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Optimization and performance evaluation of peptide-loaded monolithic poly-ε-caprolactone microspheres in mice bearing melanoma B16F1

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The objective of this investigation was to develop a bleomycin depot based on monolithic microparticulate technology to suppress tumour growth and to maintain constant plasma drug concentrations within an optimal therapeutic window over a prolonged period of time. Formulations were optimized with biodegradable poly-ε-carpolactone and evaluated *in vitro* for physicochemical characteristics, drug release in phosphate buffered saline (pH 7.4) and evaluated *in vivo* in tumour bearing mice. This investigation revealed that upon subcutaneous injection, the biodegradable depot-forming poly-ε-carpolactone microspheres controlled drug release and suppressed tumour growth kinetics significantly compared to control. A preliminary pharmacokinetic evaluation exhibited steady plasma drug concentrations during the study period. This formulation with its reduced frequency of administration and better control of drug disposition is expected to provide an economic benefit to the user compared with products currently available for chemotherapy.

1. Introduction

The ring opening polymerization of ε-caprolactone yields a semicrystalline polymer with a melting point of 59–64 °C and a glass transition temperature of −60 °C. This polymer, poly-ε-caprolactone (PCL) has been regarded as tissue compatible and used as a biodegradable suture in Europe [1]. A block copolymer of ε-caprolactone with glycolide, offering reduced stiffness compared with pure poly(glycolic) acid, is being sold as a monofilament suture by Ethicon, Inc., USA under the trade name Monacryl. We have investigated in our laboratory, the applicability of PCL for delivering anticancer agents [2], contraceptive agents [3] and a range of anti-infectives for the treatment of periodontal diseases [4, 5]. In all the cases, films of PCL have been investigated for prolonging the action of the incorporated drugs. Effect on the stability of the drug upon formulation has also been studied.

There have recently been reports of microspheric formulations of PCL where BSA has been incorporated as a model protein [6]. Although the permeability of macromolecules in PCL is low, it may be sufficient for protein delivery [7]. The major advantages of PCL include its hydrophobicity, *in vitro* stability and low cost. This polymer is under investigation for the delivery of levonorgestrel worldwide. We have made an attempt to formulate a monolithic microparticulate depot system for BLM employing PCL as the polymer matrix so as to develop a long-acting, injectable drug delivery system to provide sustained drug release over a period ranging to few months.

2. Investigations, results and discussion

This research work envisages the encapsulation type of biodegradable drug delivery systems, which aims at sustaining the release of an encapsulated peptide for periods ranging up to few months. Several unknown elements still exist when developing a stable dosage form for proteins and peptides even though extensive research has been carried out in the field of drug delivery. The work was carried out employing PCL, a synthetic biodegradable polymer, which can be formulated with a high degree of reproducibility. Because the homopolymer has a degradation time in the order of two years depending on molecular weight, copolymers have been synthesized to accelerate the rate of bioabsorption. For example, copolymers of ε-caprolactone with DL-lactide have yielded materials with more rapid degradation rates.

2.1. Formulation of PCL microspheres

The most frequently utilized method for the formulation of microspheres is the water/oil/water (w/o/w) emulsion-solvent evaporation technique. This method, also known as "in-water drying" has been further modified to enable the encapsulation of highly water-soluble peptides [8, 9]. We obtained microspheres with minimal intra-batch and batch-to-batch variation (with respect to size, entrapment efficiency etc.) by employing multiple emulsion technology. The manufacturing process was standardized for various process parameters like stabilizer concentration and type, stirring speed, polymer concentration, volume of internal phase etc. so as to obtain optimal drug entrapment and release. The method employed yielded monolithic microspheres with reproducible characteristics.

2.2. Particle size and entrapment efficiency

The method of preparation with optimized process/formulation parameters resulted in formation of discrete, spherical, free-flowing microparticles. We also investigated the effect of the emulsion stabilizer concentration, stirring speed, polymer concentration and volume of internal organic phase on the particle size. The results obtained are presented in Table 1. During the solvent evaporation process, there is a gradual decrease in the volume and subsequent increase in the viscosity of the dispersed oil/aqueous droplets. This affects the droplet size equilibrium and the droplets tend to coalesce and produce agglomerates during the early stages of solvent removal. Adding a small quantity of a droplet stabilizer (emulsifier) in the continuous phase would solve this problem. The emulsifier provides a thin protective layer around the internal phase droplets, and hence reduces their coalescence and coagulation. As the solvent is removed, the emulsifier continues to maintain the spherical shape of the internal phase droplets and prevents their aggregation, until the microspheres are hardened and isolated as discrete particles. At both the concentrations tried with the emulsion stabilizer (PVA), microspheres were obtained successfully. Increased surfactant

concentration decreased the mean diameter of the microspheres. The concentration of PVA selected for further studies was 0.1% w/v because it resulted in the preparation of microspheres of desired mean diameter for subcutaneous injection and the problem of foaming was controllable during the formulation procedure. It is reported that when microspheres are administered subcutaneously, depending on their size and hydrophobicity, they can either be phagocytosed by the macrophage population or remain in the subcutaneous tissues [10]. PVA has been established as safe excipient in pharmaceutical formulations. The microspheres reported in this study remain localised at the site of injection since they are too big to be engulfed and migrated in the draining lymph nodes by macrophages.

The nature and concentration of emulsion stabilizer and stirring speed are parameters of vital importance in the microsphere preparation step. The stirring speed provides the energy and the emulsion stabilizer decreases the interface tension between the organic droplets and the aqueous medium. An inverse relationship between stirring speed and mean particle diameter could be deduced (Table 1). For higher stirring speed and PVA concentration, the energetic conditions were appropriate for the maximum separation of w/o or w/o/w emulsion depending on the case, so that small mean particle diameters and narrow particle size distribution could be obtained.

The particle size increased with increasing concentration of polymer dissolved in a fixed volume of suitable solvent (MC) although the increase was non-significant (Table 1). Slight fusion of semi-formed particles might have resulted due to increased frequency of collision, when the polymer concentration was increased. The stirring efficiency might have been reduced due to the increased viscosity with increased polymer concentration. Moreover, the high concentration of polymer in the emulsion droplets led to an enhanced efficiency of protein entrapment, because the higher viscosity of the organic phase tends to restrict migration of the inner aqueous/protein phase to the external water phase. One of the methods followed for reducing the migration of the highly water-soluble drug from the internal aqueous phase to the external aqueous phase of the w/o/w emulsion is the use of drug retarding agents like gelatin in the inner aqueous phase. By inclusion of high bloom strength gelatin in the internal aqueous phase and subsequently cooling the primary emulsion immediately after its formation to temperatures below 15 °C, it is possible to raise the viscosity of the primary emulsion, thus preventing drug being leached out during further processing (secondary emulsification).

Increase in the volume of the internal organic phase (MC) while keeping the volume of external aqueous phase constant resulted in decrease in the mean diameter of the microspheres (Table 1). This may be due to a decrease in polymer concentration and hence viscosity, which facilitated dispersion of the organic phase as very fine droplets during the secondary emulsification stage. The mean diameter influenced the entrapment efficiency to a small extent that can be explained on the basis that larger surface area of the microspheres results in a greater drug loss during microencapsulation and washing.

Table 1: Effect of process parameters

Effect of stabilizer concentration on microsphere size:

PVA (% w/v)	Mean diameter (μm ± SD)
0.1	43.34 ± 16.47
0.5	37.39 ± 18.33

Effect of stirring speed on size of microspheres:

Speed (rpm)	Mean diameter (μm ± SD)
1000	58.83 ± 24.28
2000	49.53 ± 18.51
3000	44.37 ± 17.47

Influence of polymer concentration on microsphere size/entrapment efficiency:

Polymer conc. % w/v	Mean diameter (μm ± SD)	% entrapment
10	37.77 ± 16.45	50.26 ± 2.33
15	42.37 ± 16.22	54.11 ± 3.59
20	51.41 ± 17.85	57.46 ± 3.31

Influence of volume of internal phase on microsphere size/entrapment efficiency:

MC volume (ml)	Mean diameter (μm ± SD)	% entrapment
10	52.32 ± 18.25	57.71 ± 4.52
20	34.68 ± 16.40	55.16 ± 3.29

2.3. In vitro drug release

The release of compounds from biodegradable polymers depends heavily on the erosion of the polymer, a complex process whose mechanism is not entirely understood. The erosion of biodegradable polymers follows mechanisms specific for a certain type of polymer and is influenced by a number of parameters. Most important is the chemical degradation of bonds in the polymer chain. The rate at which the bonds are cleaved depends on the type of bonds between the monomers, the diffusivity of water in the polymer and the polymer crystallinity. The pH of the degradation medium exhibits a catalytic effect on the hydrolysis of bonds, influences the solubility of degradation products, and finally regulates their release. Degradation products such as monomers and oligomers carry functional groups that change pH inside the cracks and pores in the eroded polymer and these groups can have feedback on the erosion process. It can be concluded that the polymer erosion is a composite process that can be very complex and is specific for each polymer.

The physicochemical characteristics of PCL microspheres taken for *in vitro* and *in vivo* evaluation are elaborated in Table 2. A profile of *in vitro* release of BLM-loaded biodegradable formulations is depicted in Fig. 1. PCL-based formulation released about 26% of drug payload within 30 days with minimal burst effect. The rate of release varied from approximately 0.5 to 8.0 μg ml^{-1} day^{-1} at the end of one month. BLM (molecular weight 1580) is a small glycopeptide having a tripeptide chain attached to the pyrimidine core. Compared to most of the hydrophilic drugs, it has a relatively larger molecular weight and hence drug release via diffusion pathway is comparatively a difficult task as most of the microspheres were non-porous in na-

Table 2: Physicochemical characteristics of microspheres taken for further *in vitro/in vivo* investigations

D : P ratio	Mean diameter (μm ± SD)	Yield (%)	Entrapment efficiency (%)
1 : 40	38.35 ± 13.57	79.54	57.38 ± 4.75
1 : 60	43.51 ± 20.56	71.24	60.11 ± 3.88
1 : 80	45.08 ± 23.63	76.51	61.68 ± 3.47

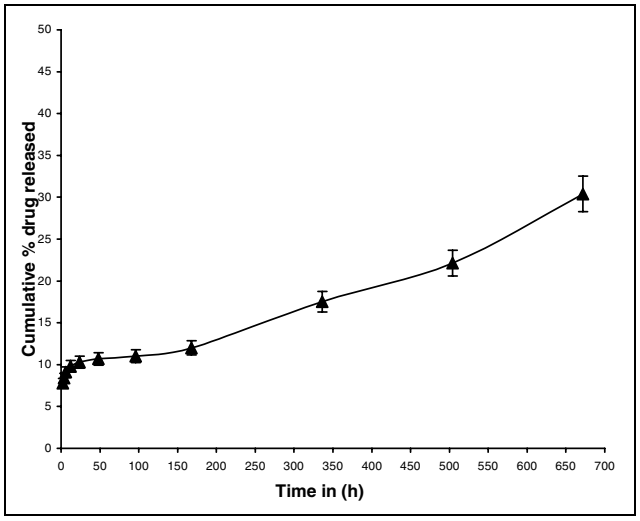

Fig. 1: *In vitro* release of BLM from PCL microspheres

ture. However, higher hydrophilicity of the drug would help to drive it through the pores formed as a result of erosion. The higher rates of release with time indicates the commencement of polymer hydrolysis and hence erosion of the microsphere system [11].

2.4. Pharmacodynamic evaluation

The anti-tumour efficacy of bleomycin sulphate in the free form as well as in the delivery system was assessed in a well-established transplantable, non-metastatic solid tumour model, namely, melanoma B16F1. We chose the solid tumour as model for evaluation since the majority of the complex problems encountered in cancer chemotherapy are in treating solid tumours and BLM exhibits potent anticancer activity against solid tumours. Further, the tumour model chosen exhibits well defined growth properties and hence, an assessment of the pharmacodynamic activity of the drug in the delivery system becomes more meaningful.

The results obtained with a single dose, single modality treatment are shown in Table 3. As the dose was immediately available for cytotoxic action with free BLM, it retarded the tumour growth at initial stages itself resulting in a significant ($P < 0.05$) increase in VDT (3.25 days against 2.88 days with control). There was significant ($P < 0.05$) increase in VDT for PCL microsphere-based depot formulations also at both the dose levels when compared against control. Similarly, the depot formulations exhibited significant ($P < 0.05$) increase in GD values compared to BLM-control at both the dose levels. We observed a PR of 10%, only for higher dose levels studied for depot formulation.

Table 3: Antitumour efficacy of BLM-loaded PCL microspheres in C57BL/6J mice bearing melanoma B16F1

Treatment (%)	VDT (days)	GD (days)	Partial Response (%)	No Response
Control	2.88 ± 0.34	–	–	100
BLM control (s.c.)				
10 mg kg^{-1}	3.25 ± 0.56	2.36 ± 0.76	–	100
PCL-BLM (s.c.)				
10 mg kg^{-1}	$4.67 \pm 0.78^{*}$	$4.96 \pm 0.81^{**}$	–	100
20 mg kg^{-1}	$6.18 \pm 1.14^{*}$	$7.37 \pm 1.18^{**}$	10	90

Control – No treatment
BLM control – Free BLM injection in sterile water for injection
Results are mean ± SD
* $P < 0.05$ compared with control
** $P < 0.05$ compared with BLM control

The pharmacodynamic behavior of formulations against melanoma B16F1 can be explained on the basis of altered biodistribution. Upon formulating as microspheres with PCL, not all the dose of injected drug was available for pharmacodynamic action immediately post-administration. Instead only a small fraction (drug adhering onto microsphere surface), which amounts to quite a negligible proportion of the injected dose, was available for immediate action. Further fractions of doses were slowly released from the formulation offering the maintenance dose. For comparing the antitumour efficacy of BLM-loaded biodegradable formulations, GD and PR obtained would be more sensible parameters, as the actual and realistic concentrations of BLM would be attained in plasma after an initial lag period. Hence, by the time the tumour volume has doubled, there would be a comparatively smaller fraction of administered dose available to exert pharmacological action. However, with GD, this inherent drawback in the experimental protocol would be overcome, as BLM levels in the plasma would start rising due to biodegradation of the polymeric backbone resulting in more of BLM at the injection site available for systemic absorption. The cytotoxic effects of BLM as revealed by increased VDT, GD and PR% in the present study with biodegradable microspheres in solid tumour models, is in good agreement with the reports of other investigations in mammalian cells *in vitro* [12, 13]. The anticancer effect of BLM at very low concentrations of 2.0 μg ml^{-1} was reported by Umezawa et al. [14]. BLM inhibited DNA synthesis in HeLa cells and showed growth inhibition of certain mouse tumours such as Ehrlich carcinoma, Sarcoma-180, Yoshida sarcoma, Rous carcinoma and Ascites hepatoma etc. The present finding, the tumour growth inhibition, evidenced by increased VDT in melanoma when treated with different doses of BLM (10 mg kg^{-1} and 20 mg kg^{-1}) corroborates the above.

2.5. Pharmacokinetic evaluation

When the data obtained with plasma drug estimation is compared with pharmacodynamic data, a good correlation could be established with anti-tumour activity and plasma drug concentration. These are the initial trends of drug release in a period of 10 days and the trends may change upon longer periods of study. BLM is a highly water-soluble drug and hence would easily get dissoluted in the invading aqueous phase and will be released from the depot through the pores or the aqueous channels formed in the polymeric microspheres. A fluctuation in the plasma levels of BLM was observed which could be due to variations in the rate of drug leakage through the pores and channels and differences in the rate of polymer erosion. However, due to influence of physiological conditions on polymer bioerosion, the rates of drug release were faster than that seen under *in vitro* conditions. The formulations were given as s.c. injection in the same volume of vehicle. The rate of BLM release into systemic circulation started rising steadily as a function of time. Hence, as with VDT of solid tumour, the formulations had significant ($P < 0.05$) effect, which could be attributed to the amount of BLM released from the formulations within the period of time taken for tumour volume to double that of treatment vol-

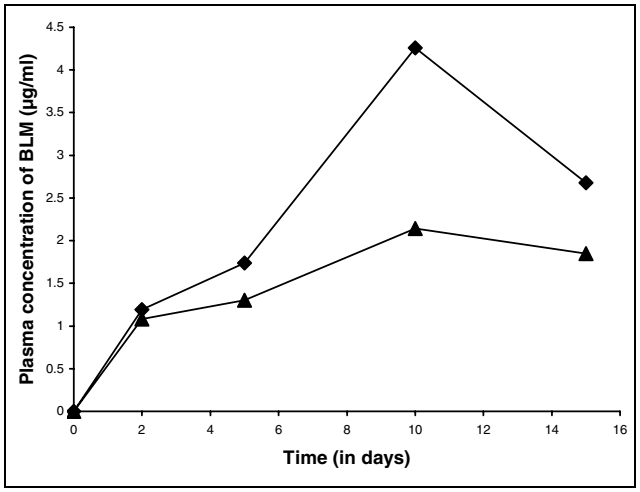

Fig. 2: Plasma concentration-time curve for BLM-loaded PCL microspheres in C57BL/6J mice bearing melanoma B16F1. (—▲—) PCL 10 mg kg^{-1} s.c. (—◆—) PCL 20 mg kg^{-1} s.c.

ume. An initial drug fraction was released quite quickly (may be attributed to the burst effect) *in vivo* so as to inhibit the tumour growth at initial stages itself wherein the tumour cells are vulnerable to drug action. BLM acting through producing single and double strand breaks in DNA by forming free radicals such as superoxide or hydroxyl radicals, was effective in causing damage to tumour cells during the initial phase of their multiplication cycle. Similarly, the concentration was maintained in the systemic circulation to cause further inhibition of growth and multiplication as evident by significantly ($P < 0.05$) increased GD values at both dose levels (Fig. 2). The effect on PR% at 20 mg kg^{-1} for PCL formulation may be due to the higher plasma drug concentrations attained with higher dosage. With higher drug payload (total concentration in injected fraction), there was a concentration gradient maintained between microsphere interior, the site of injection and the systemic circulation, through which, BLM was continuously infused into systemic circulation. However, none of the animals exhibited complete regression as the total quantity of BLM released might not have been enough to completely inhibit the growth kinetics of tumour cells which are well established by the plasma concentration.

The research envisaged in this particular project would be more meaningful if we could reformulate the microsphere-based depot system using a polymer having faster biodegradation rate (approximately one month), so that the encapsulated drug payload is delivered at the initial phase of tumour growth.

3. Experimental

3.1. Materials

Bleomycin sulfate (BLM) was purchased from M/s Khandelwal Laboratories, Mumbai, India. PCL (molecular weight 72,000) was procured from Aldrich Chemicals, USA. All the other chemicals and reagents were of analytical grade and were used as procured.

3.2. Animals

The animals (C57BL/6J mice) were obtained from the National Institute of Nutrition, Hyderabad. The institutional Animal Ethical Committee of Kasturba Medical College approved the experimental protocol for all the *in vivo* studies. The animals were maintained under controlled conditions of temperature (23 ± 2 °C), humidity (50 ± 5%) and light and darkness (10 and 14 h respectively) in polypropylene cages filled with sterile paddy husk as bedding material. They were fed balanced diet (Lipton India Ltd.) and water ad libitum. The mice chosen for experiments were six to eight weeks old, weighing between 25–30 g.

3.3. Formulation of PCL microspheres

BLM-loaded PCL microspheres were prepared by the emulsion-solvent evaporation method [15]. PCL was dissolved in an appropriate volume of methylene chloride (MC) to get a polymer solution of 40 to 60% w/v. A primary emulsion (w/o) was prepared with aqueous solution (0.1 to 0.5 ml) of the drug as internal phase and polymer solution as the external phase using a high-speed homogenizer. The primary emulsion was allowed to stabilize for about 15 min and then was poured under high-speed stirring into the bulk of the aqueous solution of polyvinyl alcohol (PVA, 0.1 w/v), previously cooled below 10 °C. After high-speed stirring for about 3 min to get a secondary w/o/w emulsion, it was agitated slowly using a mechanical stirrer for about 4 h at 25 °C. The microspheres formed were collected by filtration using a Millipore® filtration assembly fitted with Sartorius filters (0.45 µm) and washed with distilled water. Finally, they were dried in a vacuum dessicator for suitable time and stored in airtight, amber colored containers under refrigeration.

In detail, BLM-loaded PCL microspheres were prepared as follows: 1g PCL was dissolved in 2 ml of MC. Aqueous solution (100 µl) of drug containing a fixed concentration of BLM (2.5 mg) was added and homogenized to get the primary emulsion. This was poured into 400 ml of 0.1% w/v aqueous solution of PVA and homogenized at 3000 rpm for 3 min. followed by slow stirring at room temperature for 4 h.

3.4. Determination of particle size

The mean diameter and particle size distribution was determined using a particle size analyzer (Shimadzu – CIS 100, Japan; based on laser transition time principle for particle size measurement).

3.5. Determination of encapsulation efficiency

Twenty-five milligrams of drug-loaded microspheres were dissolved in 5 ml of MC. The drug was back extracted into 10 ml of phosphate buffered saline (PBS pH 7.4) by agitation for 30 min. Aqueous layer was separated and was assayed for drug content after suitable dilution with PBS (pH 7.4) at 291 nm using a UV-visible spectrophotometer. From the data obtained, drug entrapment efficiency was computed.

3.6. In vitro release studies

Weighed microspheres containing a known quantity of drug loaded microspheres were suspended in PBS pH 7.4 in stoppered flasks which were placed in horizontal shaker water bath maintained at 37 ± 1 °C at a speed setting of 25 cycles per minute. At predetermined time intervals, microsphere samples were collected from pre-labeled flasks and assayed for drug content as explained under 3.5. Amount of BLM released was estimated by material balance and a graph of time versus cumulative percentage of drug released.

3.7. Pharmacodynamic evaluation

3.7.1. Tumour induction

The mouse melanoma B16F1 cell line was obtained from the Department of Radiobiology, KMC, Manipal. For the experiments, solid tumours were induced by intradermal administration of 5×10^5 viable tumour cells on the dorsal side of the C57BL/6Jmice. Once the tumour became palpable, diameters in three perpendicular planes was measured as explained earlier [16] and tumour volume (V) was calculated: [$V = \pi/6$ ($D_1.D_2.D_3$), where D_1, D_2, D_3 are tumour diameters along three perpendicular planes].

3.7.2. Drug treatment

The drug-loaded delivery system (microspheres) was evaluated for anticancer efficacy in mice bearing solid tumours. The treatment was given in single dose modalities once the tumour size reached 100 ± 10 mm^3. The first group ($N = 6$) served as control and received no treatment. The second group ($N = 12$) received free BLM in normal saline (10 mg kg^{-1}). The third group received drug-loaded PCL microspheres suspended in a suitable vehicle (consisted of aqueous solution of 0.5% w/v sodium carboxymethyl cellulose (viscosity of 9 cps), 0.9% w/v sodium chloride and 0.1% w/v polysorbate 80). Two dose levels were explored (10 and 20 mg kg^{-1}). All the injections were given through a 21-guage needle subcutaneously.

3.7.3. Study parameters

The morphological tumour growth response was assessed on the basis of tumour regression, volume doubling time (VDT – time taken for the tumour volume to reach double the treatment volume), growth delay (GD – difference in time between treated and untreated tumours to reach five-times the treatment volume), partial response (PR – regression to 50% or

more than the treatment volume) and no response (NR – less than 50% regression in the treatment volume), calculated from the tumour volume measured thrice weekly. Survival studies were not carried out due to ethical considerations. All the animals were sacrificed once the tumour volume exceeded 1500 mm^3.

3.8. Pharmacokinetic evaluation

Pharmacokinetic studies of BLM preparations were carried out in mice bearing experimental tumour viz., melanoma B16F1. The treatment modality was similar to that followed for pharmacodynamic evaluations. Blood samples were withdrawn periodically by sino-arbital vein puncture using heparinized capillaries (at each point, blood was collected from three mice and pooled); plasma was separated by centrifugation and stored in vials under refrigeration until further analysis. BLM in the plasma was estimated by reverse phase HPLC as reported earlier [17,18].

3.9. Statistical analysis

The data obtained from above experiments was analyzed by SPSS software using an IBM computer.

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Received June 18, 2001
Accepted October 1, 2001

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