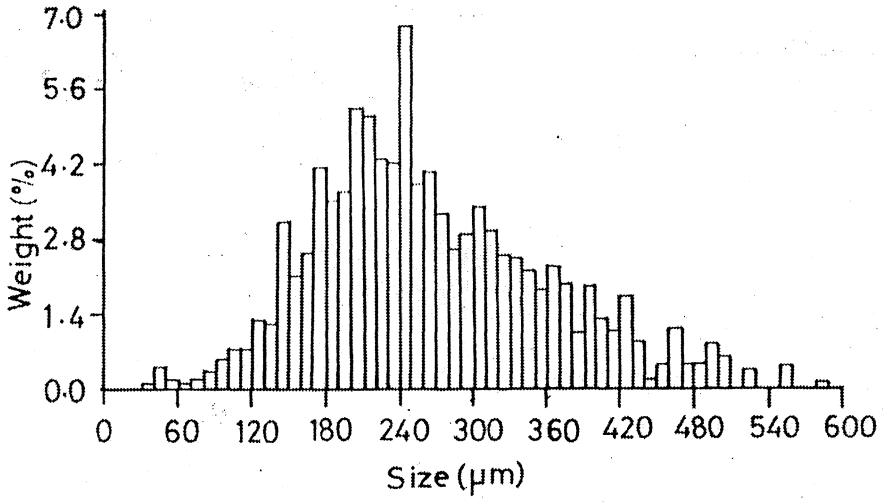


Figure 3.5a. Particle size distribution of casein microspheres prepared at a stirring speed of 500 rev/min with 1% solution of polymer with mean diameters 265 ± 96 μm.

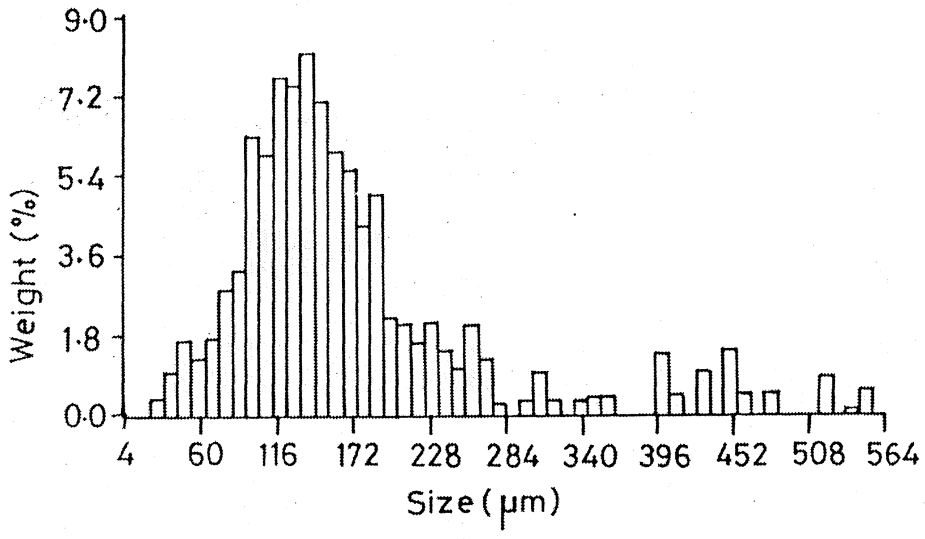


Figure 3.5b. Particle size distribution of casein microspheres prepared using 1% polymer solution at stirring speed 1500 rev/min having mean diameters 172± 97μm.

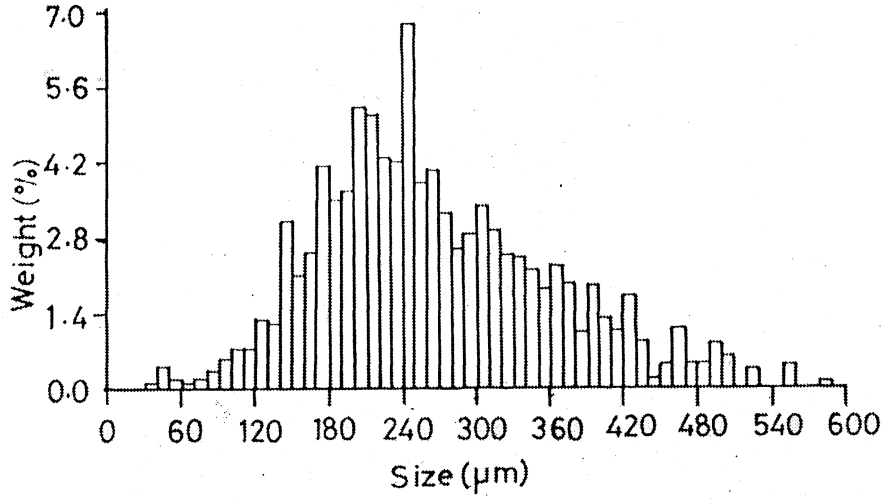


Figure 3.6a. Particle size distribution of casein microspheres prepared using 1% polymer solution at stirring speed 500 rev/min having mean diameters 265± 96μm.

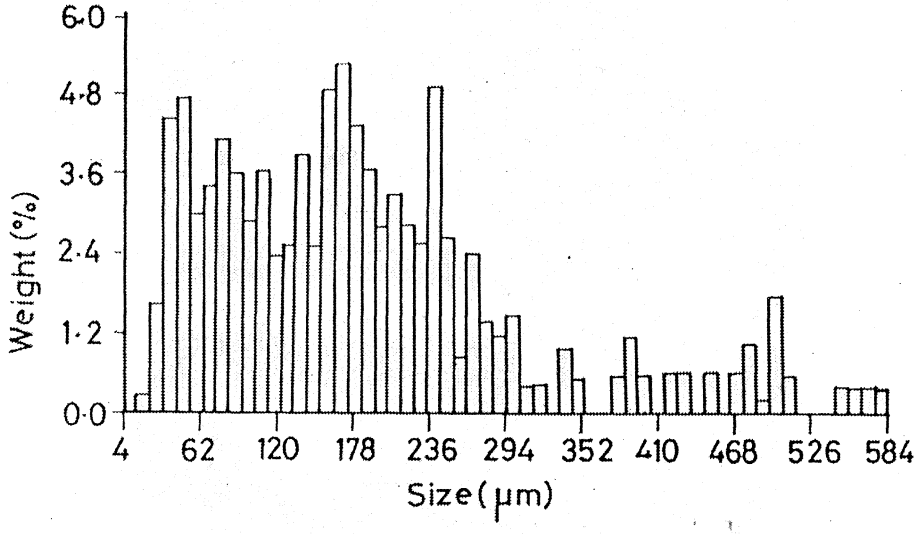


Figure 3.6b. Particle size distribution of casein microspheres prepared at a stirring speed of 500 rev/min with 4% solution of polymer with mean diameters 186 ± 121 μm.

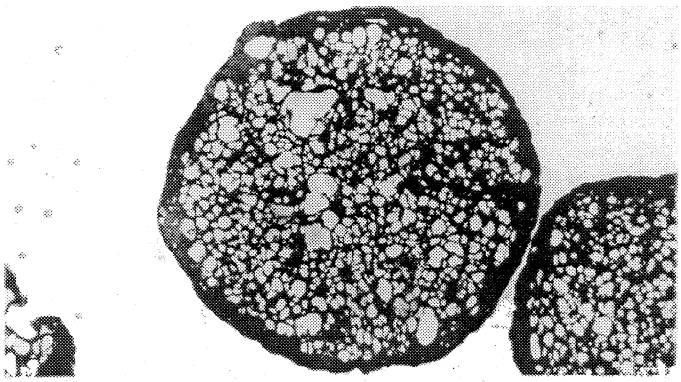


Figure 3.7a. Internal structure of casein microspheres examined by optical microscope (Original magnification 350 x).

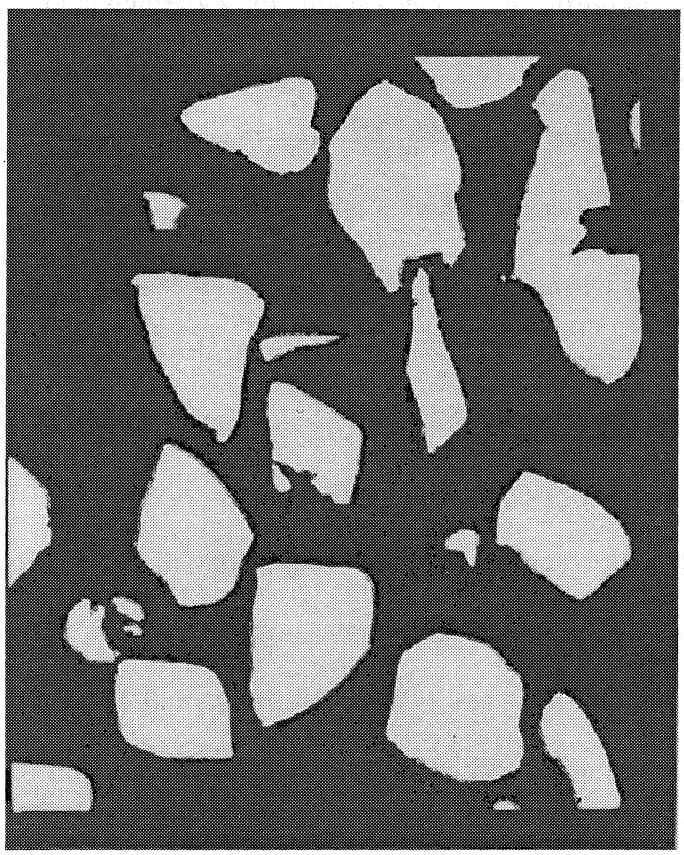


Figure 3.7b. Internal structure of casein microspheres examined by transmission electron microscope (Original magnification 3500 x) Pore size is approximately 1 μm.

Hydrophilicity of the matrix is very important in drug delivery. Hydrophilic microspheres could be easily dispersed for injection without the use of surfactants which may influence tissue reaction, drug release and drug activity. High payloads of water soluble drugs could be obtained by loading them into preformed microspheres from concentrated aqueous solutions of the drug. Wet chemical reactions for surface and bulk modification of the microspheres would readily be accomplished if microspheres possessed high hydrophilicity.

The swelling characteristics of microspheres in different fluids were determined as described in Chapter 2 (Section 2.8). EFC (%) of medium cross-linked microspheres (cross-linked using 10 mL GST) in gastric fluid, intestinal fluid, phosphate buffer (0.1 M, pH 7.4) and distilled water are given in Table 3.2. EFC (%) is a measure of hydrophilicity of the matrix. Microspheres prepared were found to absorb large amount of fluids as evidenced by their EFC. Ionization has an important role in the swelling behaviour (Castel et al 1990). High hydrophilicity of the microspheres is due to the ionization of the -COONa groups present in the microsphere matrix. Carboxyl groups present in casein gets converted into their sodium salt on dissolution in NaOH and exist as such in the microspheres. Ionization is greater in intestinal fluid and phosphate buffer beccause of the high pH of the medium. In gastric fluid, the swelling is less because of the low pH of the medium. On acidification, the sodium salt of the carboxyl functions in the proteins is converted back to -COOH. The EFC decreases due to the low ionization of the COOH group.

Table 3.2
EFC (%) of casein microspheres in the sodium salt form and H^+ form in biofluids at 37°C

Biofluid	(Sodium salt form)	(H^+ form)
Distilled water	90 ± 4.2	22 ± 3.1
Gastric fluid	45 ± 3.2	13 ± 2.7
Intenstinal fluid	81 ± 2.5	31 ± 3.4
Phosphate buffer	83 ± 3.7	29 ± 2.1

Swelling of the microspheres was also found to depend on cross-link density. Table 3.3 shows the EFC (%) of microspheres of low cross-link density, medium cross-link density and high cross-link density (5, 10 and 15 mL GST used for cross-linking) in distilled water. Decrease in swelling with increasing cross-link density is due to the tightly interconnecting structure.

Table 3.3
EFC (%) of microspheres of varying cross-link density in distilled water

Cross-link density	EFC (%)
Highly cross-linked (15 mL GST)	82 ± 3.7
Medium cross-linked (10 mL GST)	90 ± 4.2
Lightly cross-linked (5 mL GST)	94 ± 2.1

3.1.3 Degradation and Toxicity of the Microspheres

3.1.3.1 Degradation studies *in vitro*

Degradability of the microspheres is an important parameter. Biodegradability of the carrier matrix influences the drug release profiles when microspheres are implanted in the living tissue. Biodegradability is also important for

an implanted drug carrier, since it does not necessitate the removal of the drug-loaded implant after the drug is depleted and absorbed in the body.

In vitro degradation of the microspheres was determined by turbidity measurements of the solution after incubating the microspheres in phosphate buffer containing a proteolytic enzyme as described in Chapter 2, section 2.9.1.

Protease was found to digest the microspheres completely giving rise to a clear solution. Time taken to obtain a clear solution was taken as the time for digestion. Figure 3.8 shows the variation of degradation time with cross-link density. Degradation rate is also dependent on the concentration of protease solution. Figure 3.9 shows the variation of degradation time with concentration of protease solution.

The proteolytic stability of the microspheres was also checked using pepsin and pancreation, in order to examine their susceptibility to degradation during transit in the GI tract if they were to be employed for the sustaind delivery of oral drugs. Simulated gastric fluid containing 0.32% pepsin and intestinal fluid containing 1% pancreatin (USP standard) at 37°C were not found to digest the microspheres even after 24 h. Microscopic examination before and after incubation did not show any degradation. Thus, it can be presumed that glutaraldehyde cross-linked bovine casein spheres would not be succeptible to enzymatic attack during its transit in the GI tract at the enzyme concentrations present in the Gl tract.

3.1.3.2 Degradation studies *in vivo*

Microspheres cross-linked with 15 and 30 mL GST (Type I & II) and those cross-linked with 30 mL GST followed by 3 mL 25% aqueous glutaraldehyde (Type III) were used for the *in vivo* degradation studies. Such high concentrations of glutaraldehyde were employed for cross-linking the microsphere in this study inorder to examine the maximum tissue residence time achievable by the microspheres. At seven days post implantation, microspheres of all types were present in the skeletal muscle, with most of them clustered together (Figure 3.10). The spheres had a homogeneous eosinophilic appearance. The inflammatory cellular infilterate between spheres and around implanted groups as a whole consisted predominantly of fibroblasts and macrophages. Fibrocytes formed a single or double layer close to the microsphere margin. Infiltrate of neutrophils was found to invade the sphere material. Foreign body-type giant cells were seen adjacent to microspheres.

At six months post-implantation, the speres seem to have lost their shape and size. Imflammatory response is scant and consist predominantly macrophages and fibroblast (Figure 3.11). At nine months post-implantation, broken degraded masses of microspheres were seen for all three types (Figure 3.12). Microspheres still elicited a response of macrophages. Foreign body-type giant cells were still present adjacent to the spheres.

At 12 months post-implantation, it was very difficult to locate remnants of microspheres by gross examination. Microscopic examination revealed only

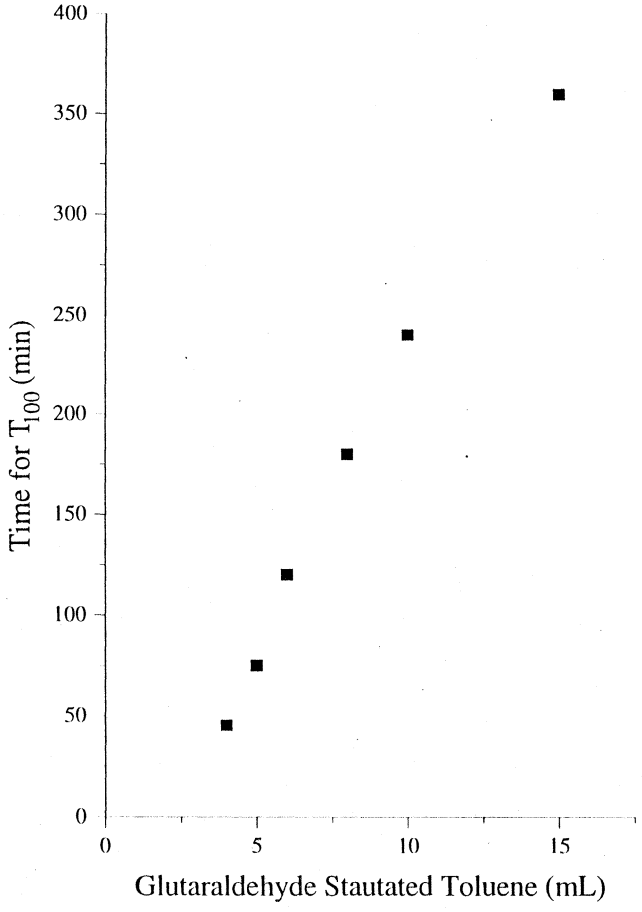


Figure 3.8. *In vitro* degradation of casein microscopheres cross-linked using various amounts of GST (using 5 mg% solution of protease) followed by turbidity measurements at 37°C demonstrating a linear relationship between cross-link density and the ease of degradation.

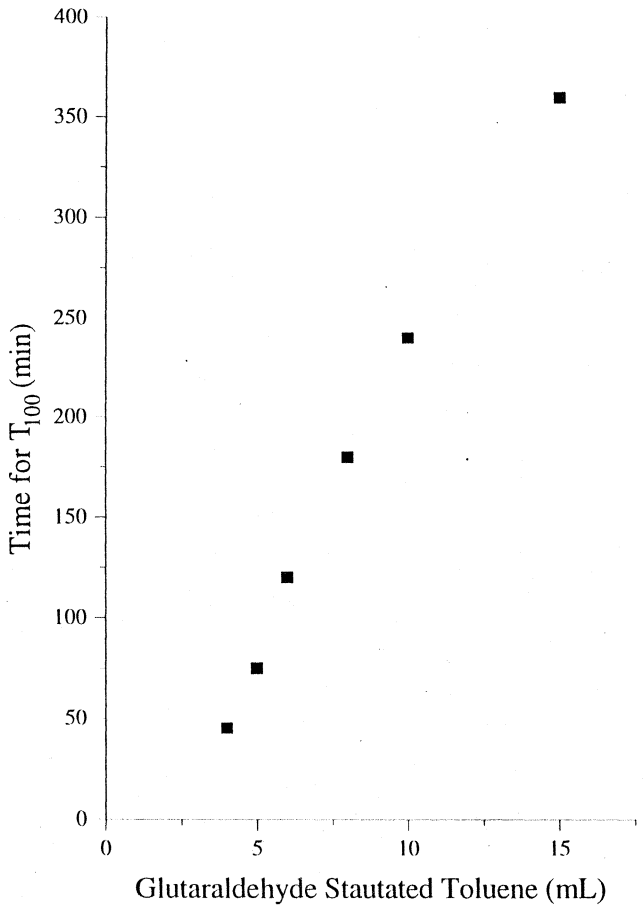


Figure 3.8. *In vitro* degradation of casein microscopheres cross-linked using various amounts of GST (using 5 mg% solution of protease) followed by turbidity measurements at 37°C demonstrating a linear relationship between cross-link density and the ease of degradation.

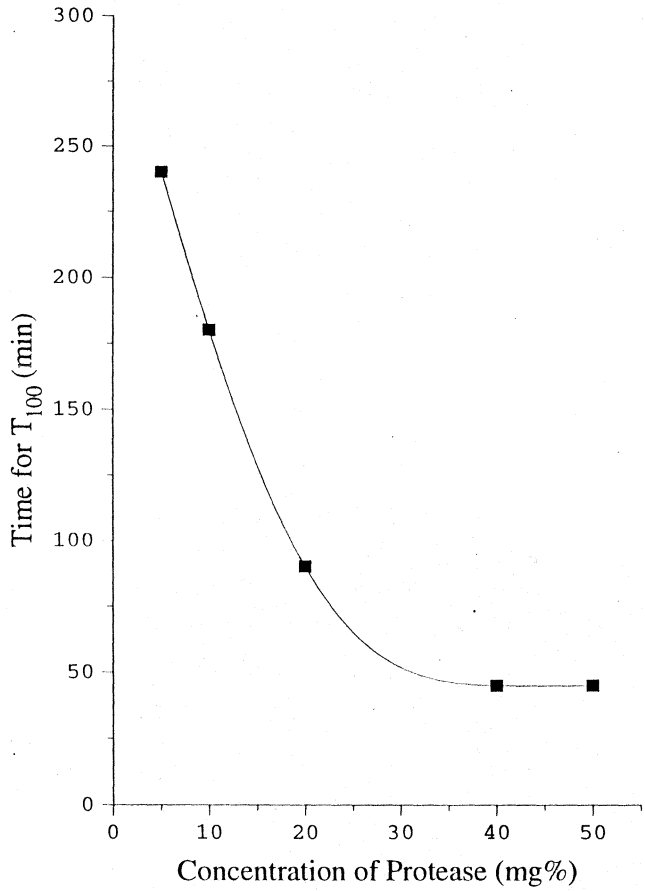


Figure 3.9. *In vitro* degradation of casein microspheres cross-linked using 10 mL GST in presence of various amounts of protease at 37°C.

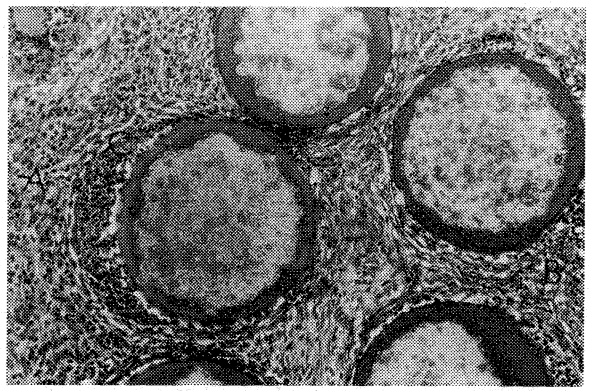


Figure 3.10. Histological section of rat skeletal muscle containing casein microspheres cross-linked with 15 mL GST 7 days post-implantation. A - fibroblast, B - macrophages and C - neutrophils.

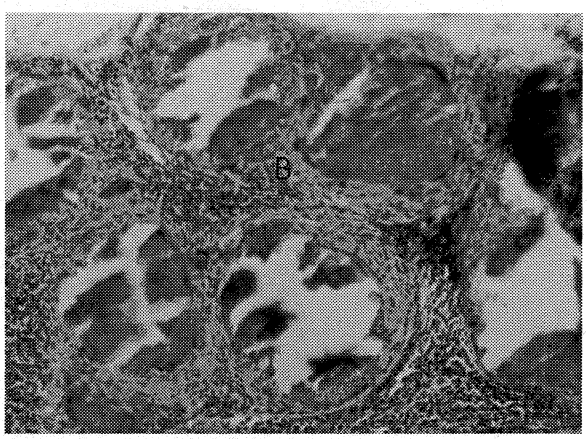
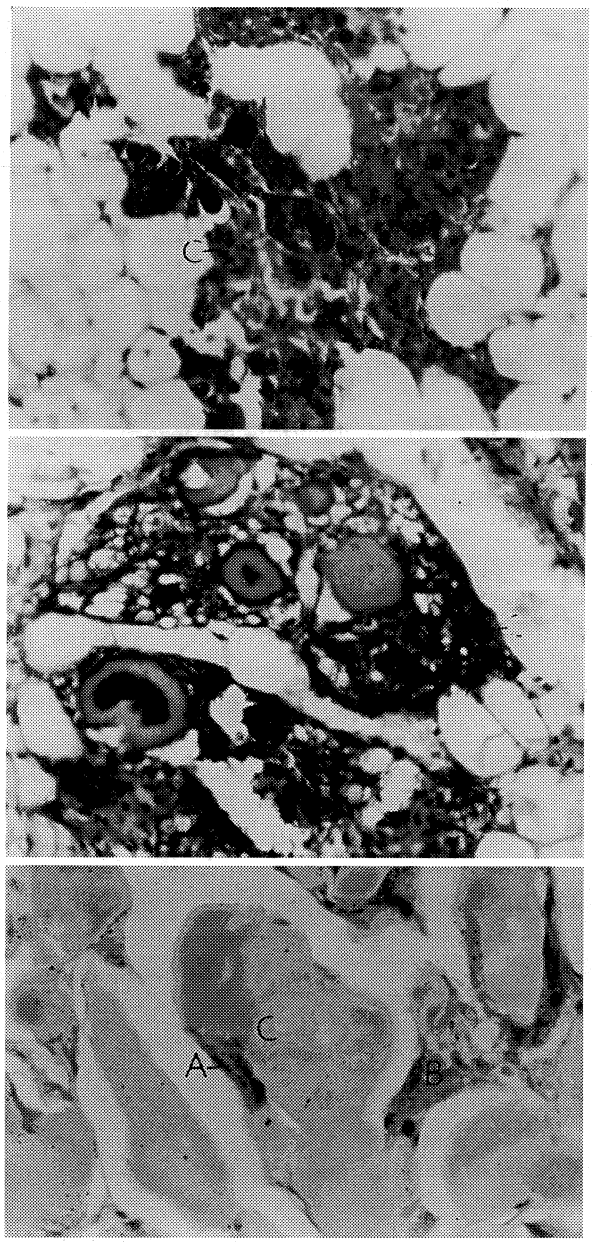


Figure 3.11. Histological section of rat skeletal muscle containing casein microspheres cross-linked with 15 mL GST six months post implantation. A - fibroblast, B - macrophages.



(1)

(2)

(3)

Figure 3.12. Histological section of rat skeletal muscles containing fragments of casein microspheres cross-linked with (1) 15 mL GST (2) 30 mL GST and (3) 30 mL GST and 3 mL aqueous glutaraldehyde nine months post-implantation. A-Foreignbody giantcell, B-macrophages, C-fragments of microspheres .

brown globular masses at the implantation site with a few fibroblasts, fibrocytes and macrophages.

The histopathological picture indicated that the microspheres were well tolerated by living tissue with no adverse tissue reactions and the *in vivo* life of the microspheres was around six to seven months. It has been reported that glutaraldehyde cross-linked albumin microspheres (cross-linked using 1% of glutaraldehyde) degrade completely in the rabbit muscle in 2 months (Lee *et al* 1981). Casein, therefore appears to have a longer *in vivo* life than that of albumin. Although spheres of 3 different cross-linking densities were implanted, it was very difficult to discern the differences in the biodegradability of the spheres. Type III spheres, which are highly cross-linked were expected to last longer in the tissue, but there appeared to be little evidence to this effect. Therefore, addition of 15 mL of GST for cross-linking the standard recipe (section 2.2.4.1) presumably introduces the maximum cross-links in the protein and introduction of further glutaraldehyde doesnot significantly alter the *in vivo* degradation characteristics of the microspheres. The extended biological life of casein microspheres compared to albumin microspheres is presumed to be due to the higher molecular weight (33000-375000) of this heterogenous protein compared to albumin which has a molecular weight of 60,000 Da.

3.1.4 Advantages of Glutaraldehyde Cross-linked Casein Microspheres in Drug Delivery

Studies on placebo spheres demonstrate the feasibility of using casein in drug delivery application. The method used for the preparation of microspheres

affords the synthesis of microspheres of wide size range. Microspheres of desired size can be prepared by optimizing the viscosity of the dispersion medium, energy of dispersion etc. High hydrophilicity of the microspheres allows the possibility of loading water soluble drugs and proteins, into preformed spheres. This has the advantage that the most native form of the therapeutic substance could be incorporated to the microsphere matrix after microspheres are prepared. The technique also eliminates possible reactions with glutaraldehyde if the drug also posseses reactive functionalities that enter into reaction with glutaraldehyde. Often, the use of ultrasound in the preparation of submicron particles denatures protein-based drugs as well as affect the activity of many drugs (Macleiod and Dunn 1968; Tabata *et al* 1993). Loading drugs and proteins into preformed microspheres avoids the effects of glutaraldehyde, ultrasound, etc.

The longer *in vivo* life of casein microspheres compared to albumin offer opportunities to extend the period of drug delivery in parenteral applications. Therefore a prolonged delivery of drugs would be possible from casein matrix compared to albumin.

Histopathological evaluation of placebo spheres in rat muscle indicate that the microspheres are well tolerated by the living tissue with no adverse tissue reactions.

Proteolytic stability of the microspheres to enzymes present in the GI tract during its transit as evidenced from the *in vitro* degradation studies suggest that glutaraldehyde cross-linked casein microspheres could be employed as a carrier for the sustained delivery of many oral drugs.

Part of this work has been published in Journal of Pharmacy and Pharmacology., M. S. Latha, A. Jayakrishnan, K. Rathinam and M. Mohanty 46, 858, 1994.

3.2 Casein Microspheres for Sustained Delivery of Oral Drugs

3.2.1 Glutaraldehyde Cross-linked Casein Microspheres for Sustained Theophylline Delivery

Rapid digestion of proteins by the enzymes present in the GI tract is the main problem in oral delivery using proteins as drug carriers. Removal of free amino group in the protein is reported to reduce their proteolytic succeptibility (Samanen 1985). Aldehyde cross-linking of casein would result in the removal of most of the free amino groups in the protein and this is expected to impart stability to the microspheres in the GI tract. *In vitro* degradation studies on glutaraldehyde cross-linked placebo casein microspheres in gastric and intestinal fluids containing digestive enzymes at the concentrations prevailing in the GI tract (USP Standard) showed that they were resistant to proteolytic degradation for 24 h and could be used for sustained delivery of oral drugs. Demonstrated in this chapter is the use of glutaraldehyde cross-linked casein microspheres for the sustained delivery of theophylline.

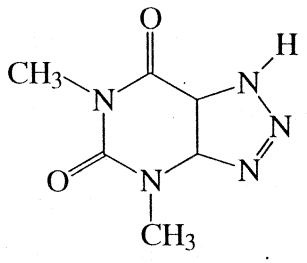


Figure 3.13. Structure of Theophylline

The chemical structure of theophylline is shown in Figure 3.13. Theophylline is used in the treatment of acute and chronic bronchial asthma (Anderson *et al* 1983). Theophylline is an inhibitor of cyclonucleotide phosphodiesterase and so causes accumulation of cyclic AMP which may in some way influence movements of calcium ions involved in smooth muscle contraction resulting in muscle relaxation. Theophylline also stimulates the release of catecholamine from the adrenal medulla and it inhibits the enzyme catechol-o-methyltransferase. This may also contribute to its bronchodilator action. The diuretic action of theophylline is brief in duration, it has relatively weak stimulant action on the central nervous system but has a more powerful relaxation action on bronchial smooth muscle than cafeine or theobromine.

Therapeutic efficacy with minimal toxicity for theophylline is within the range of 10-20 μg/mL (Soeterboek and Jonkman 1980). Conventional dosage form of theophylline is administered 3 to 4 times a day to avoid large fluctuations in plasma concentration (Mellstrand *et al* 1980). Sustained release dosage form on the other hand provides a desirable serum concentration for prolonged periods without frequent dosing thereby providing patient compliance. Even though there are several sustained release dosage forms of theophylline in the form of tablets, children are unable to swallow these tablets (Motycka *et al* 1985). Liquid preparations, on the other hand have to be administered more frequently, as the absolute bioavailability of orally administered theophylline in aqueous solution is 100% and peak serum concentration is acquired 1 h after administration (Hendeles *et al* 1977).

Motycka *et al* (1985) prepared a sustained release formulation of theophylline in the form of a drug/ion- exchange resin complex and suggested a suspension of these beads in a suitable vehicle as a prolonged dosage form for paediatric use. Although polymeric microspheres have attracted considerable attention as drug carriers (Davis *et al* 1984), only few reports have dealt with the preparation and evaluation of theophylline-loaded polymeric microspheres as a sustained release dosage form (Benita and Donbrow 1982; Lin and Yang 1987; Pongpaibul *et al.*, 1988; Thanoo *et al.*, 1992). As originally suggested by Motycka *et al* (1985), microsphere-based formulations would be particularly suitable for paediatric use. This chapter is concerned with the preparation and evaluation of theophylline-loaded glutaraldehyde cross-linked casein microspheres as a sustained release oral dosage form.

3.2.1.1 *In Vitro* Evaluation of Theophylline-Loaded Casein Microspheres

Theophylline-loaded casein microspheres of different drug payloads were prepared as described in chapter 2 (Section 2.2.7). Microspheres containing more than 50 wt% theophylline could be prepared by this method . Figure 3.14 shows the SEM of casein spheres loaded with 54% theophylline. The proteolytic stability of drug-loaded spheres was checked using pepsin, and pancreatin. Microspheres incubated in simulated gastric fluid containing 0.32 % pepsin and intestinal fluid containing 1% pancreation (USP standard) at 37°C were not found to digest the microspheres even after 24 h. Microscopic examination of the spheres before and after incubation did not show any

degradation. Addition of TCA into the incubation medium did not show any sign of protein precipitation. Thus, it can safely be presumed that glutaraldehyde cross-linked bovine casein spheres would not be succeptible to enzymatic attack during its transit in the GI tract.

Differential thermal analysis (DTA) was carried out with free drug, drug-loaded casein spheres and placebo microspheres. The plots are shown in Figure 3.15.

The endotherm of the free drug at 275°C shows the melting of the drug crystals. In the drug-loaded spheres, this endotherm still persists, though shifted very slightly to a lower temperature by few degrees proving that the drug remains as a crystalline dispersion inside the microspheres. This small shift in the endotherm can effectively be ignored as this could presumably be due to a difference in the particle size of the free drug and the drug in the protein matrix. There are several reports in the literature that the peak in the DTA curves is prone to shift on change in particle size (Wendlandt 1974). The slight shift seen in the present study is therefore believed to be insignificant. The physical state of the drug inside the matrix is reported to be important as it affects the stability of the drug and its release rate (Buri and Gumma 1985).

The particle size distribution of casein microspheres with different drug loadings determined using test sieves (Chapter 2, Section 2.10.2) is shown in Figure 3.16. The weight fraction of particles having diameter less than 300 μm is below 5% in all preparations at different drug loadings when all the other variables were kept constant. With increase in drug content, the fraction having

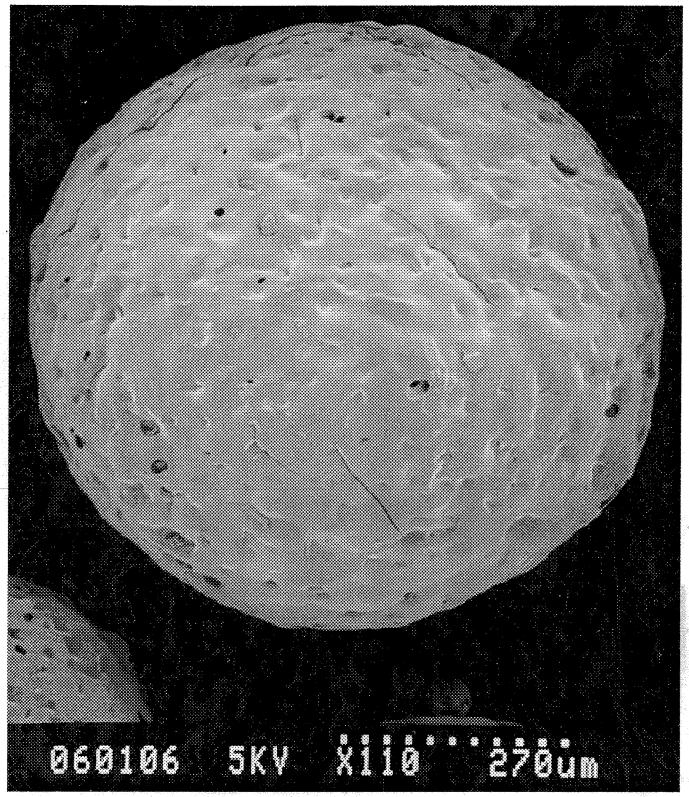


Figure 3.14. SEM of casein microspheres containing 54% theophylline

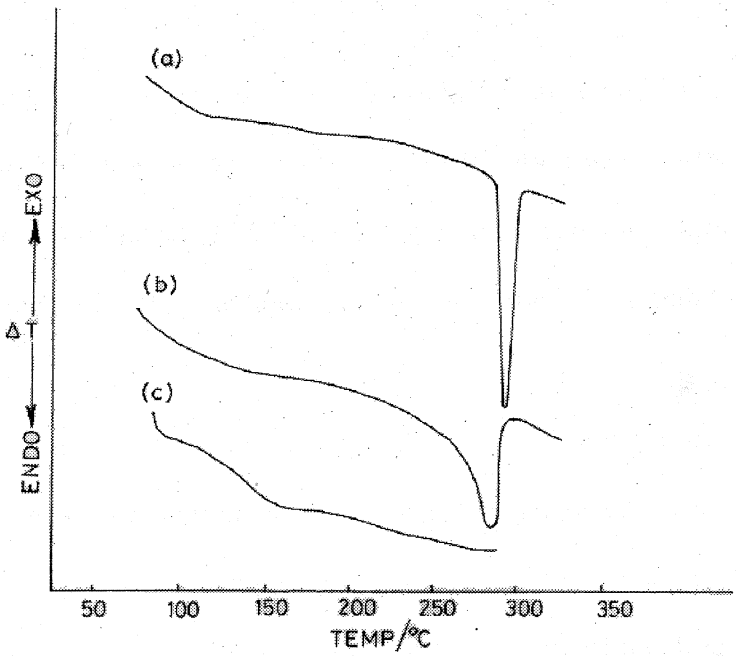


Figure 3.15. DTA profiles of theophylline (a), casein spheres loaded with 54% theophylline (b) and placebo casein spheres (c).

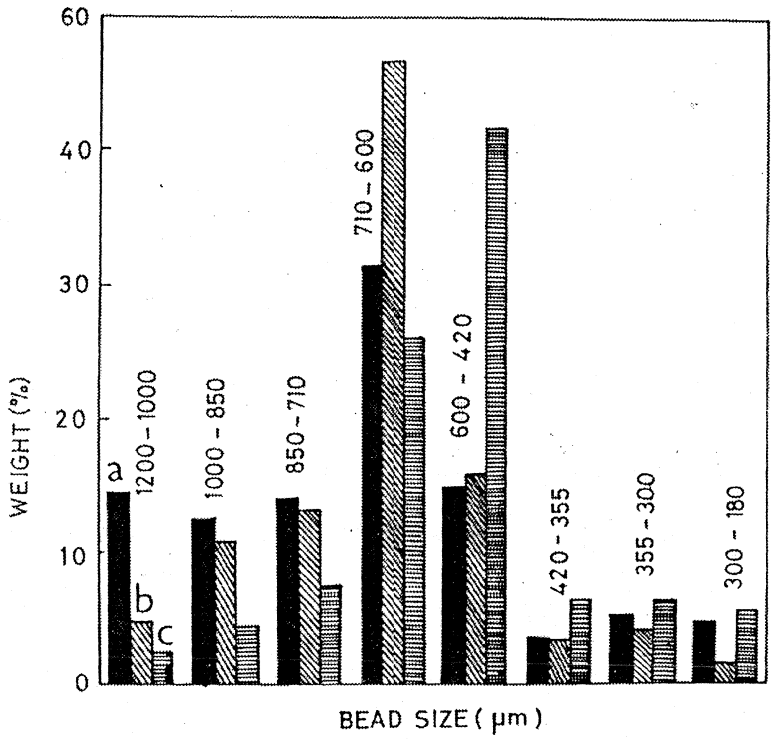


Figure 3.16. Particle size distribution of casein microspheres with different theophylline loadings prepared using 1% polyurethane as the stabilizer as a stirring speed of 1000 rev/min (a) 54%, (b) 45% and (c) 32% loading

larger particles is increased which could be attributed to a viscosity effect. Deviations, if any, in the particle size distribution were further checked using microscopy (Chapter 2, Section 2.10.3). No deviation from the distribution was detected by this method, thereby confirming the reliability of the sieving method.

As expected, the incorporation efficiency of theophylline is increased with increasing concentration of the drug in the dispersed phase (Table 3.4). As there is no chemical reaction between the matrix polymer and theophylline, the loading is achieved by simple physical entrapment of the drug.

Table 3.4
Incorporation efficiency of theophylline in casein microspheres at different initial loadings

Theoretical loading (%)	Actual loading (%)	Incoporation efficiency (%)
66	54	82
60	45	75
50	32	64

The release rates of theophylline from casein microspheres in their H^+ form in simulated gastric and intestinal fluids are shown in Figure 3.17. Microspheres of 710-850 μm size (obtained by sieving) containing 54% drug, and cross-linked with 10 mL GST were employed in the study. The rate of release into gastric fluid from acidified spheres is lower than in intestinal fluid. This is in accordance with the expectations because the matrix swells more at pH 7.4 because of the conversion of the protein into its sodium salt form thereby opening the pores more to facilitate more rapid diffusion of the drug. While 100% of the drug is released in about 5 h in intestinal fluid, it takes nearly 8 h for complete release to be achieved in gastric fluid. Determination of the swelling rates of the placebo spheres in gastric and intestinal fluid (Figure 3.19) showed that though the equilibrium swelling is attained in both fluids in the same fashion, microspheres swelled to a greater extent in intestinal fluid compared to gastric fluid as expected. The greater swelling seen in intestinal fluid is believed to be due to the ionization of the protein into its sodium salt form. When microspheres prepared from the alkaline solution of casein was acidified using hydrochloric acid after the bisulphite wash, considerable shrinkage in particle

size could be observed. The average decrease in diameter of the spheres on acidification was determined for 25 spheres before and after acidification using an optical microscope and was found to be 10± 3%. Thus the drug in acidified casein spheres is more tightly packed than in the sodium salt of casein. Further evidence to this effect is obtained when the rate of release of theophylline was examined from casein microspheres in their sodium salt form into gastric and intestinal fluids. Compared to the release from acidified casein spheres, the release was faster from spheres in the sodium salt form in both gastric and intestinal fluids (Figure 3.18).

The rate of release is also very much dependent on the cross-linking density of the spheres. Though glutaraldehyde concentration employed for cross-linking the spheres was very small as it was introduced via organic medium as a saturated solution in toluene, the effect of cross-linking density on the release rates was found to be remarkable (Figure 3.19). The introduction of the cross-linking agent via organic phase is believed to cross-link the spheres extensively on the surface with less cross-linking inside the matrix thereby providing a surface net controlling the diffusion of the drug from the matrix. While it takes 8 h for complete release of the drug from spheres cross-linked with 15 mL of GST, it took only about 5 h for complete release from spheres cross-linked with 5 mL GST. There was no noticeable difference in the release rate from spheres cross-linked with 10 and 15 ml GST. This could probably be due to the fact that the surface cross-linking density of the spheres in these two preparations was not too different.

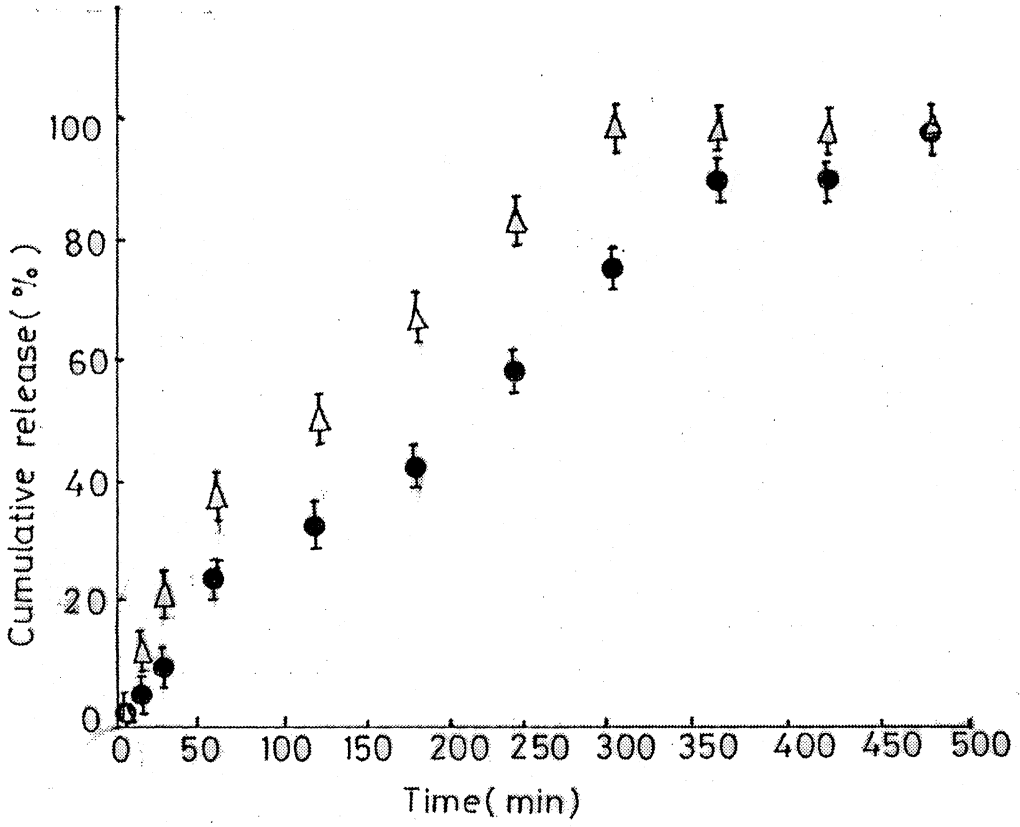


Figure 3.17. *In vitro* release profiles of theophylline from casein microspheres crosslinked with 10 mL GST (710-85 μm, 54% loading) in the H^+ form in gastric and intestinal fluids at 37°C. Into simulated gastric fluid (•), into simulated intestinal fluid (△).

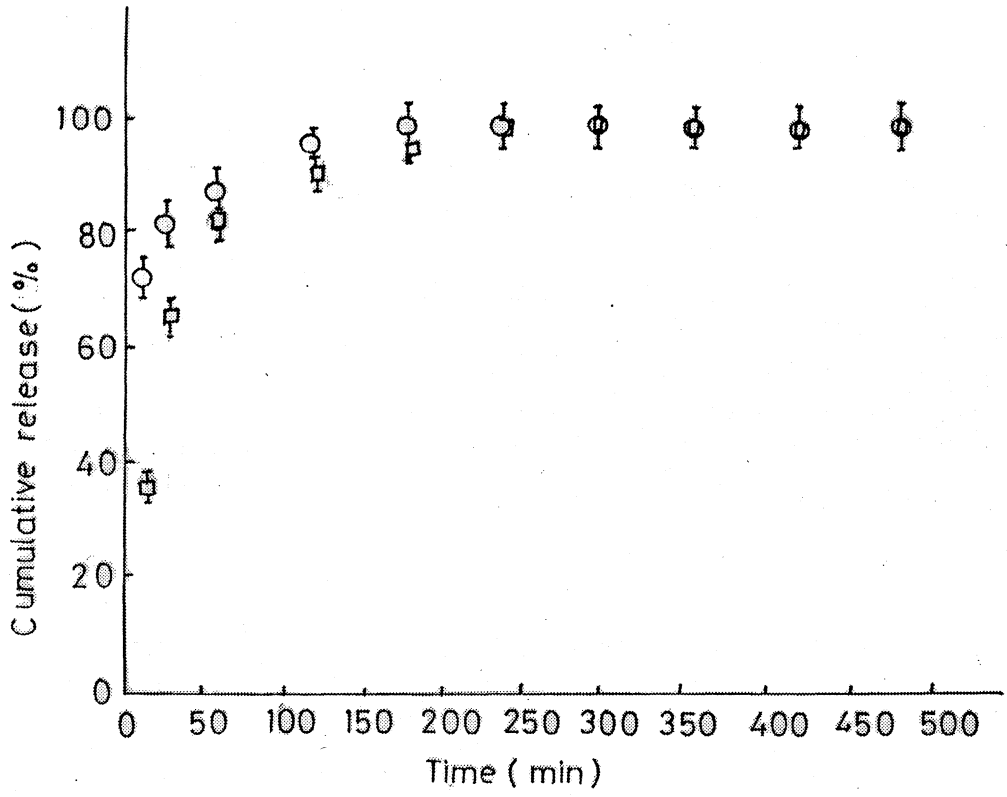


Figure 3.18.*In vitro* release profiles of theophylline from casein microspheres in the sodium salt form into (○) simulated gastric fluid and (□) intestinal fluid at 37°C.

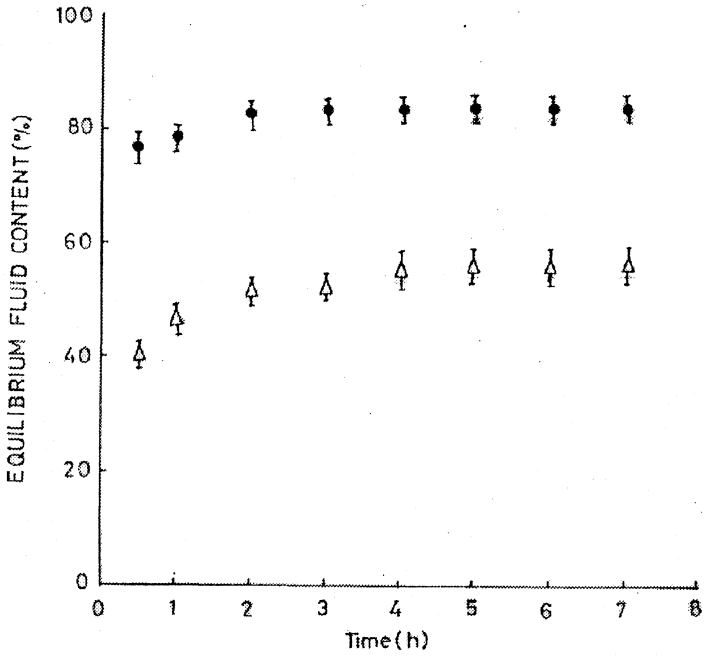


Figure 3.19. Rate of swelling of placebo microspheres in their H^+ form cross-linked with 10 mL GST in simulated gastric fluid (Δ) and in simulated intestinal fluid (•).

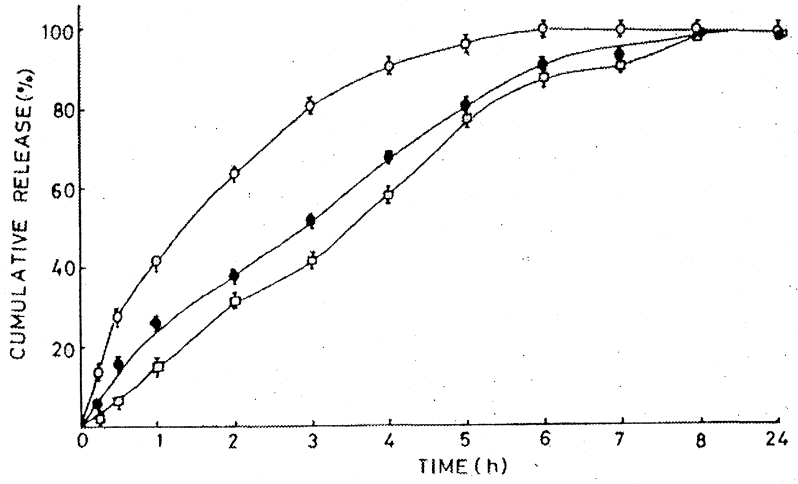


Figure 3.20. Theophylline release from acidified casein microspheres (710-850 μm sizes) 54% loading in simulated gastric fluid. Microspheres cross-linked with 5 mL (○), 10 mL (•), and 15 ml (□) GST.

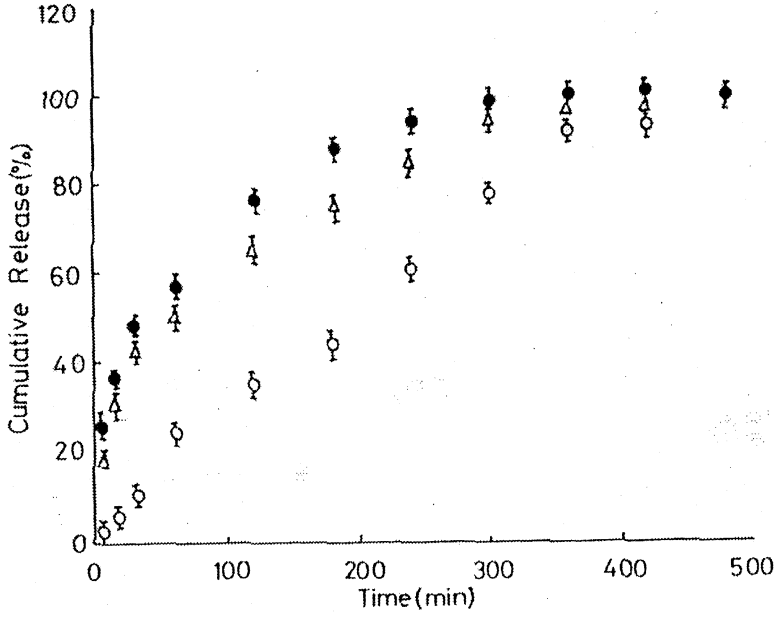


Figure 3.21. Rate of release of theophylline at 37°C from acidified casein microspheres containing 54% theophylline cross- linked with 10 mL GST, with size 350-425 μm (•), 425-600 μm (Δ) and 710-850 μm (⊛).

The rate of release was also influenced by the microsphere size to a significant extent. Data illustrated in Figure 3.21 show that release rate from smaller microspheres was faster than the rate from larger spheres in accordance with the expectation that smaller spheres by virtue of larger area of contact with the dissolution medium would release the drug at a faster rate. Modulation of the release rate would thus be possible by mixing spheres of various sizes.

The extent of drug loading also affects the release rate from the spheres (Figure 3.22). Increase in loading increases the release rate. With decrease in loading, the fraction of the matrix polymer that hinders the diffusion of the drug is increased thereby reducing the rate of release. At high loadings, however,

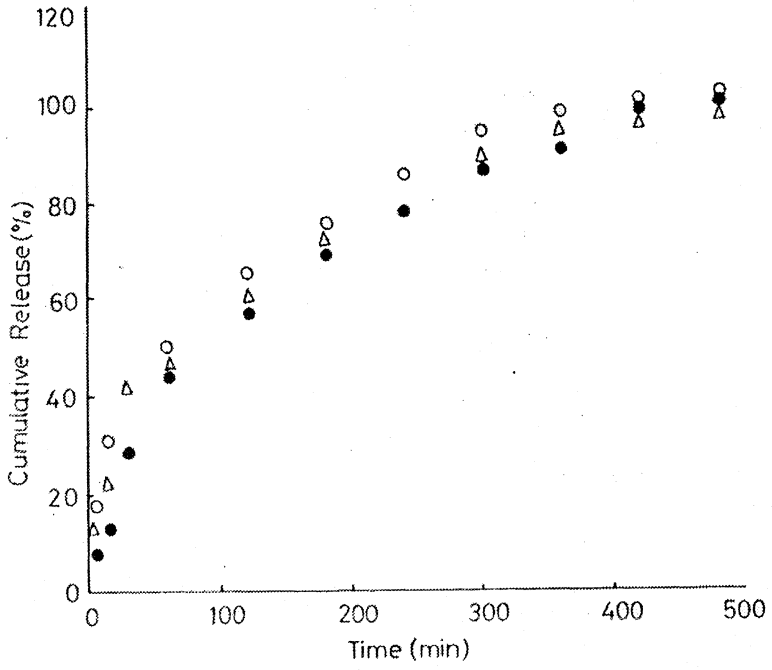


Figure 3.22. Rate of theophylline release from microspheres of 425-600 μm size, cross-linked with 10 mL GST as a function of loading at 37°C, 54% (⊛), 45% (△) and 32% (●) loaded spheres.

this effect is not very pronounced as can be seen in the case of 54 and 45% theophylline-loaded spheres.

The release of drug from a matrix type device where the release is diffusion controlled is given by the Higuchi model (Higuchi 1963), $Q = kt^{\frac{1}{2}}$, where Q is the amount released in time t and k depends on the surface area and the diffusion coefficient. Assuming that surface area and diffusion coefficient to be constant throughout the experiment, a plot of Q versus $t^{\frac{1}{2}}$ should be linear. Plot of Q versus $t^{\frac{1}{2}}$ in both intestinal and gastric fluids are given in Figure 3.23. Both plots gave a good fit with correlation coefficients 0.998 and 0.997 for gastric fluid and intestinal fluid respectively.

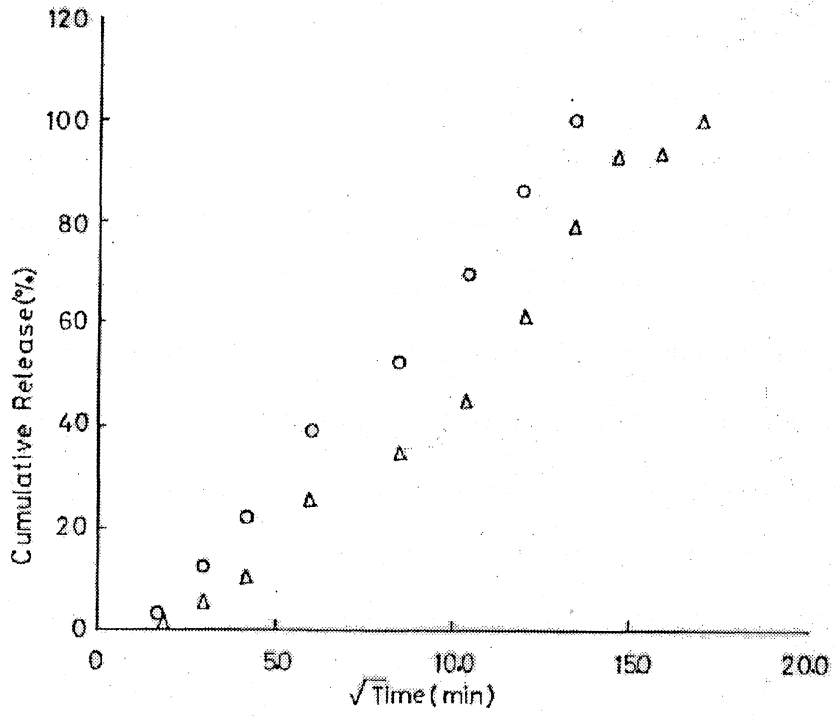


Figure 3.23. Plots of theophylline released versus square root of time in simulated gastric (Δ) and intestinal fluid (⊛) at 37°C from microspheres loaded with 54% theophylline having particle size 710-850 μm.

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3.2.1.2 Bioavailability of Theophylline from Glutaraldehyde Cross-linked Casein Microspheres in Rabbits

It is very evident from the *in vitro* release studies on microspheres with different cross-linking densities, that the rate of release *in vivo* will be modulated by the extent of surface cross-linking as the residence time of the spheres in gastric fluid will be between 3 to 5 h (Wagner 1971). Theophylline-loaded

casein microspheres cross-linked with 5, 10 and 15 mL GST were used for *in vivo* experiments. Serum concentration of theophylline after single oral administration was determined as described in Chapter 2 (Section 2.13.2).

Microspheres of 710-850 μm size having a theophylline loading of 54 $\pm$ 2% were used in all experiments. Serum concentrations obtained for theophylline powder and theophylline-loaded microspheres are shown in Figure 3.24. Maximun serum concentration in the case of powder is attained 3 h after administration. Elimination is also rapid and complete removal of the drug occurs in 24 h (Curve A). In contrast, a sustained action can be seen in the case of microsphere preparations. Microspheres cross-linked with 5 mL of GST shows maximum serum concentration at 3 h, but the rate of elimination is different from the rate observed for theophylline powder (Curve B). The peak serum concentration attained is also not too different in this case compared to the theophylline powder, but in the case of microspheres cross-linked with 10 mL of GST, the peak serum concentration is very well within the limits of better therapeutic efficacy with minimal toxicity (Curve C). Within 2 h of administration, therapeutically desirable concentration is attained and well maintained up to 12 h or even more as the fall in concentration is less rapid compared to the free drug and even after 24 h, complete elimination has not taken place. The concentration profile observed in the case of spheres cross-linked with 15 mL of GST is not too different from the one obtained with 10 mL GST cross-linked spheres (Curve D).

Mean pharmacokinetic parameters estimated (as described in Chapter 2, Section 2.13.2.2) from the serum concentration versus time curves are given in

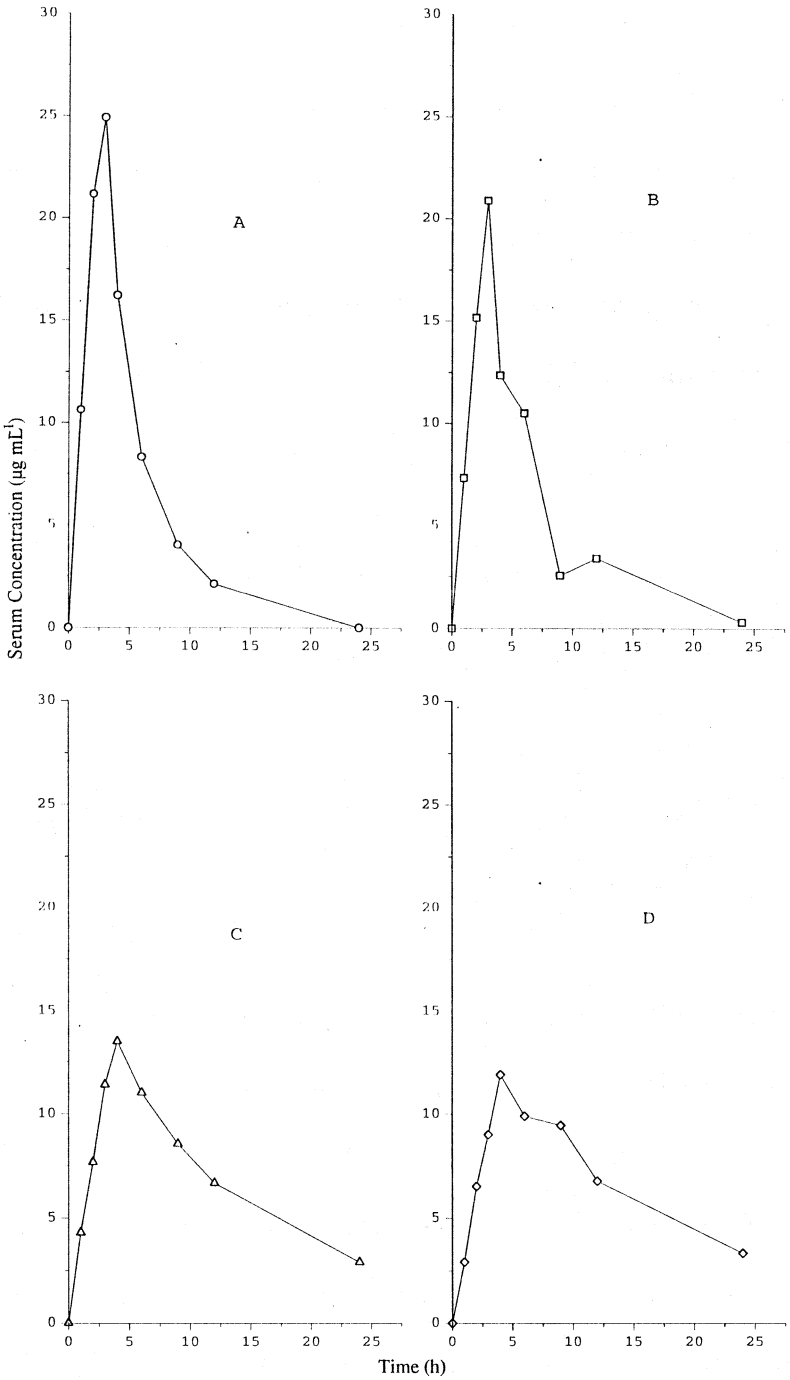

Figure 3.24. Mean theophylline concentration level after administration of theophylline powder (A), theophylline- loaded casein microspheres (710-850 μm size and 54% drug loading) cross-linked with 5 mL (B), 10 mL (C) and 15 mL GST (D).

Table 3.5. With increase in the crosslinking density, k_{el} shows a sharp decrease between spheres cross-linked with 5 mL and 10 mL GST. As seen in the case of *in vitro* and in vivo release, the difference between the 10 and 15 mL GST cross-linked spheres does not appear to be significant. The elimination half life *in vivo* for theophylline powder is about 2.5 h. In the case of microsphere preparations, it increases with increase in the crosslinking density. However, between spheres cross-linked with 10 and 15 mL GST, the difference is not very pronounced.

Table 3.5
Mean pharmacokinetic parameters of theophylline containing casein microspheres after oral administration in rabbits

Sample	max/(μg/ml)	t_{max} (h)	$K_{el}^{(h^{-1})}$	$AVC_{0-\infty}$	$t\frac{1}{2}$ (h)
Theophylline power	24.9	3	0.268	119.5	2.5
MS/5 mL GST[a]	20.9	3	0.188	127.8	3.7
MS/10 mL GST[a]	13.5	4	0.075	202.7	9.2
Ms/15 mL GST[a]	11.9	4	0.063	212.6	11.0

[a]Microspheres cross-linked with 5,10 and 15 mL GST

Correlation between *in vitro* and *in vivo* release was determined by plotting the mean percentage released in vivo at time t versus the mean percentage released in vitro at time t/k, k being the intensity factor calculated from the ratio of the mean *in vivo* over the mean in vitro time for 50% of the drug to be released (Swarbrick 1970). The advantage of using factor k is in obtaining a slope of near unity, such that both *in vivo/in vitro* release profiles will be equally represented in the plots (Yuen *et al* 1993).

Correlation was also determined between the *in vitro* and *in vivo* dissolution

times for various percentages of release estimated from the *in vitro* and *in vivo* release profiles of theophylline-loaded microspheres cross-linked using various amounts of glutaraldehyde. The percentage released *in vivo* at time t was calculated from the serum concentration-time profile. The area under the curve (AUC) of serum concentration-time profile is a measure of the bioavialability of the drug. AUC_{0-t} is taken as the amount released at time t presuming 100% absorption of the drug released since orally adminstered theophylline is known to be rapidly and completely absorbed (Simons et al., 1984; Rall 1991). The percentage released at time t was then calculated as $(AUC_{0-t}/AUC_{0-\infty}) \times 100$.

Figure 3.25 shows the plots of the percentage released *in vivo* at time t versus the percentage released in vitro at time t/k. By employing the factor k, one expects a slope of near unity such that the *in vitro/in vivo* profiles are equally represented in the plots. The value of k for the 5, 10 and 15 mL GST cross-linked spheres was estimated to be 3.75, 4.0 and 3.71. The average of 3.82 was employed in the construction of the plots. No correlation was detected between the cross-linking density and the k values. The plots are somewhat linear, the correlation coefficients for the plots being 0.991, 0.9818 and 0.9972 for spheres cross-linked with 5, 10 and 15 mL GST. The plots are not coincident and this may be attributed to the in vitro test being oversensitive in detecting the relative difference between the release rates of the three preparations and that observed *in vivo* as reported by previous investigators (Yuen *et al* 1993). There is also deviation from linearity of the plots. We have calculated the in vivo release rates from the serum concentration- time profiles presuming 100% absorption

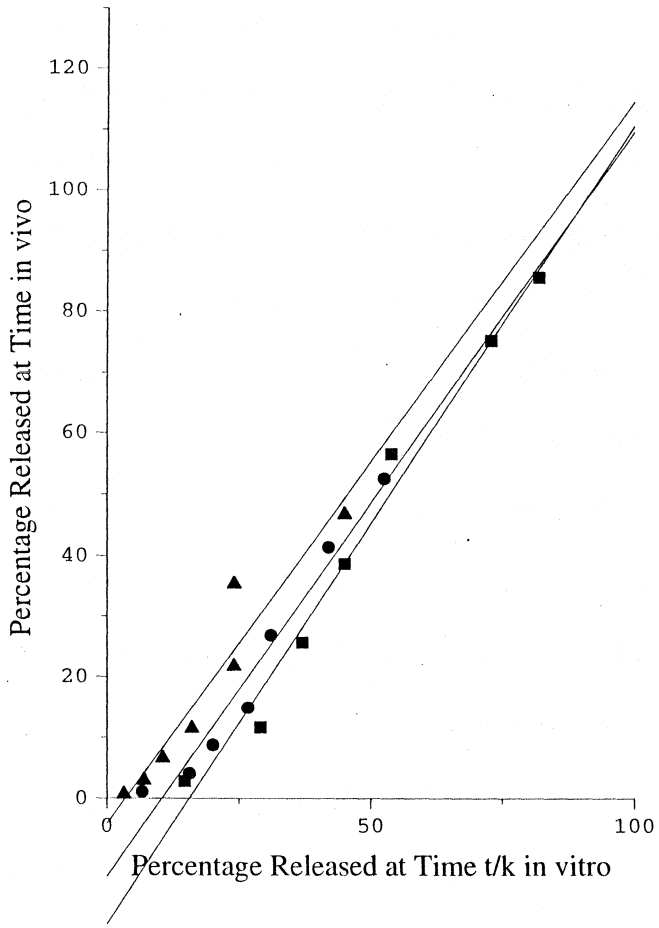

Figure 3.25. Mean percentage of theophylline released *in vivo* at time t vs mean percentage released *in vitro* at time t/k from microspheres cross-linked with 5 mL (•), 10 mL (▲), and 15 mL (■) GST.

of the drug. Even by employing a model-independent numerical deconvolution technique based on trapezoidal formula (Langenbucher and Moller 1983) to estimate the *in vivo* dissolution profiles for orally adiministered theophylline formulations, Yuen et al (1993) have encountered such deviation from the linearity of similar plots. Nevertheless, from the data presented in this study, it can be seen that a fairly good relationship exists between *in vitro/in vivo* dissolution profiles.

The plots relating to the *in vitro* and in vivo dissolution times are depicted in Figure 3.24. Over a majority of different percentages released both in vitro and *in vivo*, the plots are fairly linear. The correlation coefficients for the plots are 0.9958, 0.9985 and 0.9970 for 5, 10 and 15 mL GST cross-linked spheres. As in the case of mean percentage released *in vivo* at time t versus the mean percentage released *in vitro*, the deviation from linearity may be attributed to the slight oversensitivity of the *in vitro* method in detecting the relative difference between the release rates of the three preparations and that observed *in vivo* as reported by previous workers (Yuen *et al* 1993). Inspite of the shortcomings, the data point to the possibility of a fairly good correlation between *in vitro* and in vivo experiments.

The results reported in this study demonstrate that glutaraldehyde cross-linked bovine casein could be used in the microsphere form for the sustained release of theophylline and therapeutically desirable serum concentrations of the drug could be maintained over prolonged periods. More important is the fact that with microspheres cross-linked using 10 and 15 mL GST, the peak serum

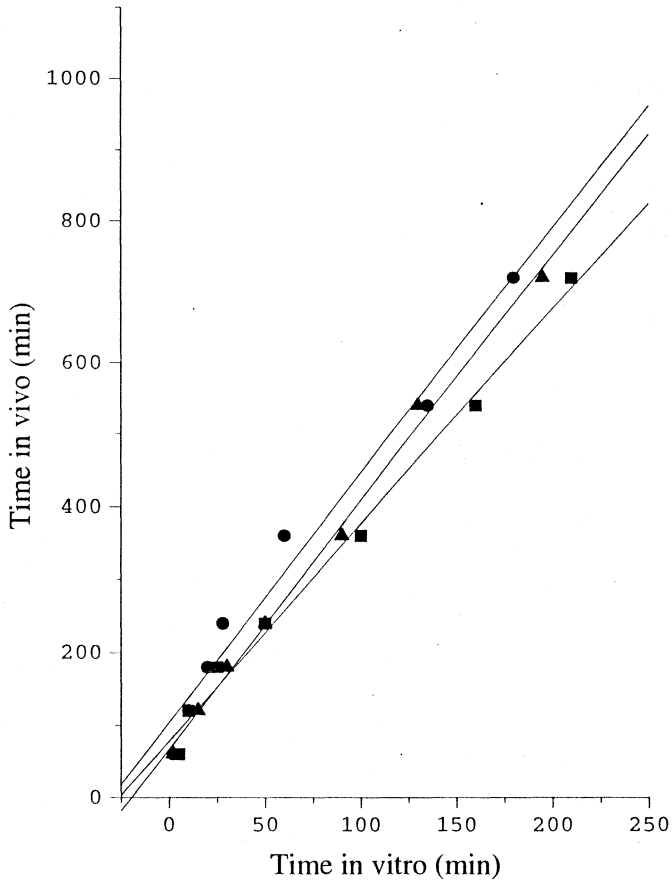

Figure 3.26. Plot of *in vivo* vs *in vitro* release times for various percentages of theophylline released from microspheres cross-linked with 5 mL (●), 10 mL (▲) and 15 mL (■) GST.

concentrations are also within the therapeutic limits. A fairly good correlation could be seen between in vitro and *in vivo* data. These observations clearly demonstrate the potential of the milk protein casein as a matrix for sustained release of oral preparations.

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3.3 Casein Microspheres for Sustained Delivery of Anti Neoplastic Agents

For successful cancer chemotherapy, total destruction of the cancer cells is necessary. Most chemotherapeutic agents are highly toxic and causes dose dependent systemic toxicities warranting discontinuation of the treatment. This results in the failure of successful eradication of cancer cells by chemotherapy (Carter 1980; Spiegel 1981; Keizer and Pinedo 1985). Acute side effects and complications are reported for radiotherapy on the other hand (Dewit et al 1983). Controlled drug delivery systems can improve the therapeutic efficacy of many chemotherapeutic agents by localizing the drug at the target cells or by introducing drug intracellularly by the cellular uptake of the system. This will reduce the dose of the chemotherapeutic agents for a given degree of therapeutic effect, reduce side effects due to the interaction of drug molecule with normal cells and enhance the potential of drugs with poor intracellular influx. Many efforts have been made in this area. Emulsions (Takahashi *et al.*, 1976; Tanigawa *et al.*, 1980), liposomes (Gabizon *et al.*, 1986; Treat *et al.*, 1989), drug conjugates (Kopecek et al., 1982; Duncan *et al.*, 1987), microspheres (Pfeiffe *et al.*, 1986; Noteborn *et al.*, 1988) etc., are some of the systems investigated for the delivery of chemotherapeutic agents. Efficacy of the system depends on several factors such as drug characterstics, the target tissue, drug release profile from the sytem etc.

Microspheres have attained considerable attention as drug carriers. Despite extensive research in this area very few systems have reached the stage of

clinical investigation. Goldberg *et al.* (1984) studied the toxicity of adriamycin after incorporating in albumin and dextran microspheres. They found that adriamycin released from the microspheres showed reduced toxicity than the free drug in CD-1 mice. Biological activity of the drug was not found to be affected by incorporation into the microspheres. Morimoto and Fujimoto (1983) have reviewed the use of albumin microspheres for the delivery of anticancer drugs.

Arterial chemoembolization of mitomycin C-loaded albumin microspheres were found to provide marked therapeutic effect with reduced toxicity in human cancer. Burger and McVie (1985) showed that cisplatin- loaded albumin microspheres were ten times more effective in localizing the drug in patients with hypervascular liver cancer. Oppenheim and stewart (1979) found that gelatin nanoparticles were taken up by certain tumour cells which include EMT 6 (mouse mammary), WEHI-3 (mouse myelomonocytic) and SP-1 (rat squamous cell).

Couvreur *et al* (1984) reviewed the preparation and characterization of poly (alkyl cyanoacrylate) particles for the delivery of antitumour drugs. They demonstrated that the nanoparticles accumulate in the neoplastic tissue. Whole body autoradiography on Lewis lung carcinoma bearing mice after administration of radiolabelled poly(isobutyl cyanoacrylate) showed high level of radioactivity in the metastasised lungs of cancerous animals with no accumulation in the lungs of healthy mice. Doxorubicin-loaded poly(isobutyl cyanoacrylate) nanoparticles were found to be more effective than free drug in their study using lymphoid L1210 leukemia of the mouse as the tumour model.

Gupta (1990) reviewed the clinical perspective of drug targeting in cancer chemotherapy. Clinical evaluation of emulsions, liposomes, starch microspheres, ethyl cellulose microcapsules and albumin microspheres have been discussed. Local administration of emulsions generally localize at the site of injection and investigations show that it is effective in localizing the incorporated drug. Inability of the system to reproducibly control the *in vivo* distribution and low stability prevented their wide clinical investigation. Liposomes offer promise in the case of brain metastases because of its lipophilic component. Trans- catheter vessel occlusion using starch microspheres has been successfully tried in patients with hepatic metastases for the delivery of 1,3-bis(2-chloroethyl)-1-nitrosourea, 5- FU, mitomycin C, adriamycin and floxuridine. Based on the data, Gupta (1990) suggested that though the drug delivery system may not be useful for the eradication of all types of neoplasms, its application for the selective *in situ* destruction of tumour tissue may obviate the need for major surgical intervention.

3.3.1 Casein as a Matrix for the Delivery of 5-FU

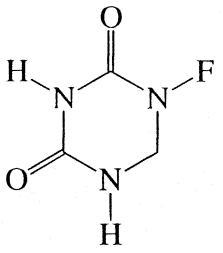

Figure 3.27. Chemical structure of 5-FU.

5-FU (Figure 3.27) was rationally developed in 1957 as a potential antitumour drug. Metabolism and mechanism of action of 5-FU is reviewed by Parker and Cheng (1990). 5-FU is an inhibitor of TMP synthetase which catalyzes the conversion of deoxyuridine monophosphate (dUMP) to TMP, which involves transfer of a methyl group from 5,10-methylene tetrahydrofolate to the number 5 carbon of the uracil ring. Replacement of the hydrogen atom at the number 5 carbon of dUMP with a fluorine atom would lead to the inhibition of TMP synthetase because the enzyme would be unable to break carbon-fluorine bond. 5-FU is used in the treatment of carcinoma of the colon, rectum, breast, stomach and pancreas (Heidelberger 1982; Waxman and Scanlon 1982). However, severe side effects have been cited for this drug. To counter the side effects, various attempts have been made. Approaches made in this direction include sythesis of monomeric prodrugs having clinical activity with

reduced toxicity (Kundu and Schmitz 1982), polymeric prodrugs fixing 5-FU onto the backbone of natural as well as synthetic polymers (Ouchi et al 1989; 1990) and microecapsulation of the drug in protein, polysaccharide and other polymeric matices (Sugibayashi *et al.*, 1977; Ghorab *et al.*, 1990; Ohya *et al.*, 1993).

3.3.1.1 *In Vitro* Evaluation of 5-FU-Loaded Casein Microspheres

5-FU is reported to be unstable in NaOH (Connors *et al* 1979) and hence *in situ* loading is difficult because casein microspheres were prepared using an aqueous alkaline solution of the protein. 5-FU loaded casein microspheres used in this study were prepared by incorporating the drug into preformed microspheres as described in chaper 2 (Section 2.2.8). Figure 3.28 shows the SEM of 5-FU-loaded casein microspheres. The amount of 5- FU incorporated into unquenched and glycine-quenched casein spheres from drug solutions of two different concentrations is shown in Table 3.6. Interestingly, there is no difference in the drug uptake of quenched and unquenched spheres and the drug uptake is a function of the concentration of the drug initially present in solution. The unquenched spheres would presumably have residual unreacted aldehyde handles on the matrix, but in the case of 5-FU this obviously has not made any difference in the drug uptake because of the absence of free amino groups on the drug (as opposed to for e.g., adriamycin) that can participate in the formation of a Schiff base linkage with the matrix.

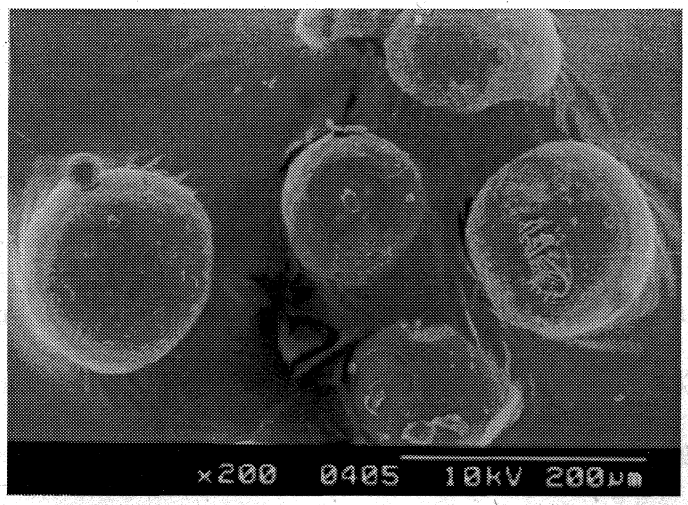

Figure 3.28. SEM of casein microspheres containing 7.1% 5-FU

Table 3.6
Amount of 5-FU incorporated into glycine-quenched and unquenched casein microspheres (75-150 μm) cross-linked with 10 mL of GST from drug solutions of two different concentrations

Conc. of drug solution (Wt%)	Amount incorporated
1.5	4.4 ± 0.15 (quenched)
3.0	7.1 ± 0.05 (quenched)
3.0	7.7 ± 0.4 (unquenched)

The *in vitro* release profiles of the drug from unquenched microspheres in presence and absence of the enzyme protease is shown in Figure 3.29. Only 10-20% of the incorporated drug is released even after 5 days from microspheres in the absence of enzyme whereas complete release is seen on digestion in presence of protease in 24 h. The release from glycine-quenched microspheres was virtually the same as the release from unquenched microspheres containing similar drug payloads (Figure 3.29). Figure 3.30 shows the release profile from

spheres of different drug payloads. A higher rate of release is seen from spheres having higher loading due to the lower hindrence of the matrix as expected. The fact that only 20% of the incorporated drug is released over the time period studied demonstrated that most of the drug incorporated is bound to the protein matrix. Similar behaviour has been observed for various drugs incorporated into protein microspheres such as albumin. Chen *et al* (1988) have reported that adriamycin incorporated into the protein matrix is covalently bound to it to some extent and the bound drug is available only on degradation of the matrix. Fujimoto *et al* (1983) have observed that the amount of mitomycin-C released over a period of 5 days from albumin microspheres is only around 20%. Sugibayashi *et al* (1979) found that the release of 5-FU from heat- denatured albumin microspheres was only 10–30% in about 24 h and thereafter remained constant for several days. The covalent attachement of drugs having amino functions onto the carrier matrix during glutaraldehyde cross-linking is a distinct possibility which however can not be ascribed to drugs such as 5-FU.

By the post-loading technique employed, one would imagine that the drug is incorporated into the spheres simply by physical entrapment, but the mechanism of incorporation seems to be very complex. Previous workers in this area have also mentioned that the mechanism of entrapment and release of drugs from protein matrices appears to be complex and poorly understood (Morimoto and Fujimoto 1983). However, no attempt seems to have been made to elucidate the mechanism of entrapment. The slow and incomplete release from the spheres in the absence of enzymes seems to be due to the entangled polypeptide chains

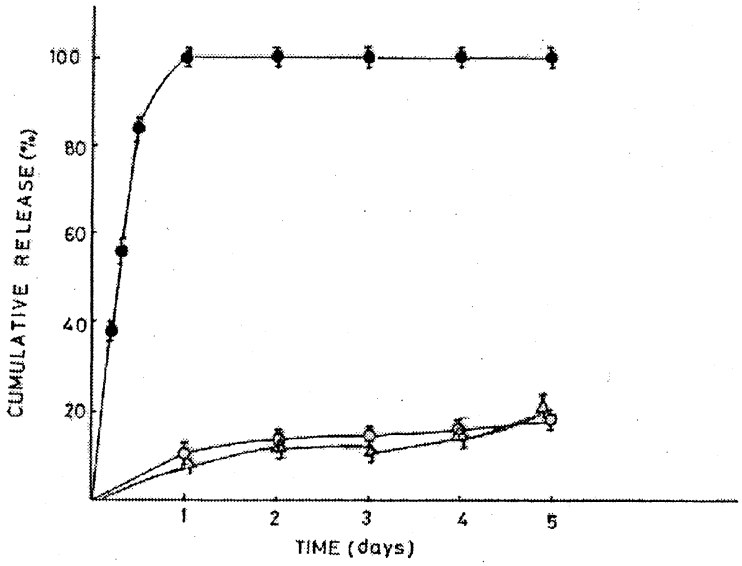

Figure 3.29. *In Vitro* release profiles of 5-FU from unquenched microspheres (•) in the presence of enzyme (△) in the absence of enzyme and quenched microspheres (⊕) in the absence of enzyme (75-150 μm, cross-lilnked with 10 mL GST and having a drug content of 7%).

in the microsphere which firmly held the drug inside the matrix. Electrolytic interaction of the protein due to the ionic functional group may also contribute to this firm entrapment. On digestion using proteolytic enzymes (Figure 3.29) the microsphere matrix opens up leading to complete release of the entrapped drug.

3.3.1.2 *In Vitro* Evaluation of Casein-5-FU Conjugate

Polymer-drug conjugates are believed to have a prolonged duration of activity. 5-FU was attached to the casein matrix using hexamethylene diisocyanate as a spacer as described in chapter 2 (Section 2.2.9). Analysis of the drug content of the conjugate showed that the loading is around 15%.

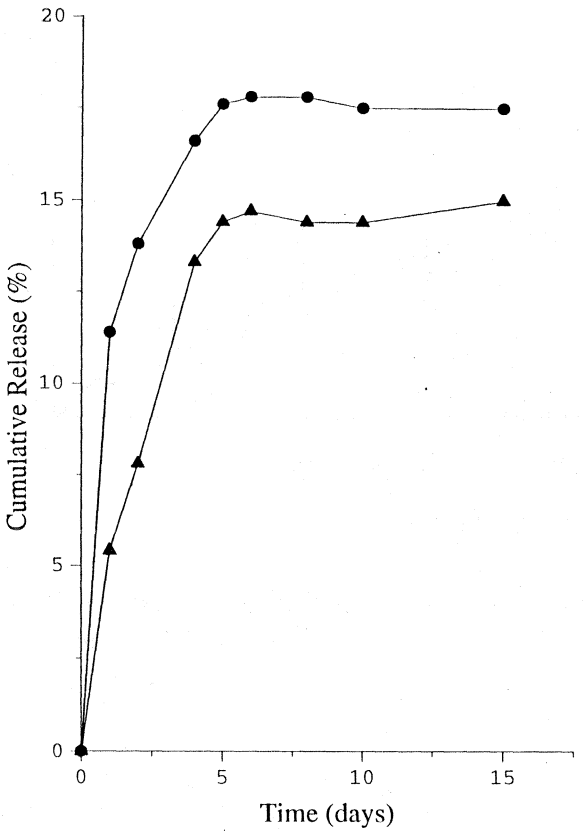

Figure 3.30. In Vitro release of 5-FU from casein microspheres (75-150 μm) cross-linked using 10 mL GST having 4.1% 5-FU (▲) and 7.1% 5-FU (●).

As opposed to the drug- loaded microspheres, the conjugate was more resistant to protease digestion. At comparable protease concentrations (0.005%), while 100% of the drug was released from the microspheres loaded with 5- FU in its native form in 24 h, only about 10% was released from the protein-5FU conjugate. Even at very high protease concentrations (0.05%) the release from the conjugate was only around 20% in 24 h. Even prolonged incubation for 5

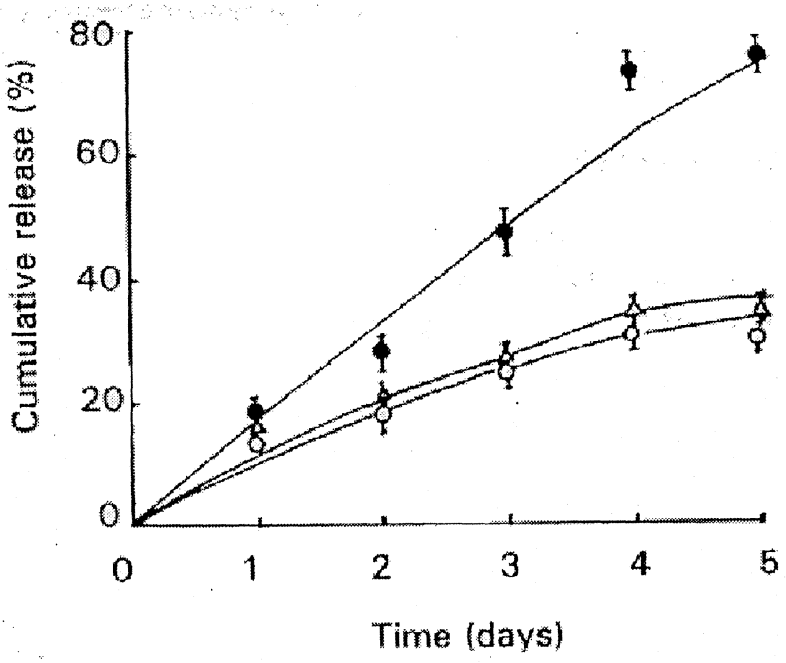

Figure 3.31. *In Vitro* release profiles of 5-FU from casein-5-FU conjugate having a 5-FU content of 15% in the absence (⊛) and in the presence of 0.005% (△), and 0.05% protease (●).

days did not release the drug completely (Figure 3.31).

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3.3.1.3 In Vivo Evaluation of 5-FU-Loaded Microspheres and Drug Conjugates Against EAC in Mice

The antitumour activity of 5-FU-loaded casein microspheres and 5-FU-casein conjugates was quantified in terms of animal survival data and change in body weight. Figure 3.32 shows the percentage of mice survived against time for mice receiving 2 mg 5-FU as free drug and 5-FU encapsulated in casein microspheres.

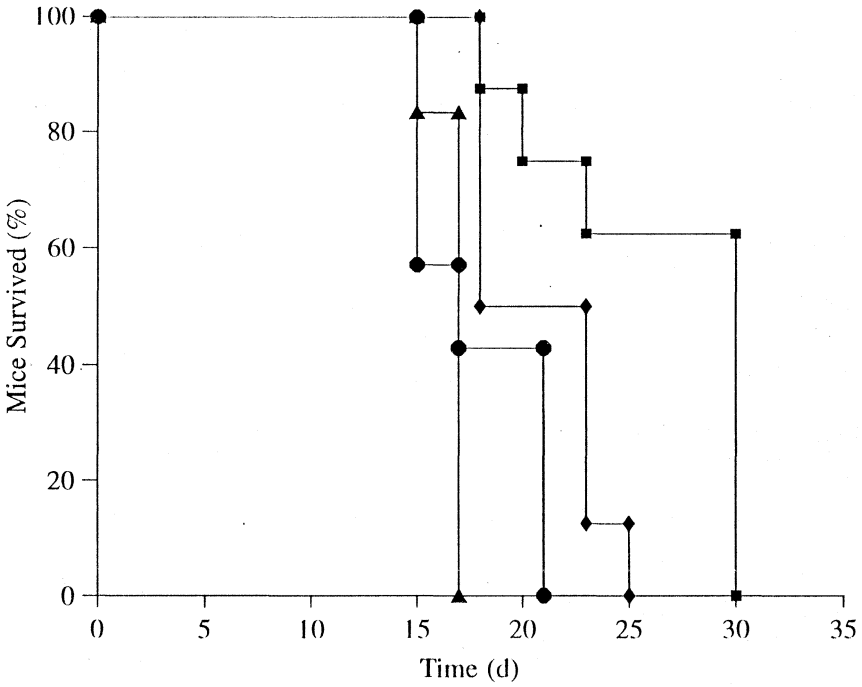

Figure 3.32. Percentage survival of mice receiving 5-FU therapy plotted against time: (♦) mice receiving no treatment, (■) mice receiving 2 mg 5-FU as free drug, (●) mice receiving placcho casein microspheres, and (▲) mice receiving 2 mg 5-FU encapsulated in casein microspheres.

For comparison, mice receiving no therapy and mice receiving placebo casein microspheres are also shown in the figure.

The mean survival time of mice receiving EAC cells with no therapy was calculated to be 17.2 ± 1.13 days which was not significantly different from the value of 15.2 ± 1.9 days calculated for those which received placebo casein microspheres ($P < 0.5$). Mice which received 5-FU therapy via microspheres modality showed a mean survival time of 15.6 ± 0.7 days which was not significantly different from the value of 22.5 ± 1.24 for those which received 2

mg of the free 5-FU ($P < 0.50$). The percent T/C ratio (calculated as the mean survival time of the treated group divided by that of the control group $\times$ 100) for mice receiving 2 mg 5-FU in casein microspheres was 91.1 as opposed to a value of 131 for the free drug.

The antitumour effect was also followed up in terms of change in body weight of the animals. Figure 3.33 shows the data. There was considerable increase in the body weight of animals untreated as well as treated with the free drug and 5-FU containing casein spheres since neither the free drug nor the microencapsulated form was found to be therpeutically effective.

With casein-5-FU conjugate, the senario was also not too different. Figure 3.34 shows the percent survival of mice against time. In spite of the fact that in *in vitro* studies, it was seen that the covalently bound drug is released in a slow and sustained fashion, in the *in vivo* system, atleast for the present cancer model, there was no therapeutic effect. The mean survival time of mice treated using casein-5-FU conjugate equivalent to 2 mg of 5-FU was 17.3 $\pm$ 1.17 days which was not significantly different from the value of 22.5 $\pm$ 1.24 days for equivalent amount of free drug. The percent T/C value was 102 as opposed to 131 for the free drug. Thus, neither 5-FU-loaded casein microspheres nor 5-FU-casein conjugate was therapeutically more effective than the free drug.

Morimoto *et al* (1980) investigated the therapeutic efficacy of 5-FU entrapped albumin microspheres against Ehrlich ascites carcinoma in rats. Their results indicated that multiple infusion of 5-FU loaded albumin microspheres was therapeutically better than infusion of the free drug. Three injections of

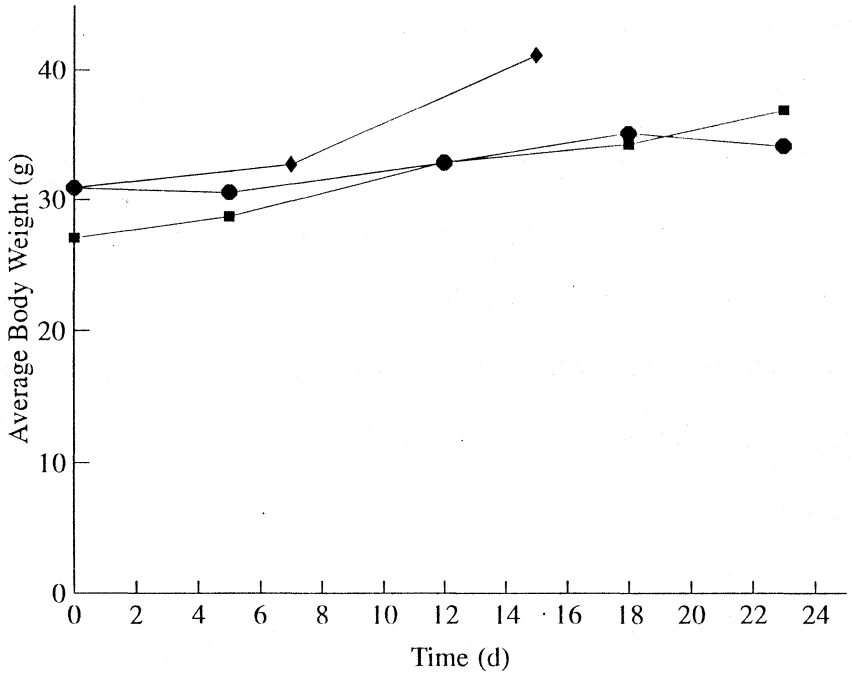

Figure 3.33. Average body weight of animals bearing EAC receiving 5-FU therapy, (♦) mice receiving casein microspheres containing 2 mg of 5-FU, (●) mice receiving 2 mg 5-FU as free drug and (■) mice untreated.

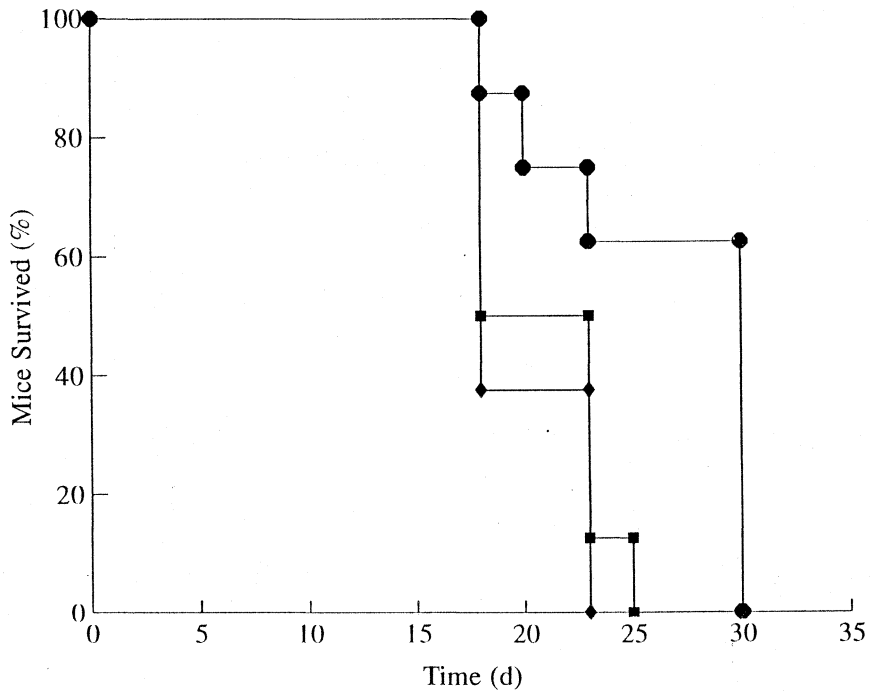

Figure 3.34. Percentage survival of mice receiving (♦) casein-5 FU conjugate

microspheres containing 5- FU were administrated and compared with three injections of the free drug after tumour inoculation. In both therapeutic modalities, the tumour volume increased as evidenced by the increase in body weight of the animals. However, survival time was found to be improved from 20.5% for the single shot infusion of microspheres to 52.5% for multiple shot infusion. The results, however, were not significant since the percentage survival of mice 24 days after tumour inoculation was less than 25%. Therefore, it is very much possible that 5-FU per se is not very effective against Ehrlich ascites carcinoma.

3.3.2 Casein as a Matrix for the Delivery of Mitoxantrone

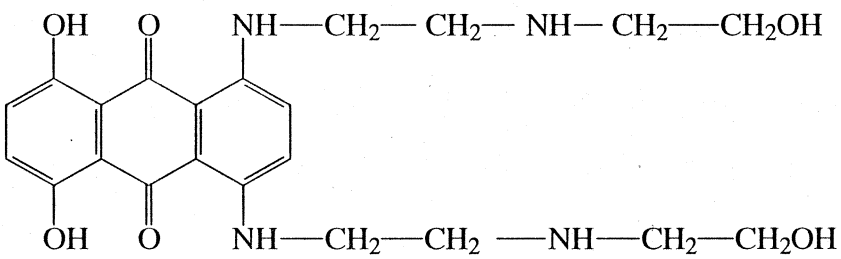

Figure 3.35. Chemical structure of mitoxantrone

Mitoxantrone (1,4-Dihydroxy-5,8-bis[(2-[(2-hydroxyethyl)amino)ethyl) amino]-9,10 anthracenedione (Figure 3.35) is a novel anti-cancer drug with a wide spectrum of antitumour activity. The anticancer activity of mitoxantrone is comparable to

that of doxorubicin, cyclophosphamide, vincristine, methotrexate, and cytosine arabinoside against p388 and L1210 leukemias and b16 melanoma and colon tumour in mice. It has lower toxicity than doxorubicin in animal models. It is reported to be well tolerated in man with a low incidence of severe nausea, phlebitis, and cardiotoxicity. Batra *et al* (1986) have reviewed the pharmacokinetics of mitoxantrone in man and laboratory animals.

3.3.2.1 *In Vitro* Evaluation of Mitoxantrone-Loaded Casein Microspheres

Microspheres loaded with mitoxantrone were prepared by post loading and *in situ* loading techniques as described in Chapter 2 (Section 2.2.10). Microspheres having two different drug loadings (10.5 and 6.5%) were prepared by the *in situ* loading technique. Loading mitoxantrone from a saturated aqueous solution of the drug into preformed casein microspheres gave rise to a drug loading of only 3.5%. Figure 3.36 shows the *in vitro* release profiles of the drug from casein microspheres having different loadings in the absence and in the presence of protease. From 10.5 and 6.5% loaded microspheres, only 40 and 17% of the incorporated drug was found to be released in 7 days in the absence of enzyme and thereafter reached a steady state. Mitoxantrone loaded into preformed, glycine-quenched microspheres was also found to be released only to the extent of 15% in 7 days and thereafter reached a steady state. As expected, a higher initial payload results in a higher release. In the presence of 0.005% protease, 100% release was seen from microspheres loaded with mitoxantrone by both techniques (*in situ* as well as post loading). Thus, it

is evident that most of the incorporated mitoxantrone is bound to the protein matrix in some way and would be available only on matrix degradation.

Previous work using 5-FU as the drug in casein microspheres has also shown similar behaviour (Section 3.3.1.3). Only 10-20% of the incorporated 5-FU was found to be released from casein microspheres in the absence of proteolytic enzymes whereas 100% drug release was observed in the presence of 0.005% protease. Similar behaviour has been observed for various drugs loaded into protein microspheres such as albumin as discussed earlier. The incomplete release of mitoxantrone not only from the in situ loaded matrix, but also from post-loaded glycine-quenched matrix suggests non-covalent attachment but strong physical binding of the drug possibly due to hydrophobic and electrostatic interactions.

3.3.2.2 *In Vivo* Evaluation of Mitoxantrone-Loaded Casein Microspheres Against EAC

The antitumour activity of mitoxantrone-loaded casein microspheres was quantified in terms of animal survial data and change in body weight. Figure 3.37 shows the percentage of mice survived against time for mice receiving mitoxantrone as free drug and mitoxantrone encapsulated in casein microspheres. For comparison, mice receiving no therapy and mice receiving placebo casein microspheres are also shown in the figure. The mean survival time of mice receiving EAC cells with no therapy was calculated to be 17.2 $\pm$ 1.13 days which was not significantly different from the value of 15.1 $\pm$ 1.9 days

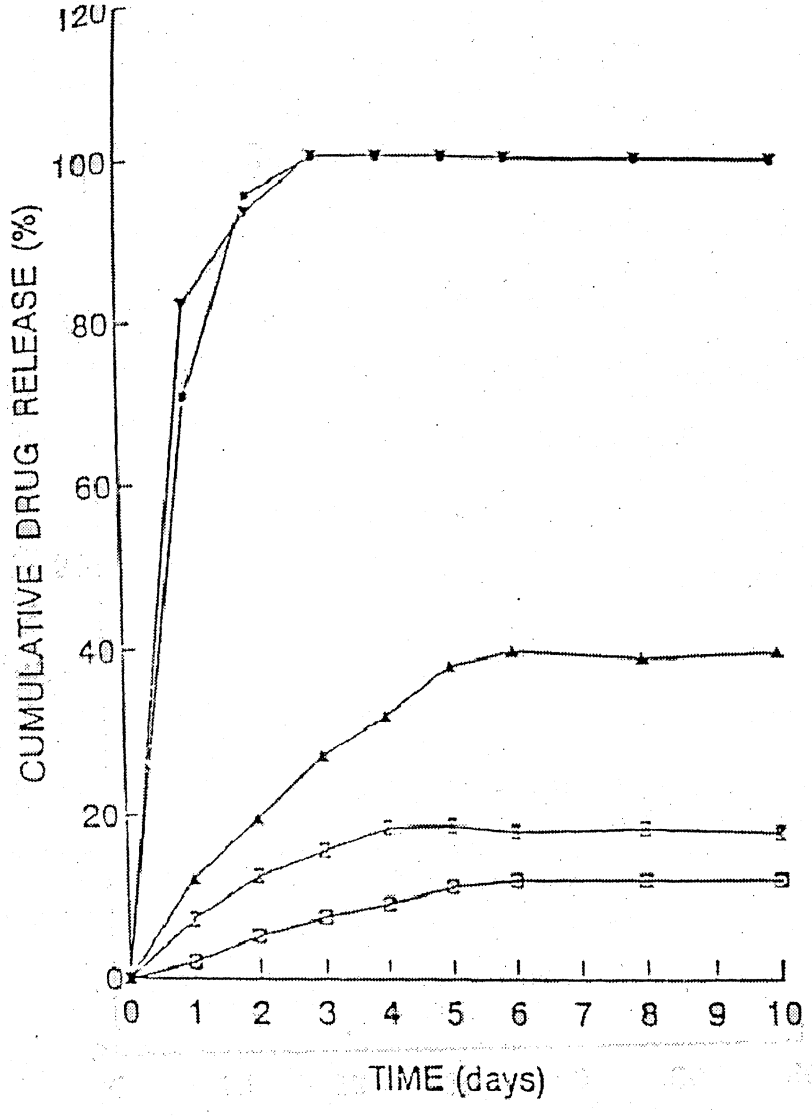

Figure 3.36. *In vitro* release of mitoxantrone from casein microspheres 10.5% in situ loaded in the presence of enzyme (■), in the absence of enzyme (▲), 3.5% post loaded in the presence of enzyme (▼), in the absence of enzyme (⊠), 6.5% in situ loaded in the absence of enzyme (⧗).

calculated for those which received placebo casein microspheres ($P < 0.50$). Mice which received mitoxantrone therapy via microsphere modality showed a mean survival time of 37.7 ± 2.9 days which was significantly different from the value of 4.6 ± 0.67 for those which received 1 mg of free mitoxantrone ($P < 0.001$). Mice receiving 1 mg mitoxantrone in casein microspheres increased the percent T/C ratio (calculated as the mean survival time of the treated group divided by that of the control group x 100) to 219.2 from a value of 26.7 for the free drug. The LD_{50} of mitoxantrone administered intraperitoneally in mice and rats is reported to be 8 to 19.7 mg/kg body weight. Therefore, the dose of 1 mg per mouse is around 1.7 to 4 times the LD_{50}. It is noteworthy that even at these doses, the microsphere modality appears to be non-toxic and therapeutically effective.

The antitumour effect was also followed up in terms of change in body weight of the animals. Figure 3.38 shows the data. While there was considerable increase in the body weight of animals untreated as well as treated with the free drug and placebo casein spheres, there was no significant change in the body weight of animals receiving mitoxantrone therapy via microsphere modality. Change in body weight of animals treated with the free drug is not shown in Figure 3.38 since the mean survival time of this group was very small (4.6 days). Microspheres loaded with mitoxantrone limited the increase in body weight of animals due to tumour growth and prolonged the life span of animals bearing EAC. Drug toxicity was apparent in the animals treated with the free drug. It is thus evident that sustained release of mitoxantrone from casein microspheres at

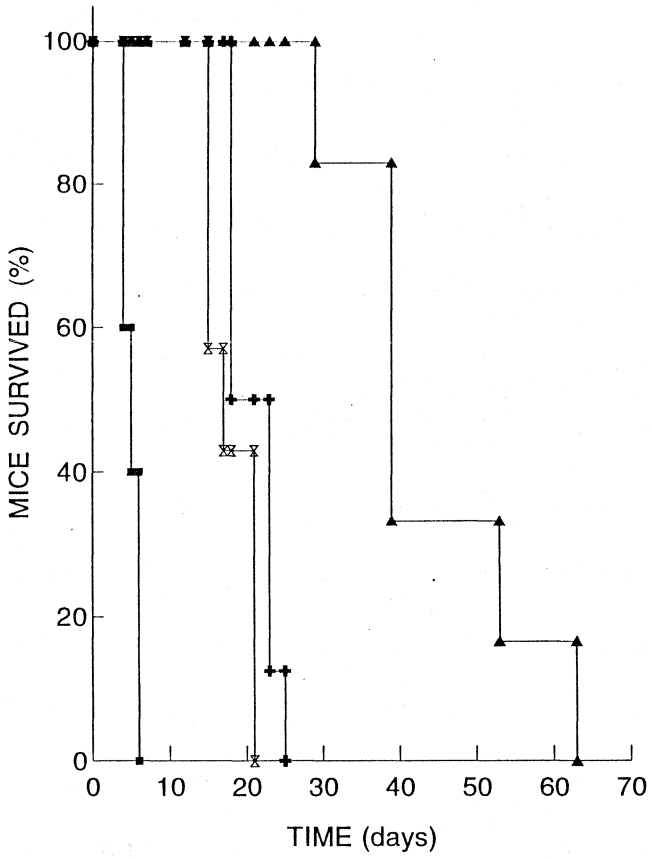

Figure 3.37. Percentage survival of mice receiving mitoxantrone therapy plotted against time, (+) mice receiving no-treatement, (■) mice receiving 1 mg mitoxantrone as free drug, (⧖) mice receiving placebo casein microspheres, and (▲) mice receiving 1 mg mitoxantrone encapsulated in casein microspheres.

therapeutically desirable concentrations is responsible for the tumour regression and prolongation of life span.

Miyazaki *et al* (1986) investigated the antitumour effect of doxorubicin containing fibrinogen microspheres on Ehrlich ascites carcinoma. With doxorubicin which has antitumour activity comparable with mitoxantrone, the percent T/C ratio observed by Miyazaki et al for fibrinogen microspheres containing doxorubicin was approximately thrice that of free doxorubicin administered at a dose corresponding to 2 to 5 times the LD_{50} of the drug. In the present study, the percent T/C ratio for mitoxantrone-microsphere modality is approximately 8 times that of the free drug at comparable doses. Recent work by Chen *et al* (1987) have shown that the therapeutic efficacy of doxorubicin- containing casein microspheres was greater than that of albumin microspheres containing the same drug against mammary carcinoma in Wistar rats. They have observed that the casein system showed greater potency per unit amount of the drug than did the albumin system. It appears that the slow release of mitoxantrone from the casein microsphere matrix consequent to matrix biodegradation is responsible for the good therapeutic effect seen in this study. Thus, the data obtained in this investigation suggest that bovine casein microspheres would be potential carriers for sustained delivery of certain antineoplastic agents.

This work has been published in Drug Delivery, M.S. Latha, P.G. Latha, A. Subramoniam and A. Jayakrishnan, 3, 75, 1996.

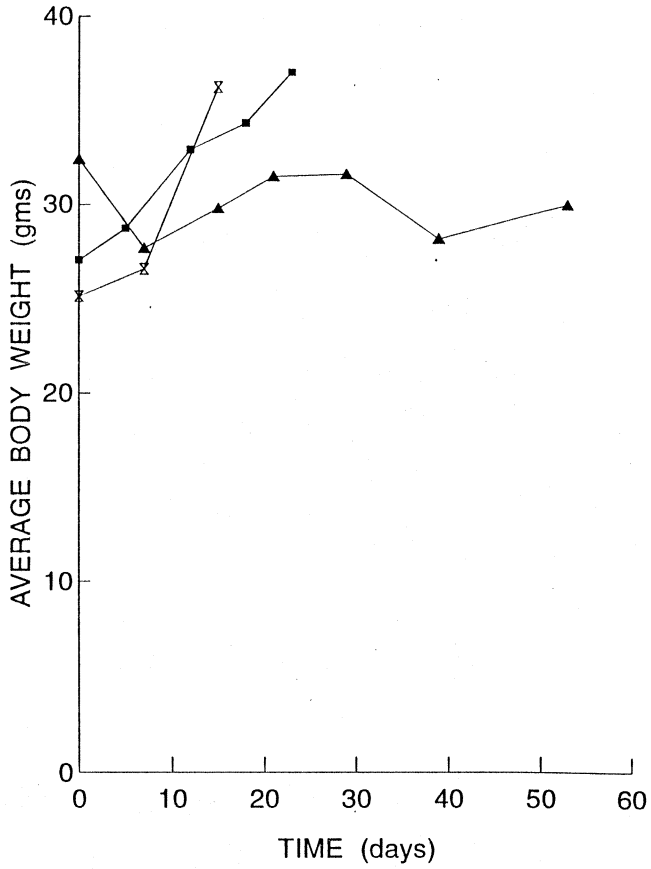

Figure 3.38. Average body weight of animals bearing EAC receiving mitoxantrone therapy (▲) mice receiving casein microspheres containing 1 mg of mitoxantrone, (⧖) mice receiving placebo microspheres, and (■) mice untreated.

3.4 Glutaraldehyde Cross-Linked Casein Microspheres for Sustained Delivery of Progesterone

Progesterone is a lipophilic drug used in the control of habitual abortion and to suppress or synchronize estrus. The chemical structure of progesterone (Pregn-4- ene-3,20-dione) is shown in Figure 3.39.

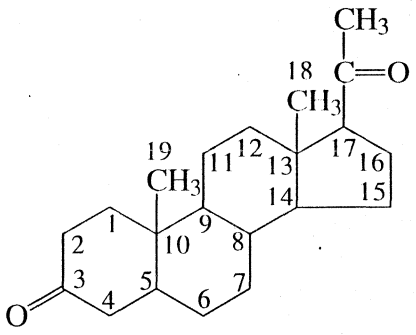

Figure 3.39. Structure of progesterone.

Unlike synthetic progestins, progesterone is attractive for fertility control because it occurs in high concentrations under natural conditions without any known side effects. Oral use of progesterone is hampered because it is not orally active except at high doses and has a short biological half-life. However, if progesterone can be delivered systemically in a sustained release pattern, it is possible to regulate fertility.

Polymeric drug delivery systems for fertility control have attracted considerable attention. Sustained release dosage form affords an optimal pharmacological

response with a minimum quantity of steroid. Dziuk and Cook (1966) showed that implants of contraceptive steroids (estradiol, progesterone and others) in poly(dimethyl siloxane) capsules were effective in synchronizing estrous cycles in sheep. Garret and Chemburker (1968a; 1968b; 1968c) found that while membranes made from nylon and cellulose esters were almost impermeable to progesterone, poly(dimethyl siloxane) was permeable. Inspite of considerable research in this direction, only two products have attained commercial status: Progestasert^{R} , a T-shaped intrauterine device designed to provide a constant release of progesterone through a rate controlling membrane of ethylene vinyl acetate, and Norplant^{R} , a silicone- based device for the delivery of levonorgestrel. Both polymers being non-biodegradable, the devices have to be removed after depletion of the drug. Silicone-based devices has run into problems because of reports on the role of silicones in auto- immune disease. Use of biodegradable delivery devices obviate the need for surgical removal of the implant.

Many biodegradable polymers have been evaluated for the delivery of steroids (Dunn *et al.*, 1981; Baker 1980; El-Nokaly *et al.*, 1993; Orienti and Zecchi 1993; Lescure et al., 1994). Pitt *et al* (1980) found that progesterone release from poly(ε-caprolactone), a synthetic biodegradable polymer is diffusion controlled and sustained action can be achieved since it degrades slowly in the body. Beck et al (1979) prepared injectable poly(lactic acid) microcapsules for the sustained delivery of progesterone. Progesterone was released by diffusion and the drug concentration was sustained for 30

days. Vaginal estrous cycle in rats and cyclic ovarian function in baboons were inhibited for 1 month. The results demonstrated the feasibility of using poly(lactic acid) microcapsules containing progesterone as a once-a-month injectable contraceptive. Deluca *et al* (1993) prepared progesterone- poly(lactic acid) microspheres with total drug content between 20% and 50%. The microspheres showed a four fold reduction in peak serum concentration at a dose of 1.0 g when compared with drug solution . Injectable drug reservoirs from glutamic acid/leucine copolymers in the form of tubes and solid rods were developed by Sidman (1977) to provide controlled release of progesterone for 6-12 months. Nuwayser and Gebelnick (1977) prepared an injectable suspension of microcapsules made from poly(lactic acid) and releasing progesterone at a rate of 150 μg/day for nearly 10 months. Anderson *et al.*, (1976) incorporated norethisterone into poly(lactic acid) and a fairly linear release was obtained for 3 months.

Proteins have received very little attention as carriers for long term delivery of drugs because of their fairly rapid degradation rates *in vivo*. Glutaraldehyde cross-linked albumin microspheres were found to degrade completely in about two months on intramuscular injection in rabbit (Lee *et al* 1981). Glutaraldehyde cross-linked gelatin microspheres also have similar degradation profiles *in vivo*. Biological life of glutaraldehyde cross-linked casein microspheres, however, were found to be more than six months on intramuscular implantation in rabbits (described in section 3.1.3.2). Even after nine months remnants of the microspheres could be seen at the injection

site. This longer biological life of casein microspheres makes it an attractive carrier for the long term delivery of active agents. Lee *et al* (1981) prepared progesterone-loaded albumin microspheres and studied the bioavailability on intramuscular injection in rabbit. Sustained release was seen only for over twenty days *in vivo*. This chapter deals with the preparation and evalation of progesterone-loaded casein microspheres.

3.4.1 *In Vitro* Evaluation of Progesterone- Loaded Casein Microspheres

Progesterone-loaded casein microspheres cross-linked with glutaraldehyde were prepared as described in Chapter 2 (Section 2.2.11). Microspheres were prepared with an initial loading of 10 and 20% steroid. Because of the high solubility of the drug in organic solvents, loss of drug during microsphere preparation was high. The actual loading achieved in the microspheres and the corresponding incorporation efficiency are given in Table 3.7. Microspheres of 60-300 μm size were prepared by mechanical stirring. For intramuscular injections it is better to have larger spheres (75-150 μm). Larger spheres remain at the implantation site for longer duration of time and act as a depot system resulting in the sustained delivery of the drug.

Table 3.7
Incorporation efficiency of progesterone in casein microspheres at different initial loadings

Theoretical loading (%)	Actual loading (%)	Incorporation efficiency (%)
20	9.97	49.8
10	6.12	61.2

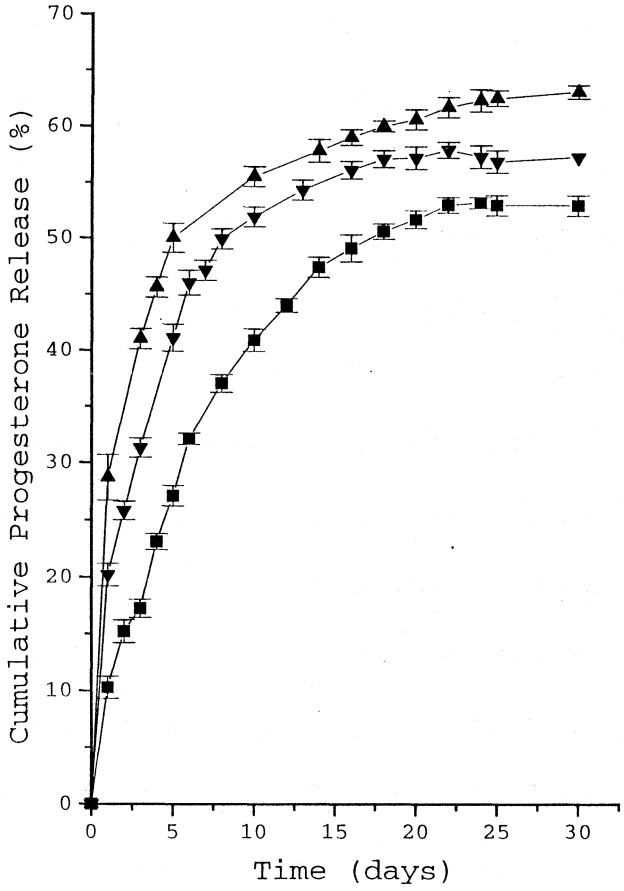

Figure 3.40. Progesterone released into phosphate buffer at 37°C from casein microspheres (150–180 μm) having 10% drug payload cross-linked with various amount of GST 5 ml (▲), 10 ml (▼) and 15 ml (■).

Figure 3.40 shows the *in vitro* release of progesterone from microspheres cross-linked with different volumes of GST (150-180 μm size) having a drug payload of 10%. Rate of release was found to be dependent on the volume of GST used for cross-linking. Rate of release is lower for microspheres cross-linked with larger volume of GST. The decreased rate of progesterone release with increasing concentration of GST is believed to be due to the increasing barrier for drug diffusion by the additional cross-links formed at higher concentration of the cross-linking agent. After the initial burst of 30% a sustained release is obtained upto 25 days from microspheres of low cross-link density (5 mL GST). Only about 60% is released even after 30 days. Initial burst is reduced to 10% with high cross-link density (15 mL GST). Maximum released in this case is about 53% in 22 days. Microspheres of medium cross-link density (10 mL GST) gave release profile in between these two.

Figure 3.41 shows the release profile of progesterone from microspheres of two different sizes. Microspheres of 75-150 μm and 150-180 μm (seperated by sieving) were used for the study. A difference in release rate is seen with change in particle size. Larger rate of release is seen with microspheres of smaller size. This is due to the larger area of contact of the microspheres with medium for the same weight of the microspheres at smaller size.

Figure 3.42. Shows the variation in drug release with drug loading. Drug release was found to be faster from microsphers having higher payload. This is due to the lower hindrance of the carrier at higher loading. Complete release is obtained from microspheres in presence of (.005%) of protease in about 4 days.

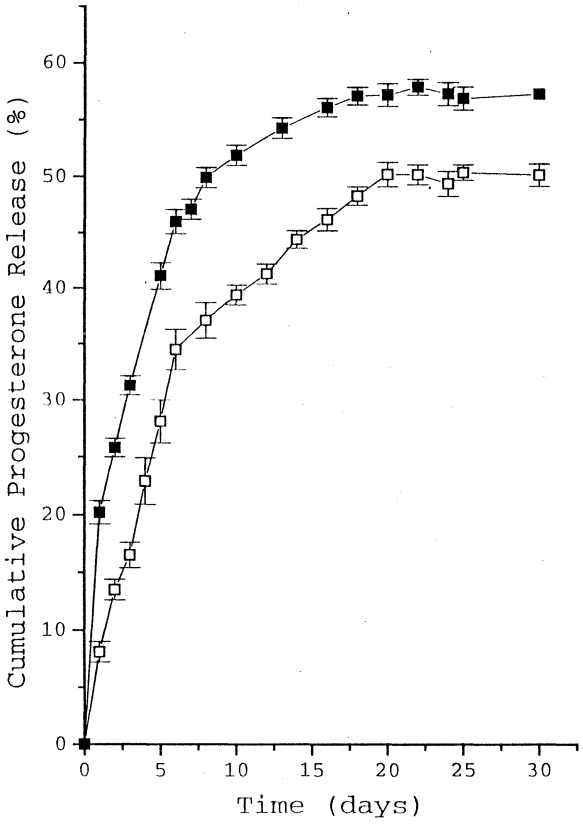

Figure 3.41. Progesterone released into phosphate buffer at 37°C from casein microspheres of different size cross-linked with 15 ml GST having 10% drug payload 75–150 μm (■), 150–180 μm (□).

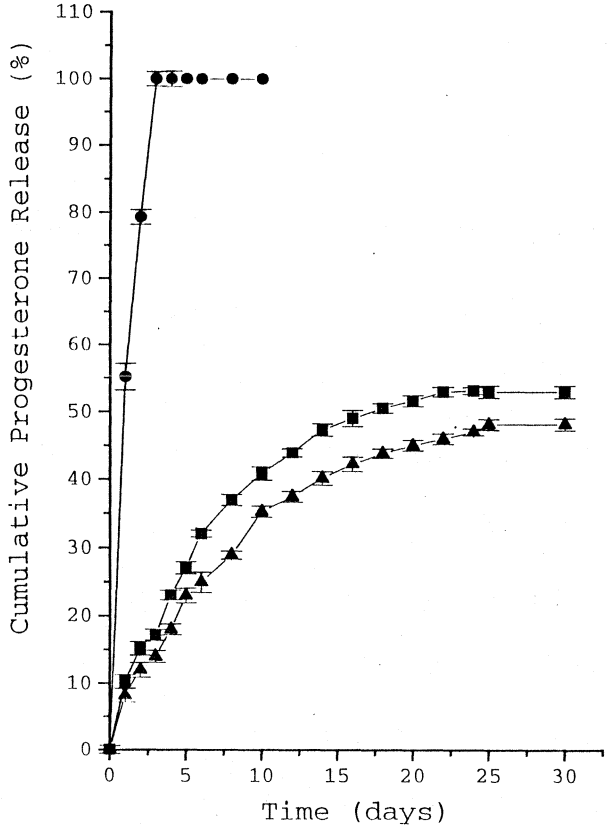

Figure 3.42. Progesterone released into phosphate buffer at 37°C from casein microspheres (150–180 μm) cross-linked with 15 ml GST having different drug payloads and in the presence of protease (•) from 10% loaded in the presence of 0.005% protease, (■) 10% loaded in the absence of protease, (▲) 6.0% loaded in the absence of protease.

Protease digest the microspheres completely releasing all the incorporated drug. Although the rate of release *in vitro* in the presence of protease will not be comparable with the rate of release *in vivo*, as the concentration of enzymes in vivo is not precisely known, the total release of the steroid on digestion of the matrix shows that all the drug will be avalibale *in vivo* on degradation of the microspheres.

3.4.2 Bioavailability of Progesterone-Loaded Microspheres in Rabbits

Bioavailability of progesterone from the microspheres was determined after injecting into rabbit as described in Chapter 2 (Section 2.13.4). Microspheres containing 10 wt% progesterone of size 75-150 μm and cross-linked with 10 ml GST were used for the *in vivo* experiments. Male rabbits were used for the experiments since female rabbits naturally have progesterone at varying concentration in their blood. Figure 3.43 shows the plasma concentration of progesterone from casein microspheres as well as from free drug plotted against time. The restive concentration of progesterone in rabbits was around 0.7 ng/mL. It can be seen from the figure that a steroid concentration of 1-2 ng/mL is sustained for about 5 months. It has been reported that serum concentrations greater than 6 ng/mL do not provide any additional benefit and those below 2 ng/mL were not therapeutically effective (Deluca *et al* 1993). Plasma concentration from powdered steroid on the other hand is not sustained beyond 4 days and there is a burst effect reaching over 20 ng/mL. The burst effect seen with casein microspheres is very small (3.75 ng/mL in 6 h).

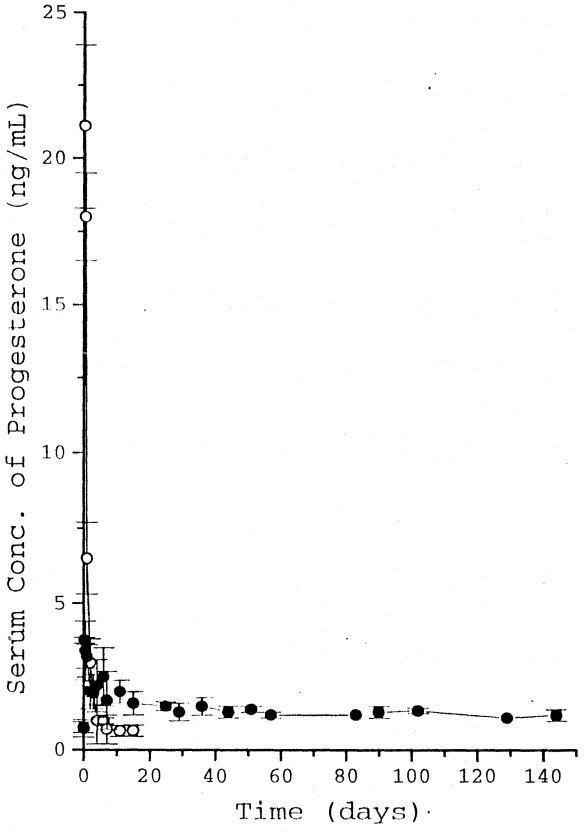

Figure 3.43. Mean plasma concentration after intramusculatar administration of (⊛) powdered progesterone and (•) casein microspheres containing 10% progesterone. Microspheres of 75-150 μm size cross-linked with 15 mL GST having 10% payload was injected.

The *in vivo* data demonstrate that sustained delivery of the steroid progesterone via casein microspheres is possible for prolonged periods. With a high payload of the steroid in the microspheres, it should be possible to maintain higher serum concentration. Very important is the observation that no significant 'burst effect' is seen with the microsphere modality unlike the free steroid where 'burst' peaks over 20 ng/mL. Also seen with the microsphere modality is the absence of peaking and troughing of the concentrations during the entire period of the study. Serum concentration is maintained throughout the period at a constant level. There was very little correlation between the *in vitro* observation and those seen *in vivo*. Degradation of the matrix followed by release of the incorporated steroid is presumably responsible for the prolonged release seen *in vivo*.

Sustaining progesterone concentrations for about 5 months *in vivo* has important implications in fertility control. By maintaining desirable serum concentrations of this steroid, one can control fertility. This observation suggests that 2 injections a year would be sufficient to exercise fertility control in a significant way. Although only 1-2 ng/mL progesterone concentration is sustained while therapeautic range is 2-6 ng/mL, it could be possible to maintain the desirable concentration by the administration of a larger amount of microspheres or by using microspheres of higher loading. However, the data obtained suggest that glutaraldehyde cross- linked casein microspheres would be a potential carrier for long term delivery of anti-fertility steroids.

3.5 Calcium Caseinate: A New Matrix for Sustained Drug Delivery

Glutaraldehyde cross-linked casein microspheres have been shown to be effective as a drug carrier for sustained delivery of oral and parenteral drugs. However glutaraldehyde cannot be employed as a cross-linking agent to stabilize the protein solution dropletes if the drug also posessed reactive functional groups that enter into reaction with the aldehyde. Activities of drugs such as methotrexate, epinephrine, salbutamol etc., are affected by aldehyde cross-linking (Gupta and Hung 1989b). Furthermore, it is important to remove the residual glutaraldehyde in the microspheres by suitable washing procedures because of its toxicity. Casein reacts with calcium chloride to form calcium caseinate, which has been used as a glue in the paper and wood industry since the early 20th century. This property of the milk protein has not been exploited for drug delivery sofar, although calcium cross-linked starch (Kost and Shefer 1990) and alginic acid (Lim 1984) have been investigated as potential encapsulating agents. Calcium cross-linking of casein is a slow reaction but gives rise to a stable linkage (Salzberg 1965). If calcium cross- linking of casein can generate a matrix from which the diffusivity of an entrapped drug molecule can be controlled, it might prove to be a potential sustained drug delivery system. In contrast with glutaraldehyde cross-linking, calcium cross-linking poses no toxicity problems. Calcium caseinate, a high protein product of edible quality and a source of dietary calcium is also an important fortifying agent for special diets.

Casein is amphoteric in nature with an isoelectric point 4.6. It forms salts with acids below this pH and with alkalies above this pH. Calcium salts react with casein to form calcium caseinate. Although the chemistry of calcium cross-linking of casein is not clearly known, it is believed to be salt formation using the carboxyl group of casein (Salzberg 1965).

3.5.1 Calcium Caseinate Beads as an Oral Delivery System

3.5.1.1 *In Vitro* Evaluation of Theophylline-Loaded Calcium Caseinate Beads

Calcium caseinate beads were prepared as described in Chapter 2 (Section 2.3.1). The shape of the beads formed was found to be dependent on the molarity of calcium chloride solution and the height of injection from the surface of the solution. Calcium chloride solutions (2 M) was found to give good beads when injected from a height of 9 cm from the surface of the solution with 20% casein solution containing 50, 60 or 66% theophylline. The size of the bead was found to be 1.7 $\pm$ 0.16 mm when measured using optical microscope (40 beads' average). Higher concentrations of calcium chloride was found to flatten the beads irrespective of the height from which the solution was dropped from the needle. Cross-linking using 4 and 6 M calcium chloride was therefore conducted after dropping the casein solution initially into a 2 M solution, decanting the same and then equilibrating with 4 and 6 M solutions of calcium chloride. Figure 3.44 shows the SEM of the surface of the bead loaded with 40% theophylline. The surface appears to be rough and porous.

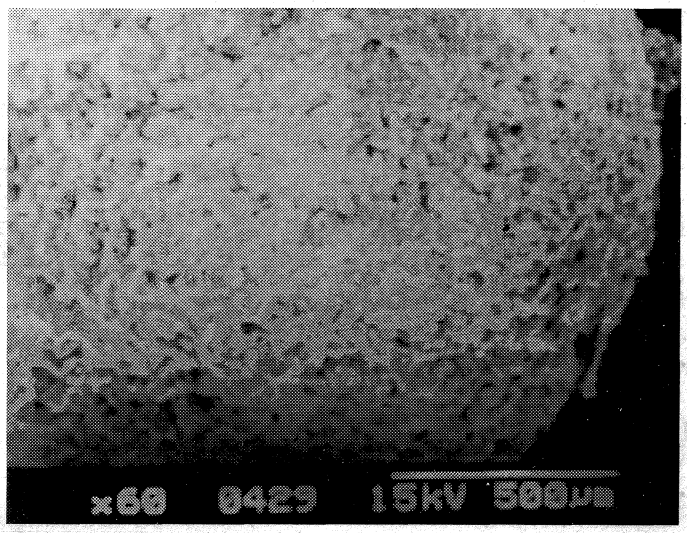

Figure 3.44. SEM of calcium caseinate bead containing 40% theophylline.

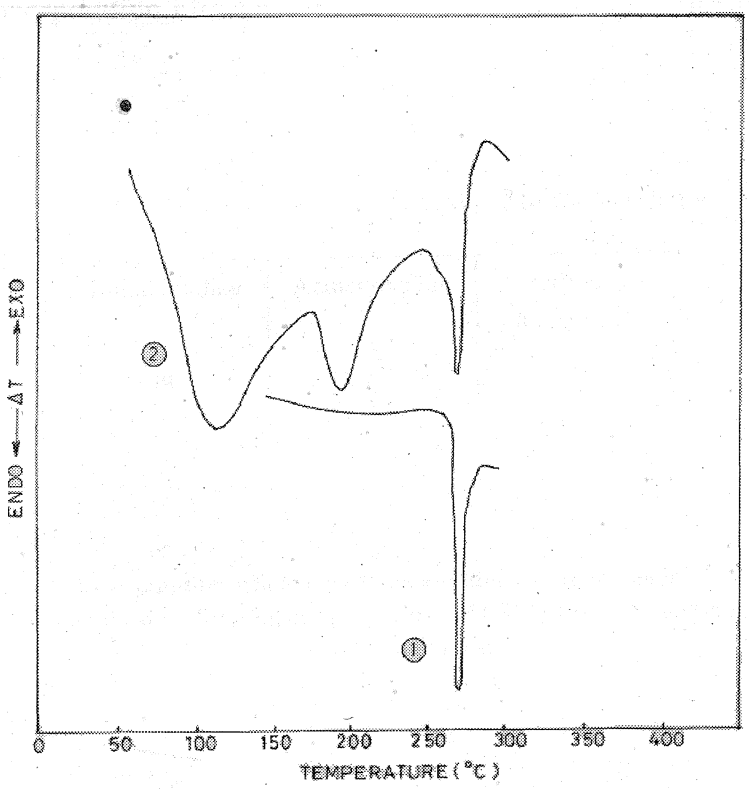

Figure 3.45. DSC profiles of theophylline (1), Calcium caseinate beads loaded with 41% theophylline (2).

The incorporation efficiency of theophylline in the beads at different initial loadings and at different concentrations of calcium chloride used for cross-linking is shown in Tables 3.8 and 3.9, respectively. The incorporation efficiency is found to increase slightly with the increase in the molarity of calcium chloride used for cross-linking. Though the increase does not seem to be remarkable compared to the increase in the concentration of calcium chloride employed for crosslinking, the upward trend in the incorporation efficiency may be attributed to the increase in the cross-linking density of the beads which entraps the drug more tightly in the matrix thereby reducing the loss during the equilibration process. Incorporation efficiency is also found to increase with increase of loading.

Table 3.8
Incorporation efficiency of theophylline in casein beads at different initial loadings cross-linked using 2 M $CaCl_2$ solution

Initial loading	Actual loading	Incorporation efficiency (%)
66	41 ± 2.4	62
60	37 ± 1.4	62
50	26 ± 1.4	52

Table 3.9
Incorporation efficiency of theophylline in casein beads prepared using different concentrations of $CaCl_2$ for cross-linking at 50% initial loading

Molarity of $CaCl_2$ solution	Actual loading (%)	Incorporation efficiency (%)
2 M	26 ± 1.39	52
4 M	28 ± 1.20	56
6 M	32 ± 0.90	64

The physical state of the drug inside the matrix affect the stability and release rate (Buri and Gumma 1985). The DSC plots of theophylline and casein beads loaded with 41% theophylline are shown in Figure 3.45. The endotherm of the free drug at 275°C shows the melting of the drug crystals. In the drug-loaded beads, this peak persists showing that drug remains in the crystalline state. There is an additional peak at 195°C in calcium caseinate which may be attributed to the degradation of the matrix at this temperature. The peak at 100°C could be attributed to the presence of water in the beads. Instability above 100°C will not affect its use in drug delivery.

The *in vitro* release of theophylline into gastric fluid from the beads cross-linked using three different concentrations of calcium chloride is shown in Figure 3.46. Only about 20 % is released in 8 h from beads having 26% theophylline loading prepared using 2 M calcium chloride. This does not increase further even up to 24 h. With increase in the concentration of calcium chloride, the cumulative amount released is decreased further. As the cross-link density increases the drug diffusion decreases resulting in a slower release.

The rate of release into gastric fluid is found to be dependent on drug loading. Figure 3.47 shows the change in release profile with drug loadings. As loading increases, the ratio of matrix weight to drug weight decreases and hence hindrance to drug release from matrix also decreases. This leads to an increase in release rate with increase in loading.

As opposed to the slow release seen in gastric fluid, the release into intestinal fluid was found to be very rapid. Figure 3.48 shows the release profile

from beads having approximately the same theophylline content cross-linked using three different concentrations of calcium chloride. Irrepective of the cross-linking density, theophylline release into intestinal fluid was virtually same. Complete release is seen from all preparations in about 3 h. Beads were found to disintegrate and dissolve slowly in intestinal fluid. The pH of the medium is responsible for the slow dissolution of the beads seen in intestinal fluid.

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3.5.2 Calcium Caseinate Granules as an Oral Delivery System

Calcium caseinate beads prepared were found to be fragile and disintegrated rapidly in intestinal fluid although the matrix remained intact in gastric fluid. Very little drug release was seen in gastric fluid whereas in intestinal fluid, 100% of the incorporated drug was released in ca 3 h. It was therefore interesting to examine the behaviour of granules from a practical point of view. Granules are very easily prepared and from a manufacturing standpoint, they are more attractive than microbeads which are more difficult to prepare.

3.5.2.1 *In Vitro* Evaluation of Theophylline- Containing Calcium Caseinate Granules

Calcium caseinate granules were prepared as described in Chapter 2 (Section 2.3.2). Granules with 66, 60 and 50% initial loadings were prepared. A

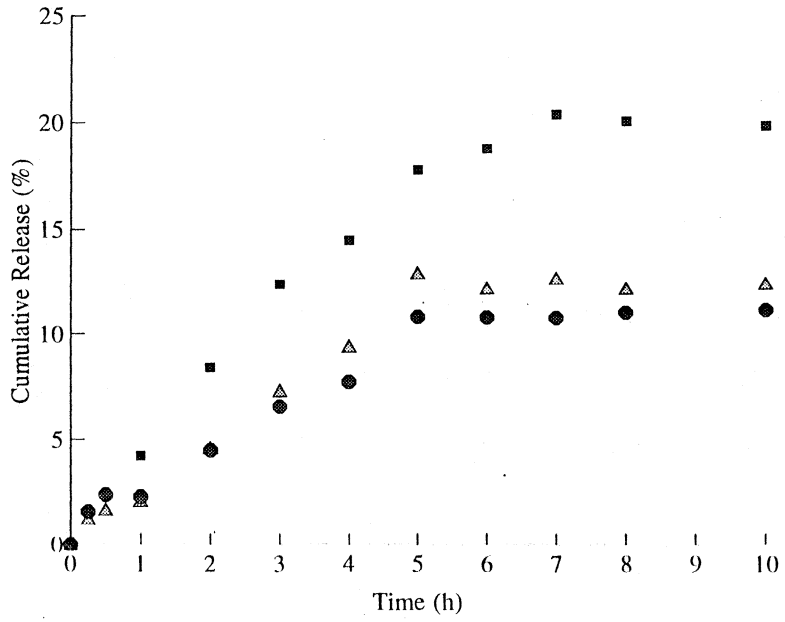

Figure 3.46. *In vitro* release profiles of theophylline in simulated gastric fluid from calcium caseinate beads cross-linked using (□) 2 M $CaCl_2$, (▲) 4 M $CaCl_2$ and (●) 6 M $CaCl_2$.

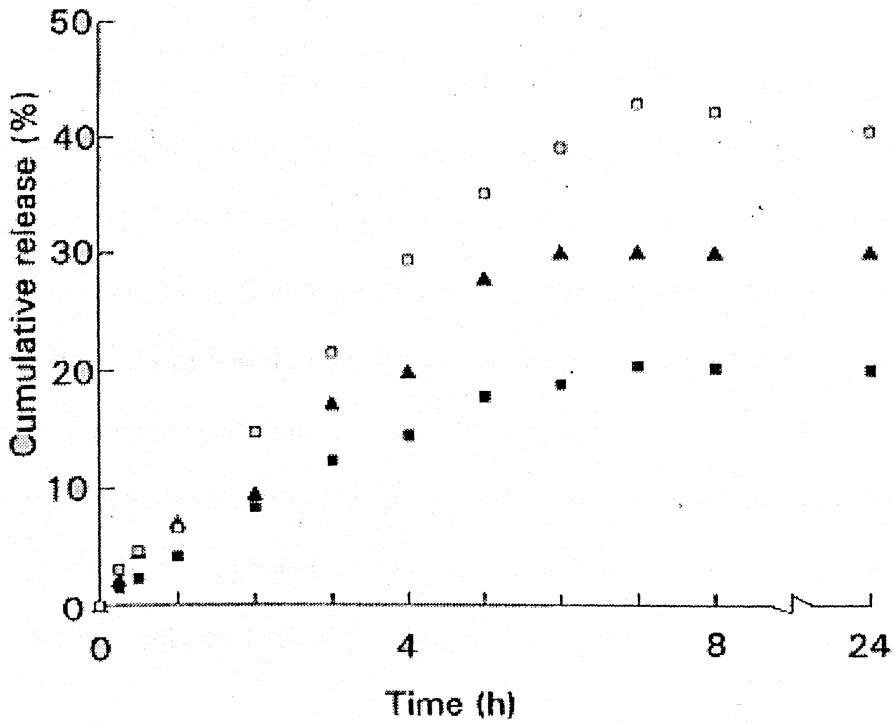

Figure 3.47. In vitro release of theophylline into simulated gastric fluid from beads cross-linked using 2 M $CaCl_2$ as a function of drug loading, 41% (□), 37% (▲), 26% (■) loading.

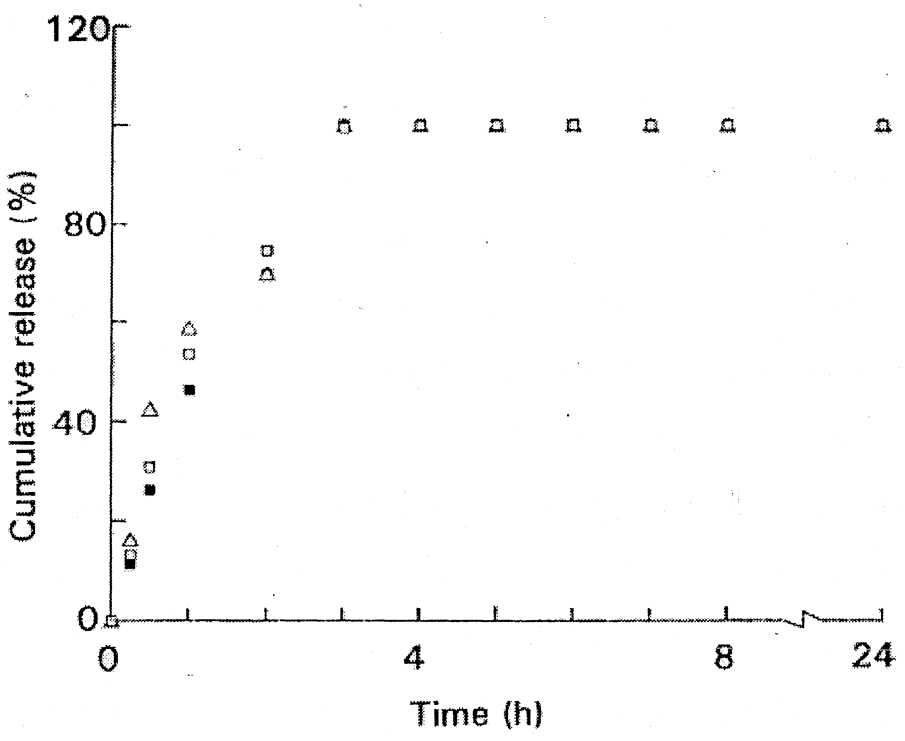

Figure 3.48. *In vitro* release of theophylline from beads cross-linked using different concentrations of $CaCl_2$ into instestinal fluid: 2 M (△), 4 M (□) and 6 M (■) $CaCl_2$.

maximum loading of 33% was obtained by this method. The incorporation efficiency of theophylline in the granules at different initial loadings is shown in Table 3.10. Figure 3.49 shows the release profile of theophylline into simulated gastric and intestinal fluid from granules of 250-300 μm size containing 33% theophylline. As opposed to the release profile observed in intestinal fluid from calcium caseinate beads where 100% release was seen within 3 h, from granules the release into both media was found to be slow. The release into intestinal fluid nevertheless, is greater in comparison with the release into gastric fluid. It is seen that only ca 17% of the drug is released into gastric fluid in 3 h. An

equilibrium seems to have attained by 3 h as there was very little increase in the release rate with time thereafter. This amount is not too different from the value observed in the case of beads into gastric fluid (ca 20%). The release into intestinal fluid was ca 35% in 5 h and does not change even after 24 h. Again, an equilibrium was established in this fluid by 5 h. The higher cumulative release seen in intestinal fluid is believed to be due to the ionisation of the matrix in the alkaline pH of intestinal fluid facilitating diffusion of the drug from the matrix. However, the ionisation does not seem to be total as the matrix did not disintegrate and dissolve in this fluid unlike the calcium corss- linked beads. Equilibrium swelling of granules was examined in both fluids and found that the granules remained hard even in intestinal fluid unlike the beads which fully dissolved in about 3 h.

Table 3.10
Incorporation efficiency of theophylline in calcium caseinate granules at different initial loadings cross-linked using 0.02 M $CaCl_2$

Initial loading (%)	Actual loading (%)	Incorporation efficiency (%)
66	33	50
60	28.6	47
50	26.0	52

Theophylline release from granules in the presence and absence of an enzyme such as protease is shown in Figure 3.50. Even in presence of 0.005% protease, complete release was not observed even after 24 h. Only about 60% of the incorporated drug is released in 8 h and thereafter the concentration remained the same.

Thus, the method of cross-linking the matrix appears to have pronounced effect on the drug release from the matrix. When an alkaline solution of casein is injected into calcium chloride solution as microdroplets and allowed to equilibrate, presumably, the cross-linking reaction was confined only to the surface. On the other hand, when casein solution was mixed with molar excess of calcium chloride for preparing granules, the resulting product was a hard mass. Therefore, it appears that extensive cross- linking of the matrix takes palce by intimate mixing of the solutions of the protein and calcium chloride.

3.5.2.2 Bioavailability of Theophylline from Calcium Caseinate Granules

Figure 3.51 shows the serum concentration of theophylline from granules and the free drug after single oral administration in rabbits. A very rapid absorption is seen with the free drug. A peak serum concentration of ca 25 μg/mL was attained in 3 h after administration. On the contrary, with theophylline loaded granules, the peak serumconcentration of ca 16 ug/mL was attained 8 h after administration. Therapeutic efficacy with minimal toxicity for theophicity for theophylline is within the range of 10-20 μg/mL (Soeterboek and Jonkman 1980). Therapeutically desirable concentration was achieved in the case of granules ca 3 h after administration and does not lead to toxic levels at any time point thereafter. In the case of theophylline powder, the peak serum concentration attained in 3 h is higher than the desirable limit for the drug. At the end of 12 h, theophylline is still detected in the serum, and the concentration in animals administered with granules is roughly twice the amount seen in animals

administered with the free drug. Even though this concentration (5.3 μg/mL) is not sufficient enough to be therapeutically effective, it is noteworthy that until 10 h desirable concentrations are maintained in the case of granules whereas after 6 h, the serum concentration observed in the case of free drug is below therapeutic level. In the case of free drug, there was no detectable concentration after 24 h whereas granules gave a serum concentration of 2.7 μg/ml. Mean pharamacokinetic parameters for theophylline powder and theophyllineloaded casein granules are shown in Table 3.11.

Table 3.11
Mean pharmacokinetic parameters of theophylline containing calcium caseinate granules after oral administration in rabbits

Sample	C_{max}(μg/mL)	tmax$^{(h)}$	Kei$^{h^{-1}}$	$AVC_{0-\alpha}$	$t\frac{1}{2}$
Theophylline power	24.9	3	0.268	119.5	2.5
Grannule	15.7	8	0.245	124.3	8.5

The data obtained in this preliminary study shows that calcium cross-linked casein granules could be an interesting drug delivery matrix for sustained oral delivery. The release profile seen *in vivo* points to the possibility of modulating release so as to obtain therapentically desirable concentrations of many drugs over prolonged periods. The consumption of bovine milk being universal, calcium caseinate appears to be a potential carrier for sustained delivery of oral drugs.

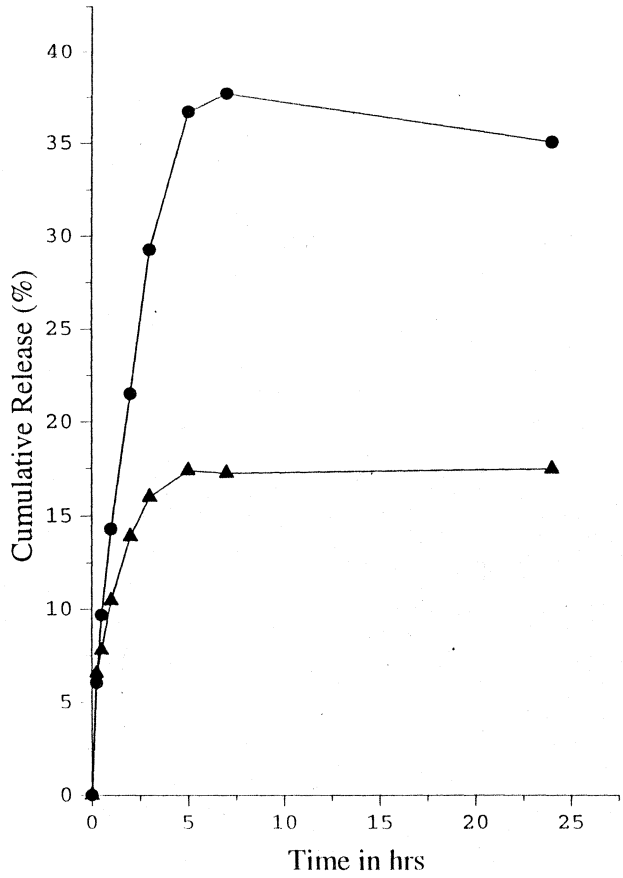

Figure 3.49 *In vitro* release of theophylline from calcium caseinate granules (250-300 μm size and 33% loading) in to simulated gastric fluid (▲) and intestinal fluid (●) at 37° C.

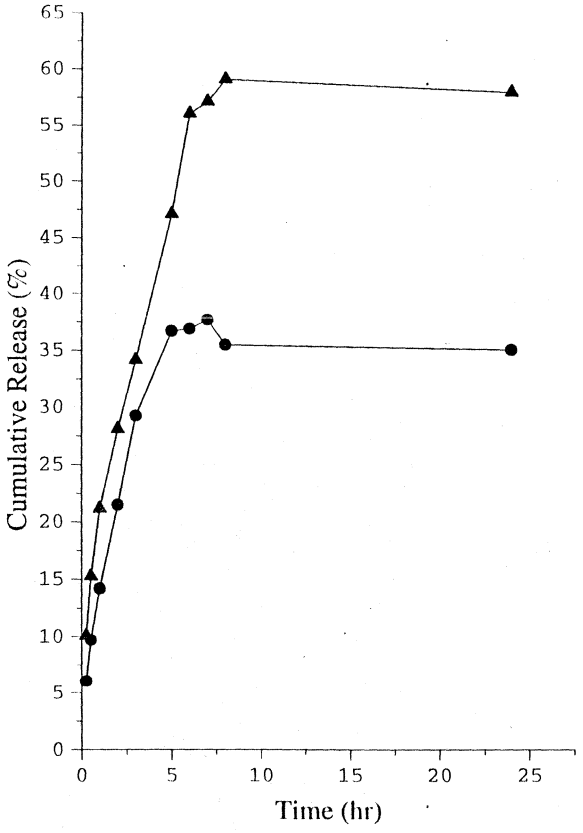

Figure 3.50. *In vitro* release profiles of theophylline from granules (●) in the absence and (▲) in the presence of enzyme

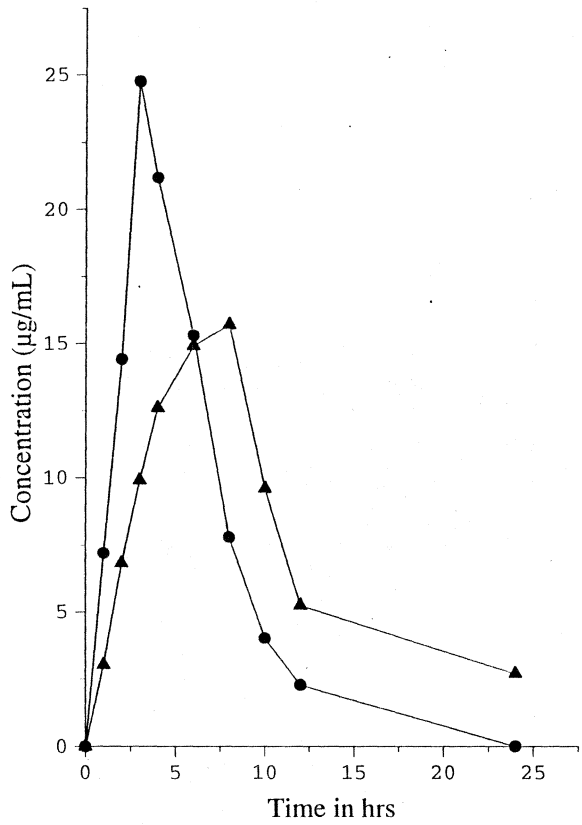

Figure 3.51. Mean theophylline concentration level in serum after oral administration of theophylline powder (○) and theophylline loaded calcium caseinate granules (●).

CHAPTER 4

CONCLUSIONS AND FUTURE PROSPECTS

CONCLUSIONS AND FUTURE PROSPECTS

A new method for the synthesis of casein microspheres using very low concentrations of polymeric dispersing agents has been developed. The method involves the use of around 1% solution of a biomedical grade aliphatic polyurathane in a mixture of hexane and dichloromethane as the dispersion medium, as opposed to a 25-30% solution of polymeric dispersing agents employed by previous workers to effect steric stabilization of protein solution droplets. Microspheres of desired size could be prepared by changing the method of dispersion, stirring speed, concentration of polymer solution etc. Microspheres possessed excellent sphericity, were completely non-aggregatory and very hydrophilic. The versatility of the method is also demonstrated by the synthesis of albumin microspheres of good spherical geometry. Serum albumin microspheres have been investigated to a considerable extent for drug delivery. The use of serum albumin microspheres as a drug carrier raises several questions regarding their safety these days because it is prepared from outdated blood where possibility of contamination with viruses such as HIV and hepatitis B

cannot be ignored. Milk being an integral part of our daily diet, preparation of casein microspheres for drug delivery application assumes importance. The significant advantages of the method include the avoidance of surfactants which become adsorbed on the particles and influence tissue reactions and drug release, and the ease of removal of the polymeric stabilizer from the final product. The method may find application for the preparation of a wide range of protein and polysaccharide microspheres for medical use.

Casein microspheres synthesised were found to be resistant to proteolytic enzymes present in the GI tract during its transit. Using theophylline as a model drug it was demonstrated that the microspheres could be used as a carrier for sustained oral delivery. The release could be modulated by manipulating various parameters during the microsphere preparation such as cross-linking density, particle size and the extent of loading. Microspheres in their acid form were employed for this particular study since release from microsphers in their sodium salt form into both gastric and intestinal fluids were found to be rather rapid. Microspheres in their acidified from are more suitable for oral drug delivery since they have to transact through both acid and alkaline pH in the GI tract.

The bioavailability of theophylline from casein microspheres studied in a rabbit model showed good *in vitro/in vivo* correlation. Sustained theophylline release could be maintained from the microspheres well within therapeutic limits as opposed to the free drug. Casein being abundant and inexpensive it appears to have good potential as a carrier for sustained delivery of many oral drugs.

Casein microspheres prepared were found to be highly hydrophilic. Taking advantage of the high swelling nature of such microspheres, 5-FU and mitoxantrone were loaded into preformed microspheres thereby incorporating the drugs in the microsphere matrix in their most native form. Drug release data *in vitro* showed that only a small fraction of the drug is released even after 5 days under sink conditions suggesting that the drug is bound to the matrix in a complex fashion even after loading into preformed spheres. Entrapped drug was found to be released completely only on digestion of the protein matrix.

The antitumour effect of 5-FU loaded microspheres, 5-FU-casein conjugate and mitoxantrone- loaded microspheres was evaluated against EAC in mice. While no significant antitumour effect was seen both in the case of 5-FU loaded microspheres and 5-FU-casein conjugate, with mitoxantrone loaded microspheres, significant therapeutic efficacy was observed in comparison with the free drug. The mean survival time of mice receiving 1 mg mitoxantrone was 4.6±0.67 days as opposed to 37.7±2.9 days for mice receiving equivalent amount of drug in microspheres. The percentage T/C ratio (mean survival time of the treated group divided by the control group X 100) for animals receiving treatment via microsphere modality was 219.2 days as opposed to 26.7 days for those receiving therapy with free drug . It is speculated that 5-FU *per se* may not be therapeutically effective against EAC. In fact, data reported by previous workers using 5-FU and 5-FU loaded albumin microspheres against this carcinoma support our speculation. 5-FU-casein microspheres and 5-FU-casein conjugate may be effective against some other tumours.

The biocompatibility of casein microspheres prepared by glutaraldehyde cross-linking was evaluated by intramuscular implantation in rats over a one year period. The study demonstrated that the microspheres were well tolerated by the living tissue without any adverse reactions. Very important was the observation, that the microspheres have an *in vivo* life of more than 6 months intramuscularly. This period is about three times the *in vivo* life of albumin microspheres reported earlier. Thus casein appears to be a potential protein-based drug carrier for the prolonged delivery of many drugs.

Progesterone-loaded casein microspheres were prepared and bioavailability studies in rabbits showed that a steroid concentration of 1-2 ng/ mL of plasma was sustained for about 5 months. Therapeutic range for the drug is 2-6 ng/ mL. With a higher payload of the steroid in the microspheres, it should be possible to maintain higher serum concentration. Very important was the observation that serum concentration is maintained throughout the period at a constant level.

During the course of investigation, it occured to us that calcium cross-linked milk protein could be a potential drug carrier for oral drugs. Although calcium cross-linked starch and alginate were investigated for drug delivery, calcium cross- linked casein was not investigated for drug delivery sofar. Therefore, calcium cross-linked casein beads and granules were prepared and evaluated using theophylline as the model drug for oral delivery. *In vitro* release studies with beads showed that only a small fraction of the drug was released in gastric fluid whereas complete release was obtained in intestinal fluid. The matrix would be suitable for delivering drugs that are only absorbed within the confines

of small intestine and for protecting drugs that are unstable at the gastric pH. Since calcium cross-linked casein beads were found to be fragile, an attempt was also made to prepare calcium caseinate grannules containing theophylline. Unlike the beads prepared by calcium cross-linking, the granuules were found to be hard. *In vitro* release studies conducted show that a sustained release was possible that from such granules. A preliminary *in vivo* study in rabbits demonstrated that sustained theophylline concentration were maintained for many drugs.

Since casein microspheres are highly hydrophilic and swelling in nature, it may be possible to load other proteins and polypeptides in their most native form from their aqueous solutions into preformed, precleaned microspheres. Thus, glutaraldehyde cross-linked casein microspheres could be investigated as a possible matrix for sustained delivery of protein and polypeptide-based drugs.

Casein microspheres prepared could be used as drug carriers for intracellular drug targeting in macrophage associated diseases such as tuberculosis, leishmaniasis etc. Microspheres of small size ($<5\mu$m) should be ideal for targeting drugs such as primaquine, rifampicin etc to the macrophages. Once phagocytosed by the macrophages, lysosomal enzymes would digest the protein matrix releasing the drug.

In addition to drug delivery applications, casein microspheres could possibly be used for scintiscan imaging by labelling them with radioactive labels such as Tc^{99m}. Presently serum albumin microspheres are employed for this purpose. Casein microspheres will be a cheaper and a safer alternative to albumin

microspheres. This could be a potential immediate application of casein microspheres and deserves investigation.

Casein microspheres could also be prepared by crosslinking the protein with other cross-linking agents such as terephthaloyl chloride, 2,3-butanedione, formaldehyde etc. Polymeric cross-linking agents like starch dialdehyde could also be used for cross-linking. The effect of such crosslinking agents on the stability of the matrix and the release profiles of the drugs encapsulated would be interesting problems for future investigations.

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APPENDICES

Appendix A: List of Publications

The following publications have already resulted from the material presented in this thesis.

1. "Glutaraldehyde cross-linked bovine casein microspheres as a matrix for the controlled release of theophylline: *In vitro* studies", M. S. Latha and A. Jayakrishnan, *J. Pharm. Pharmacol.* **46**, 8-13, 1994.
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Appendix B: List of Abbreviations

DCM	-	Dichloromethane
DSC	-	Differential Scanning Calorimetry
DTA	-	Differential Thermal Analysis
EAC	-	Ehrlich Ascites Carcinoma
ELISA	-	Enzyme-linked Immunosorbent Assay
5-FU	-	5-Fluorouracil
GI	-	Gastro Intestinal
GST	-	Glutaraldehyde Saturated Toluene
HLB	-	Hydrophilic Lipophilic Balance
PBS	-	Phosphate Buffer Saline
SEM	-	Scanning Electron Micrograph
TCA	-	Trichloro Acetic Acid