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ORIGINAL ARTICLE

THE POTENTIAL ADVANTAGES OF NANOTECHNOLOGY IN ANAPLASMOSIS THERAPEUTICS: PREPARATION OF OXYTETRACLINE-LOADED PMMA NANOPARTICLES FOR ORAL DELIVERY

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Abstract:

In recent days, nanoparticles-based drug delivery systems have considerable potential for treatment of several infectious diseases. The concept of nanoscale devices has led to the development of biodegradable self-assembled nanoparticles being engineered for the targeted delivery of therapeutic molecules. Biodegradable nanoparticulate carriers have become the promising nanoscale drug delivery system. The aim of the present investigation was to formulate and characterize the Oxytetracycline-loaded polymeric nanoparticles. Oxytetracycline, an antianaplasmic agent, was encapsulated by the nanoprecipitation method using poly(methyl methacrylate). Zeetasizer, Atomic force microscope, differential scanning calorimetry and FTIR were used to characterize the nanoparticles. The average diameters of the nanoparticles ranged between 200-250nm and the zeta potential was -32 mV. The drug loading capacity and loading efficiency of nanoparticles varied between 33.7% - 72.2% and 44.5% - 76.0%. The release profiles exhibited a biphasic phenomenon indicating controlled release of drug. The investigation suggests that the resultant nanoparticles would promise to be a good candidate for oral administration.

KEYWORDS:

Oxytetracycline; Nanoprecipitation; PMMA nanoparticles; Controlled release .

INTRODUCTION

Anaplasmosis, an infectious disease caused by Anaplasma marginale to pose serious health problems in cattle around the world. A death loss of 20 or 30 percent is more common and mortality rate may range between 5-10 percent in newly infected herds. Continued drug resistance in infectious organism(s) has become challenging task in prevention and control of the disease. Younger animals may be off feed and moderately anemic, but usually recover rather quickly. Animals less than 1½ or 2 years of age seldom show symptoms, although they may become infected and serve as a reservoir of infection for other herd members. Anaplasmosis is spread from infected to susceptible cattle by ticks and biting insects. Biting insects spread the disease by transmitting infected blood from infected to susceptible animals. The

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infectious organism invades and destroys red blood cells, causing anemia, weakness, and sometimes death. A high body temperature (103-104o F) is associated with pyrexia with increased lachrymation and salivation. Death often occurs within 24 to 48 hours of the onset of symptoms and may be the first indication that anything is wrong in a herd. Abortion after recovery from clinical disease is common.

The anaplasmosis organism is susceptible to Oxytetracycline (Terramycin) or Chlorotetracycline (Aureomycin) antibiotics. Unfortunately, cattle showing clinical signs usually respond to high dosages of Oxytetracycline given by injection. In some cases, however, 60 or 70 percent of the red blood cells in cows with clinical disease are infected. These cattle may die in spite of treatment because the tetracyclines only prevent spread of the organisms between red blood cells. They do not kill the organism once it is inside the cell. Early recognition and treatment is important. When clinical disease is diagnosed, immediate treatment of affected animals with high dosages (10 mg Oxytetracycline per pound of body weight or more for 3 to 5 days) is recommended. In many herds, feeding Oxytetracycline at the rate of 0.1 to 0.25 mg or chlortetracycline at 0.5 mg per pound of body weight daily for 30 days throughout the vector season. Although vaccines are available for anaplasmosis, unfortunately vaccines also do not eliminate the infection from a herd. They only prevent the development of clinical disease. Vaccinated cattle that are carriers of anaplasmosis will remain carriers, even though they will not develop the disease themselves. Testing and treating in the late fall or early winter precludes possible further spread by insect vectors. Some of these drawbacks including inadvertent use of high dosages of Oxytetracycline could be minimized via emerging nanotechnological tools to benefit the targeted farmers. As a matter of fact, the diagnosis and treatment of infectious diseases in veterinary area have not been greatly improved with the recent developments in nanobiotechnologies. The advent of microspheres technology suffered from several demerits, such as low drug encapsulation efficiency, high polymer consumption, and difficulties in the inclusion of drugs for therapeutic preparations In fact, biodegradable and biocompatible drug-loaded nanoparticles can be designed for their robustness, high carrier capacity, to administer through variable routes include oral application and injection. A targeted drug delivery system using nanotechnology strategies must supply drugs selectively to its site of action in a manner that provides maximum therapeutic activity (thought controlled drug release), prevent degradation or inactivation during transit to the target site and protect the body form adverse reactions. This cost effective strategies would reduce the burden to targeted farmers and the side effects in diseased animals. These properties of nanoparticles would to allow slow and sustained release of drug from the matrix. This would enable drug bioavailability, reduce dosing frequency, and may resolve the problem of non adherence to prescribed therapy, which is one of the major obstacles in the control of infectious diseases. This article highlights the preparation and characterization of Oxytetracycline-loaded nanoparticles formulated for the oral delivery.

REVIEW OF LITERATURE

Anaplasmosis in cattle is an arthropod-borne disease caused predominantly by the intraerythrocytic rickettsia Ana-plasma marginale21. Anaplasmosis is characterised by mucosal pallor, depression, inappetance, general weakness and a rapidly rising parasitaemia. Vertical transmission of anaplasmosis has been documented, although its significance in the spread of the diseases is unclear The clinical findings in congenital anaplasmosis in calves presented to the Onderstepoort Veterinary Academic Hospital with clinical signs of congenital anaplasmosis, which was unresponsive to treatment, are described. The nature of the immunity to anaplasmosis is not well understood. The tetracycline drugs effectively inhibit the multiplication of Anaplasma in erythrocytes and have been widely used for the treatment of anaplasmosis, with parasitaemia usually decreasing to 1 % or lower within 4-7 days. an oxytetracycline regimen proposed by the World Organization for Animal Health for elimination of persistent Anaplasma marginal infections in cattle. Now a day's nanodrugs are successfully using for treatment many disease with least/no side effect. Hence current study focused on preparation of oxytetracline-loaded pmma nanoparticles for oral delivery for the better treatment.

MATERIALS AND METHODS

Poly(methyl methacrylate) (PMMA) and Oxytetracycline (OTC) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai). Polyvinyl alcohol (PVA) was purchased from Sigma-Aldrich, New Delhi, India. Acetone (AR grade) was obtained from Thomas Baker (Chemicals) Ltd., Mumbai, India. Water used for all experiments was triple distilled water or Milli-Q water. All other chemicals were of highest purity available and were used without further purification.

PREPARATION OF NANOPARTICLES

PMMA nanoparticles in the size range of 200–250 nm were produced by nano precipitation method (H. Fessi et al., 1989). In a typical procedure, 20 mg of the PMMA polymer and a weighed amount of OTC were dissolved in 10mL of acetone. The organic phase was added drop wise into aqueous phase containing 0.01% PVA as stabilizer under sonication (Vibra-Cell™ Ultrasonic Processors, Newton, USA). Drug-free nano particles were also prepared by omitting the drug.

SEPARATION OF UNINCORPORATED DRUG FROM NANO PARTICLES

Raw nano particles were purified by cross flow filtration (using a lab scale tangential flow filter (TFF) system (Millipore, India) fitted with Pellicon XL 50 ultra filtration device with a molecular weight cut off 50,000Da (Biomax polyethersulfone, Millipore) to remove the free drug (OTC alone) and stabilizer (PVA). Filtration was performed at feed pressure 2.07 bar (30 psi) and retentate pressure 0.69 bar (10 psi) by adding volumes of water which were collected as filtrate fractions. The amount of eliminated free Oxytetracycline in each fraction was determined by using UV spectrophotometer at 264 nm.

FREEZE-DRYING

In order to determine the exact solid content of the nanoparticles, lyophilization was carried out in a freeze-drier Labconco (Labconco Corporation, USA) and dried under high vacuum (0.05 mbar) for 48 h. The accurate amount of nanoparticles in the dried powder was assessed by weighing the vials after freeze-drying. The dried samples were stored at -20 °C before analysis. The nanoparticles recovery which is also referred to as nanoparticles yield in the literature was calculated using following equation

$$\text{Nanoparticles recovery (\%)} = \frac{\text{Mass of PMMA nanoparticles recovered}}{\text{Mass of polymer, drug and Stabilizer}} \times 100$$

PHYSIOCHEMICAL CHARACTERIZATION

Particle size and Zeta potential

The average particle size and zeta potential of the NPs were measured using a Zeetasizer Nano-NZ (Malvern Instruments Ltd., UK)). Freshly prepared particles suspension (800 µl) was placed in a clear disposable zeta cell (DTS-1060C, clear disposable zeta cell) without dilution. The measurement was carried out using a light source 4mW HeNe laser (633 nm) at a fixed angle 173°. The following parameters were used for experiments: medium refractive index 1.330, medium viscosity 0.8872 cP, a dielectric constant of 78.54 and temperature 25°C. Each size measurement was performed at least 12 runs. All measurements were carried out in triplicate directly after nano particles preparation, and the results were expressed as mean size ± S.D.

Surface topography of nano particles

Nanoparticles topography was studied with the atomic force microscope (AFM) using Nanoman (Veeco Instruments Inc. of Plainview NY, USA) at VINL, JNCASR, Bangalore. The nanoparticles were first diluted in deionised water to an appropriate concentration and then ultrasonicated for 15-30 min. A few drops of the suspension were placed on a freshly cleaved mica surface and allowed to dry at room temperature. The images were captured using was non-contact AFM mode and silicon tips with spring constant of 20-80N/m. Images were computed from the changes in vertical position. Nano particles surfaces and topography could be observed with high resolution by controlling the contrast. This general method was followed for all samples.

Oxytetracycline content in the nanoparticles

Freeze dried nano particles were added in to acetone and methanol (1:2). The mixture was centrifuged at 10,000 rpm for 10 min in cold centrifuge (Sigma, USA) and the supernatant was analyzed by HPLC system (Shimadzu Corporation, Kyoto, Japan).

In order to determine the content of OTC a reverse phase HPLC method was used. The mobile

phase was 20 mM KH2PO4, methanol and orthophosphoric acid (70:30:0.2 V/V/V). The HPLC system consisted of a model LC-10AT VP pumps and SPD-10AV VP UV detector (Shimadzu Corporation, Kyoto, Japan) linked to an injection valve with 20µl sample loop. The analysis performed at a flow rate of 1.5 ml/min with the UV detector at 264 nm. A reverse phase Lichrosphere® C18 column (250 × 4 mm, 5µm) from Merck was used. Data acquisition and processing were accomplished with a personal computer using Class VP software.

OTC content and entrapment efficiency were calculated according to the following equations.

$$\text{Oxytetracycline content (\%W/W)} = \frac{\text{Mass of OTC in PMMA nanoparticles}}{\text{Mass of PMMA nanoparticles recovered}} \times 100$$

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Mass of OTC in PMMA nanoparticles}}{\text{Starting mass of OTC}} \times 100$$

Fourier Transformed Infrared Spectra

FT-IR spectra (Spectrum Perkin Elmer RX 1) of OTC alone, OTC-loaded PMMA nano particles, OTC-free PMMA nano particles and PMMA polymer were recorded in potassium bromide pellets, and the spectrum was recorded between 4000-500 cm-1 using a high energy ceramic source.

Thermal Analysis

The thermal behavior of the nano particles was analyzed using a PERKIN-ELMER Diamond DSC instrument (PERKIN-ELMER Diamond DSC, USA). Approximately 5 mg of nano particles were accurately weighed into a 40 µl hermetic aluminum pan and sealed. An empty pan was used as a reference. The samples were heated from 30 to 300°C at a rate of 50°C per minute and readings were taken on this first heating ramp. Indium was used as the standard reference material to calibrate the temperature and energy scale of the apparatus. All experiments were carried out in triplicate.

In vitro release study

The dialysis bag diffusion technique was used. Four milliliters of the nanosuspension were placed in the dialysis bag (Mw cutoff 12,000, Spectrum Medical Industries, Inc., US), hermetically sealed and the dialysis bag immersed in 100 ml 0.1N hydrochloric acid medium (pH 1.3) under sink conditions. The entire system was kept at 37°C with continuous magnetic stirring at 200 rpm. At predetermined time points 1ml of the sample was withdrawn and immediately another 1ml of fresh medium was added. OTC concentration was determined using HPLC-UV at 264 nm. The experiment was carried out in triplicate.

Statistical analysis

Results are given as mean ± standard error of the mean (SEM) or ± standard error (SE). Means were compared with the Student's test. Differences are considered significant at a level of $p<0.05$.

RESULTS AND DISCUSSION

Morphological evaluations

AFM was used to visualize the surface morphology of the freeze-dried OTC-loaded and OTC-free PMMA nano particles before the in vitro drug release study was undertaken. Shown in Figure 1(a) and (b) are the surfaces of the OTC-loaded and OTC-free PMMA nano particles, respectively. Surfaces of all PMMA nano particles were free from erosion. This confirms the greater satiability of PMMA nano particles to water uptake and subsequent hydrolysis and degradation. The AFM proved to be a valuable tool to study the damage and influence of degradation of erodable surfaces of polymeric nano particles during preparation and processing.

Particle size and zeta potential

Nano particles are characterized by their mean particle diameter and their size distribution. The average diameters, zeta potentials, pH values of blank nano particles and OTC-loaded nano particles are listed in table 1. The average diameters of nano particles from different preparation ranged from 230 to 242 nm. The polydispersity indices obtained from the measurements were around 0.1 or lower indicating narrow deviations in sizes. Although it is now well known that the in vivo fate of nano particles is primarily a function of their size, the shape of the nano particles may also determine their toxicity.

These measures are confirmed by atomic force microscopy that allowed both the determination of the particle size and shape of the particles (Fig. 1). These average diameters were similar to those that were observed with particles reported in previous studies with the nano precipitation method (L. Marchal-

Heussler et al., 1992). The particles were sufficiently small to avoid sedimentation.
The zeta potential of the nano particles was negative. High potential values should be achieved in order to ensure a high-energy barrier (S. Benita, and M.Y. Levy., 1993) and favor a good stability. Muller (R.H. Muller., 1991) considered that a zeta potential of about -25 mV allows an ideal stabilization of nano particles because the repulsive forces prevent aggregation upon ageing. Accordingly, -32 mV, which is the value obtained with OTC-loaded PMMA nano particles, favour the best stability among the designed nano particles.
The cross flow filtration method using a lab scale tangential flow filter system allows the separation of both drug-free and drug-loaded nano particles. It was found that a high entrapment efficiency of OTC was obtained with all forms. The percent of theoretical encapsulation was 70.2 %.
At present, freeze-drying is the method of choice to remove water from the system in nano particles formulations. Generally, a high nano particles recovery is required to reduce the manufacturing costs by considering their size and morphology, which are important for quality control and biodistribution.
In our formulations, OTC-loaded PMMA nano particles and OTC-free PMMA nano particles were recovered (~80 %) in dried form. During this process, we were able to notice enhanced hydrophilicity and redispersion of the freeze-dried nanoparticles, which may be due to the presence of PVA as surfactant.
The importance of enhanced nano particles drug incorporation efficiency has been emphasized earlier. Since high nano particles recovery is required for reducing manufacturing costs and its size and morphology important for quality control and biodistribution (S.J. Douglas et al., 1987)
Characterization of the physicochemical state of the drug and the polymer in nano particles
The physical state of both the drug and the polymer were determined since this will have an influence on the in vitro and in vivo release characteristics of the drug. Different drug/polymer combinations may coexist in the polymeric carriers, such as i) amorphous drug in either an amorphous or a crystalline polymer, and ii) crystalline drug in either an amorphous or a crystalline polymer (M. Jenquin and J. McGinity., 1994). Also, a drug may be present either as a solid solution or a solid dispersion in an amorphous or crystalline polymer.
Characterization by Fourier Transform Infrared (FTIR) was specifically carried out to determine the adsorption of the drug in the given nano particles. The FT-IR spectrum of the OTC-free PMMA nano particles, OTC alone, PMMA alone and OTC-loaded PMMA nano particles clearly indicates the carbonyl group at 3020 cm-1. There is little else in these spectra except the peaks for the various C-H and C-O stretches and deformations (2952 cm-1, 1440 cm-1, 1215 cm-1, 1728 cm-1). In addition to the peaks seen in the OTC-free nanoparticles there was a new peak with the OTC-loaded PMMA nanoparticles at 1528 cm-1 which corresponds to the NH2 group of the drug and the peak at 1041 cm-1 corresponding to the ketonic group of the drug, thus confirming the presence of OTC (Table: 5.3). The peaks for the CH3, CH2 and O=C-O-C=O stretching appeared at a similar position as in the PMMA alone but with lower intensities. This could probably indicate the effect of polymer drug interactions leading to change in vibration energies of free groups. The decrease in intensity of the carbonyl group peak could also indicate its deviation from the free state.
Differential scanning calorimetry
The melting peak of OTC was absent in OTC-free PMMA nano particles but present in OTC-loaded PMMA nano particles. In Figure 4, the Tg of the OTC-free PMMA nano particles was around 340 °C. OTC alone showed an exothermic peak at 210 °C However, this peak did not appear in the curves for the OTC-loaded PMMA nano particles. Based on thermogram data the melting point of OTC in OTC-loaded PMMA nano particles shown to be 210°C (Figure 4 (A)) is a thermogram of the OTC alone with a small peak at 210°C indicating that there was OTC in the sample. But in an admixed sample containing PMMA polymer and free-OTC the Tg of the polymer was diminished due to the presence of dissolved OTC in the formulation acting as a plasticizer. This would suggest that a very small loading of OTC on to the PMMA matrix could significantly diminish the Tg of the PMMA polymer.
In vitro release study
It needs to be verified in vitro that nano particles are able to release incorporated OTC in order to achieve a biological effect. Membrane diffusion techniques are the most widely used experimental methods for the study of the in vitro release profiles of OTC incorporated in nano particles. The in vitro release profile of OTC from PMMA nano particles in 0.1N HCl (pH 1.3) and 10 % polyethylene glycol 400 solution is presented in Fig: 5.13, which shows a very rapid diffusion of OTC with the PEG solution: 50% of OTC is released after 1.5 h and 100 % after 8 h. In 0.1N HCl 50 % of OTC is released in 1.5 h and 92 % release after 12 h. This release profile shows a biphasic phenomenon. The effect of acidic and neutral pH conditions on OTC release seems minor. Drug release from nano particles is usually a biphasic phenomenon although on some occasions a triphasic profile is also seen. Firstly there is an initial rapid removal of the drug from nano particles possibly related to loss of drug associated loosely on the surface of the nano particles. This initial release is rapid and uncontrolled and is termed the burst release

The selection and optimization of drug release studies is a problem with colloidal drug delivery systems. Other solid dosage forms (e.g. tablets, capsules, microspheres) can be readily separated from the surrounding bulk medium. This is not the case for colloidal polymeric dispersions and it is generally necessary, as for the determination of the percent of theoretical encapsulation of the drug, to separate the release medium from the polymeric suspension by means of either ultracentrifugation or separation membrane. The dialysis technique is a very popular method to study the release of drugs from colloidal suspensions. The release studies were carried out in an aqueous environment with pH 1.3 and pH 6.8 to mimic the in vivo condition, e.g. gastric pH and intestinal pH, since the aim of the study was to administer the nano particles by the oral route. The limits of this dialysis technique have been discussed by Washington, who reported that the colloidal dispersions were not diluted inside the bag. The release rate of the drug and its appearance in the dissolution medium is governed by the partition coefficient of the drug between the polymeric phase and the aqueous environment in the dialysis bag and by the diffusion of the drug across the membrane as well. This technique allows the comparison of different formulations. These findings would suggest that it is possible to engineer different release profiles from drug-loaded PMMA by blending different molecular weight fractions of the same polymer. From investigations, we also found that the manufacturing technique of the polymeric nano particles can greatly influence the release profile and that it can annul the significant effects of drug loading, molecular weight as well as polymer composition. We would like to investigate this concept further by evaluating the mechanism of nano particles formation during different coating techniques. PMMA seems to be a potential candidate to use as biocompatible coating agents from which one can achieve local drug delivery.

CONCLUSIONS

We demonstrated that a water-soluble anti-anaplasmosis agent (OTC) based PMMA nano particles can be prepared and characterized. In vitro release kinetics study shows that OTC-loaded PMMA nano particles exhibited biphasic phenomenon indicating a slow and sustained release of cargo (almost 92 % release at the end of 12 h in acidic pH). The UV peaks for OTC remained unchanged indicating retention of therapeutic activity in the formulation. Evaluation of OTC-loaded PMMA nano particles formulations were found to be a potential and cost-effective for sustained oral delivery system in terms of particle size distribution and optimum drug loading capacity characteristics. We achieved maximum in vitro release rates of OTC corresponding to systemic therapeutic levels necessary to treat the given infectious diseases for a prolonged period of time. Whether the local therapeutic concentration necessary to treat anaplasmosis in vivo can be achieved with such a system remains to be evaluated but the release profiles obtained in this study appear adequate. Based on these findings we can conclude that OTC-loaded PMMA nano particles are promising formulations for oral delivery.

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