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Poly [D, L-lactide-co-glycolide] Microspheres as a Delivery System of Protein Ovalbumin Used as a Model Protein Drug

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ABSTRACT

The purpose of this study was to develop protein containing microspheres by multiple emulsification (water-in-oil-in-water, w/o/w) technique at different homogenizing speeds using biodegradable polymer poly (D,L-lactide-co-glycolide) (85:15/PLGA). Here, ovalbumin was used as a model protein drug. Although, many studies available with PLGA-based protein loaded microparticles, none of them provides sufficiently convincing technologies for manufacturing protein-loaded microparticles with all the standardized process parameters such as amount of protein loading, protein-release, stability of the protein, polydispersity and zeta potential. Therefore, further research is required in the field. Various process parameters such as protein polymer interaction, particle size, surface morphology, drug loading, protein-polymer ratio, zeta potential, polydispersity, protein stability and protein release characteristics were studied here. *In vitro* protein release study showed that release profile of ovalbumin from biodegradable microspheres varied due to the change in homogenizing speeds during multiple emulsion preparation technique. Drug loading efficiency varied from 21.82 to 36.54%. The protein biodegradable microspheres were spherical in shape. The stability studies of protein were investigated at different temperatures for 30 days by using a Fourier Transform Infrared (FTIR) spectroscopy following ICH guidelines. The FTIR analysis showed the structural integrity of ovalbumin in PLGA microspheres. PLGA microspheres containing ovalbumin as a model protein could be useful for the controlled delivery of similar protein drugs.

Key words: Microspheres, protein delivery, ovalbumin, sustained release, stability study

INTRODUCTION

Proteins are an integral part of the body as they carry out important physiological and biological processes such as ligands for signaling, enzymes for biotransformation reactions, receptors for pharmacological response elucidation, antibodies in immune system interactions, transcription and translation (Hurwitz *et al*., 1975; Sinha and Trehan, 2003). Due to recent advent of recombinant DNA technology, a wide range of protein drugs such as vaccine, cytokines, enzymes, hormones, growth factors are now commercially available in a large quantity as therapeutic agents (Park *et al*., 1995). In formulating protein/peptides for therapeutic uses various difficulties are encountered (Mukherjee *et al*., 2008). When, protein is administered orally rapid degradation occurs due to proteolytic enzymes in the gastrointestinal tract and its macromolecular size cannot cross quickly the biological barrier which makes oral bioavailability low (Kang and Singh, 2001;

Susan *et al.*, 2005). Their short biological half-lives, usually in the range of minutes to few hours, require frequent injection regimens and cause considerable discomfort to patients, especially when long-term or chronic treatment is necessary (Kim *et al.*, 2005). One of the important ways to improve biological half-lives of proteins from minutes to several hours to month is the controlled systemic delivery of proteins (Kim *et al.*, 2005; Mukherjee *et al.*, 2008). Biodegradable microspheres have been extensively studied as a controlled and sustained delivery systems for protein and peptide drugs (Yeh *et al.*, 1995). Poly (D,L-lactide-co-glycolide) (PLGA) is well characterized biodegradable polymer (Kim *et al.*, 2005; Susan *et al.*, 2005) approved for human use as surgical sutures, implantable devices and drug delivery systems by US Food and Drug Administration. The PLGA is a copolymer of lactides/glycolides. Depending on lactide/glycolide molar ratio, various verities of PLGA are available in the market. Generally, PLGA (50:50 molar ratio) based microspheres, microparticles, nanoparticles have been studied widely but very few studies have been reported with PLGA (85:15 molar ratio). Further, none of the available studies related to manufacturing and standardization of process parameters is sufficient enough to bring them in a large scale industrial production. Thus more studies in this area are required. In present study, we used PLGA (85:15, lactide/glycolide molar ratio) to develop controlled release protein delivery systems of biodegradable microspheres. The purpose of the present study was to develop protein (ovalbumin)-loaded microspheres with biodegradable polymer, poly (D,L-lactide-co-glycolide) (PLGA) and standardization of various process parameters such as homogenizing speed during preparations, particle surface morphology and surface charges, particle size and *in vitro* protein release to obtain microspheres with maximum protein-loading and minimum polydispersion with a maximally sustained protein release pattern.

MATERIALS AND METHODS

Material: Ovalbumin and poly [D, L-lactide-co-glycolidic acid] (85:15) (PLGA) were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Polyvinyl alcohol (PVA, M.W. 1, 25,000) was obtained from S.d Fine-Chem. Ltd., Mumbai, India. Dichloromethane (DCM) was purchased from E. Merck Ltd., Mumbai, India. All others chemicals used were of analytical grade.

Methodology: The study was designed and the formulations were developed and experiments were conducted during 2006-2008. Microspheres were prepared by double emulsification-solvent evaporation technique. Precisely, PLGA (250 mg) was dissolved in 2 mL dichloromethane (DCM). The PVA solution (0.5 mL) [2.5% w/v PVA in water] prepared earlier containing 6 mg protein (ovalbumin) was homogenized for 4 min at 10,000 rpm to produce w/o type emulsion. This was then added drop-wise in 75 mL 1.5% w/v PVA solution in 400 mL glass tube and was homogenized for 6 min at 10,000 rpm to produce w/o/w type of emulsion. Finally, it was stirred on a magnetic stirrer overnight to evaporate dichloromethane. Other samples were produced by using the same method only varying homogenizing speeds (HS). The different HS used were 7000, 8000, 9000 and 10000 rpm and the samples prepared were S_7, S_8, S_9 and S_{10}, respectively. Suspension was centrifuged at 16,000 rpm for 40 min at 4°C to separate the microspheres. Samples were frozen at -20°C for 3 h. After pre-freezing, they were freeze-dried for 48 h in a 10 mL freeze drying vial (initial self stemperature -15°C and were decreased to -40°C). Finally, samples were removed.

Evaluation and characterizations of microspheres

Drug-excipients interaction: The pure protein, excipients and mixture of protein with the excipients were mixed separately with IR grade KBr in the ratio 1:100 and corresponding pellets

were prepared by applying 5.5 metric tones of pressure in a hydraulic press. The pellets were scanned over a wave number range of 4000 to 400 cm^{-1} in Magna IR 750 Series II (Nicolet, USA) FTIR spectroscope as described earlier (Mukherjee *et al.*, 2006).

Yield and % yield: Obtained microspheres were weighed and (%) yield was determined using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of microspheres obtained}}{\text{Total weight of drug and polymer used}} \times 100 \quad (1)$$

Loading efficiency of the formulations: Accurate weight of microsphere sample (5 mg) was taken into 1 mL 5% SDS-0.1 M NaOH solution in an effendorf tube and shaken in an incubator shaker at 37°C till, it got clear solution. After centrifugation at 3000 rpm for 4 min the supernatant was collected and analyzed (Lowry *et al.*, 1951). Amount of the protein was calculated from the calibration curve. Protein-loading percentage and protein-loading efficiency were calculated using the following formula (Pralhad and Rajendrakumar, 2004).

$$\text{Loading efficiency} = \frac{\text{Weight of protein in microspheres}}{\text{Weight of protein taken initially for preparation of formulation}} \times 100 \quad (2)$$

Scanning Electron Microscopy (SEM): The surface morphology of the microspheres was analyzed with a scanning electron microscope (JSM 6100 JEOL, Tokyo, Japan) (Ramesh and Ravichandran, 2008). Experimental samples were coated with gold and examined by a scanning electron microscope. Same procedure was done of the experimental microspheres after *in vitro* protein release of 30 days in Phosphate Buffer Saline (PBS), pH 7.2.

Particle size distribution and zeta potential study: The weighed amount of microsphere samples were suspended in PBS, pH 7.2 and particle size distribution and zeta potential were analyzed using a particle size analyzer as per manufacture's protocol (Malvern Instrument Limited, UK).

***In vitro* protein release study:** *In vitro* protein release study (Kang and Singh, 2001; D'Souza and DeLuca, 2005) was carried out by keeping numbers of prelabled (such as day 1, 2 and 3 etc.) micro-centrifuge tubes in which microspheres (5 mg) were suspended in 1 mL of Phosphate Buffer Saline (PBS), pH 7.2 in each case and shaken in an incubator shaker at 37°C. At predetermined time intervals, the samples were taken out of the incubator and centrifuged at 3000 rpm for 3 min. The supernatant was removed completely and the amount protein released was determined (Lowry *et al.*, 1951). Amounts of protein were determined from the calibration curve. *In vitro* protein release studies were carried out in triplicate for each batch of microspheres. The cumulative % release of ovalbumin from PLGA microspheres were plotted against days.

Stability study: Microsphere samples were incubated in incubators at different temperatures at 4, 25 and 40°C, respectively for 30 days. The analytical changes in FT-IR spectra, if any, for the protein and the excipients were determined by FTIR spectroscopy.

RESULTS AND DISCUSSION

Drug excipient interaction was initially assessed using FTIR-spectroscopy. When, the Fig. 1-3 were compared the data indicate that there were some interactions in the wave number ranges between 3700-2800, 1800-1600, 800 and 600 cm^{-1}. The wave numbers 3700-2800 cm^{-1} are the IR stretching vibration regions of functional groups -OH,-CH (aromatic), -CH (alkene), -CH (alkane) and -NH. Again 1800-1600 cm^{-1} wave number region is the IR stretching vibration region of the functional groups of C = N, C = C (olefenic) and C = O (keto). The 800-600 cm^{-1} wave number region is the IR stretching vibration region of the functional groups C-Cl, C-Br and C-C

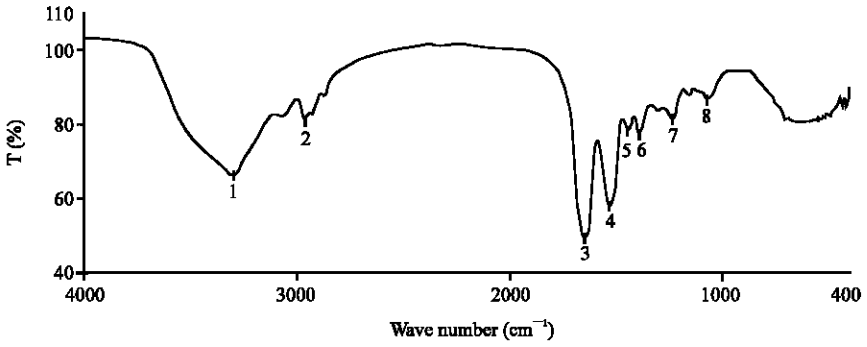

Fig. 1: FTIR spectra of ovalbumin

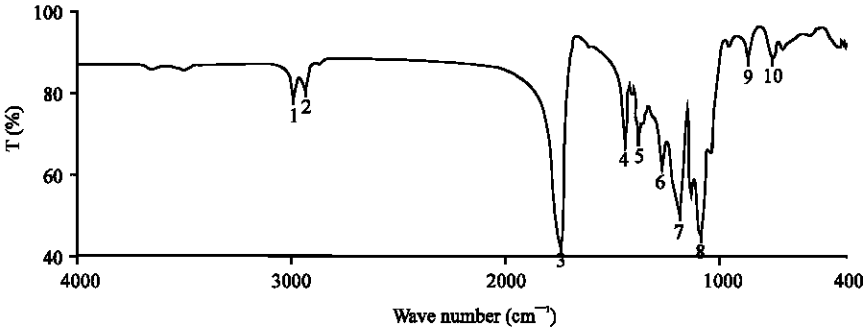

Fig. 2: FTIR spectra of excipients

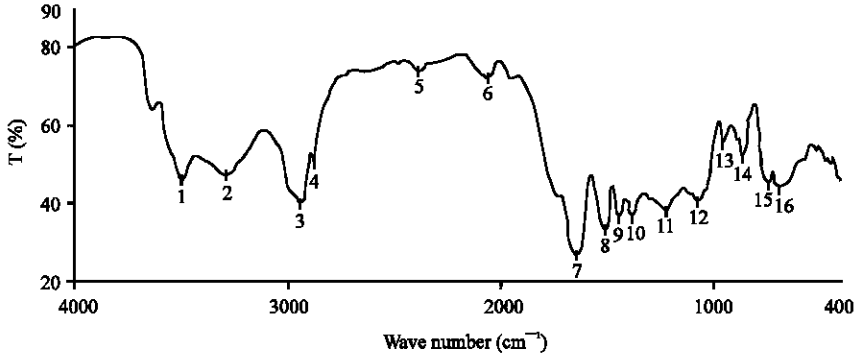

Fig. 3: FTIR spectra of ovalbumin and excipients

Table 1: Yield and percentage yield of the experimental formulations

Formulation code	HS (rpm) at which formulation prepared	Amount of PLGA used in formulation (actual) (g)	Amount of ovalbumin (mg)	Yield (%)	Loading efficiency (%) (% w/w) (Mean±SD, n = 3)
S_7	7000	0.2540	6	60.23	36.54±1.88
S_8	8000	0.2550	6	47.05	26.95±1.36
S_9	9000	0.2520	6	40.08	24.89±1.36
S_{10}	10000	0.2495	6	36.32	21.82±1.06

Table 2: Particle size (Z-average), polydispersity indices (PDI) and zeta potentials of different experimental samples

Sample	T°C	PDI	Z-average (nm)	Zeta potential (mV)
S_7	25	0.308	3229	-0.9671
S_8	25	0.205	4487	-3.6990
S_9	25	0.361	2837	-4.9090
S_{10}	25	1.000	1434	-6.4870

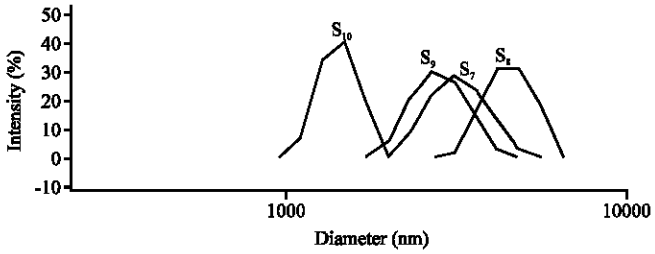

Fig. 4: Particle size distribution patterns of experimental microspheres

(Mukherjee *et al*., 2006). Thus, physical interactions between the amino group of the ovalbumin protein and the keto group of PLGA might take place in the region between 3700 and 2800 cm^{-1} by formation of weak bonding such as Van der Waal force of attraction or dipole moment or weak hydrogen bond since, in this region no shifting of characteristic peak was detected. Further, the characteristic peaks of the protein (Fig. 1) and the excipients (Fig. 2) were predominantly present in the spectra of the protein-excipient mixture (Fig. 3). Variation in peak length may be due to the variation of amounts of substances in the physical mixture tested for FTIR spectra.

The yields of various formulations (Table 1) were between 36.32 to 60.23%. Hence, loss of yield was observed in microsphere preparations using PLGA and ovalbumin. Loading efficiency as measured in terms of percentage varied between 21.82 and 36.24. More loading efficiency was detected with less homogenizing speeds.

Polydispersity index was found to vary with the homogenizing speed. The PDI values were varied as 0.308, 0.205, 0.361 and 1.0 in S_7, S_8, S_9 and S_{10}, respectively (Table 2). The particle size distributions of the microsphere samples were carried out using particle size analyzer. The average particle sizes were between 1.43 to 4.48 μm (Fig. 4). Zeta potentials were in negative and varied from -0.6 to -6.4 (Fig. 5). The highest value was obtained for S_7 and the lowest value was for S_{10} of the experimental formulations. The zeta potentials of all microsphere samples were negative in charge (Table 2).

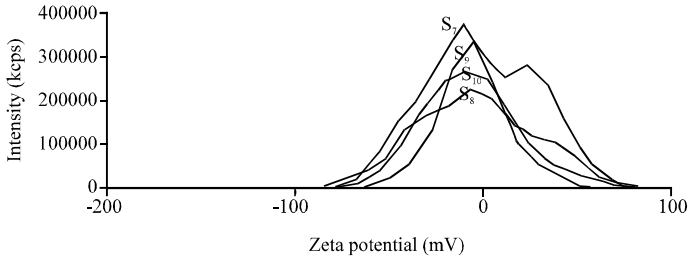

Fig. 5: Curves show zeta potentials of various experimental formulations

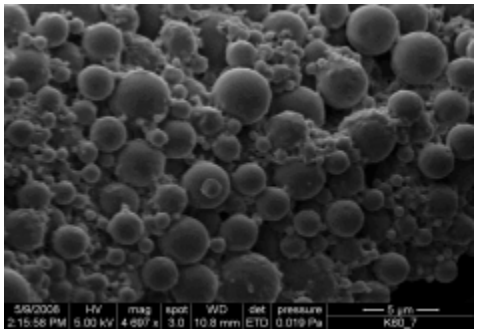

Fig. 6: Scanning electron microscopy of PLGA microspheres (S_{10})

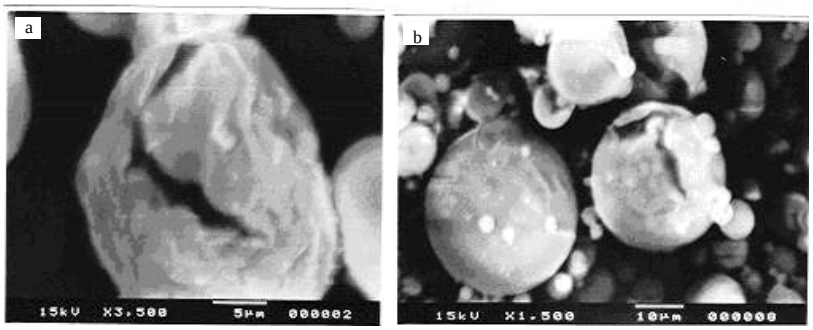

Fig. 7: (a, b) Scanning electron microscopy of PLGA microspheres (S_{10} at different magnifications) after *in vitro* release

The SEM photograph (Fig. 6) shows PLGA microspheres containing ovalbumin (Ramesh and Ravichandran, 2008). The size of the PLGA microspheres varied between 1.4 to 4.5 µm. Figure 6 shows that the PLGA microspheres containing ovalbumin were spherical. Figure 7 shows the SEM photograph of the PLGA microspheres 30 days after *in vitro* release of ovalbumin. Some small openings were seen on the surface of PLGA microspheres. In greater magnification, the holes are prominent and that the protein might have released through those areas from the core (Fig. 7a, b).

Ovalbumin released from the experimental microspheres were found to follow biphasic release (Fig. 8). Initially all the formulations showed some burst release in first couple of hours and this

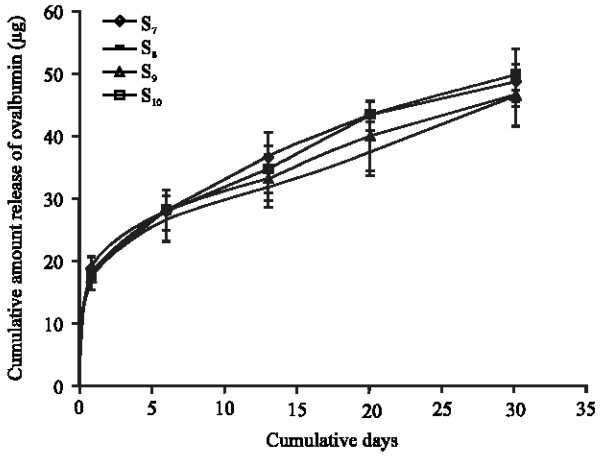

Fig. 8: Cumulative amount of ovalbumin released from various formulations. Data show Mean±SD (n = 3)

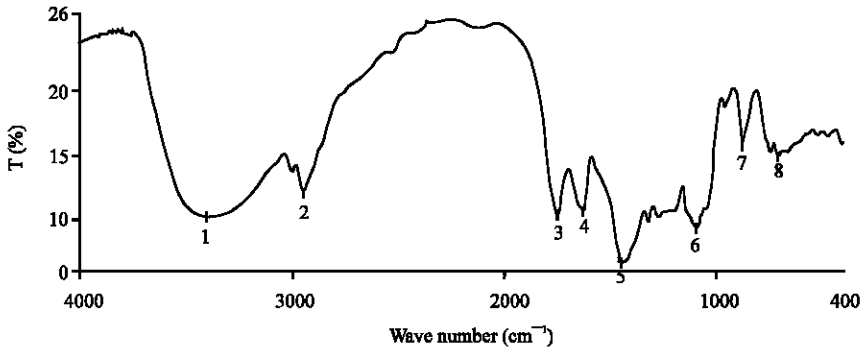

Fig. 9: FTIR spectra of ovalbumin microspheres (after formulation)

was followed by sustained protein releasing pattern. Cumulative ovalbumin release pattern varied by 20% in the formulations S_{10} as compared to S_8 and S_9.

For stability study of protein, the samples were kept at different temperatures such as 4, 25 and 40°C for 30 days following ICH guidelines and spectra of protein and excipients were determined by FTIR spectroscopy. Figure 9-12 represent the IR spectra of the formulation FP-4 (S_{10} which was kept at 4°C for 30 days), FP-25 (S_{10} which was kept at 25°C for 30 days) and FP-40 (S_{10} which was kept at 40°C for 30 days), respectively. Figure 9 represents the spectrum of the mixture of PLGA, ovalbumin and PVA. Figure 10-12 were compared with the Fig. 9. No variation of spectra suggests that the protein remained stable in the formulations.

Biodegradable polymeric matrix has been found promising for delivering proteins over a long period of time. The use of biodegradable poly (D,L-lactic-co-glycolic acid) (PLGA) microspheres for the delivery of peptides and proteins has been widely reported (Mehta *et al.*, 1996). Processing conditions employed during preparation of microspheres determine the properties of the microspheres, such as the size, morphology, encapsulation efficiency and drug distribution (Yang *et al.*, 2001). Here, we developed microspheres of PLGA containing ovalbumin as a model protein drug by varying various process parameters. In the present study, variation of processing

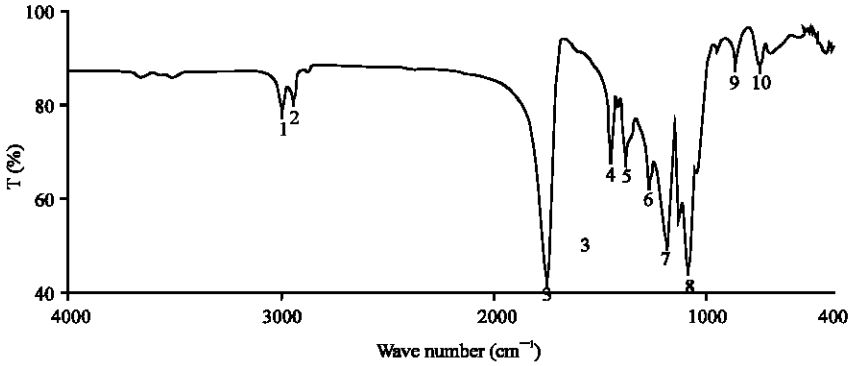

Fig. 10: FTIR spectra of formulation (30 days at 4°C)

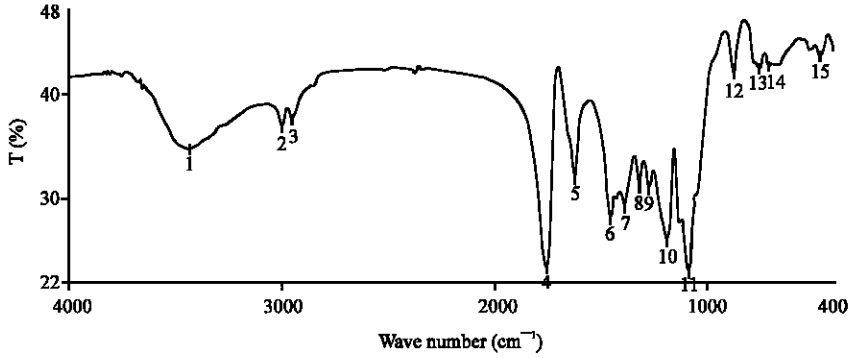

Fig. 11: FTIR spectra of formulation (30 days at 25°C)

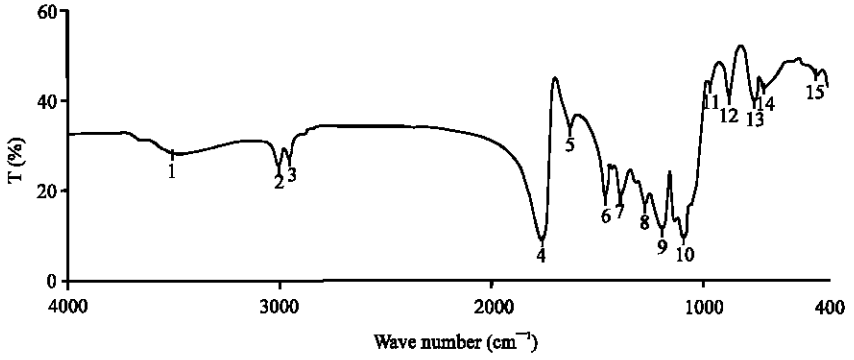

Fig. 12: FTIR spectra of formulation (30 days at 40°C)

parameters such as speed of homogenization and quantities of excipients was found to cause variation in yield (%) and protein loading efficiencies.

For the development of formulations, drug-excipient interaction is an important study which predicts the availability of the drug from the formulation, its release pattern and stability of the formulation (Mukherjee *et al.*, 2007). Slow release can be achieved due to binding the protein molecules physically to the microsphere matrix (Mukherjee *et al.*, 2007). The polymeric matrix

should have reactive functionalities to which the drug can be bound through functionalities available on the drug. There are various methods such as DSC, IR spectra, FTIR spectra, TLC etc., used frequently to study the drug-excipient interaction (Mukherjee *et al.*, 2007). The FTIR spectrum is one of the latest and very accurate data which can clarify drug-excipient interactions at the level of various functional groups existing between the protein and excipient molecules. No chemical interaction is suggested as the characteristic peaks of protein as well as excipients were present. However, studied physical interaction could be beneficial for sustaining the release of protein from the formulations.

Ovalbumin-loaded PLGA microspheres had a loss in yield. The loss of yield might be mainly due to the adherence of primary emulsion on the inner wall of the homogenizer which was not fully recovered due to its sticky nature.

The loading efficiency decreased according to an increase in speed of homogenization amongst the formulations studied. The variation of loading efficiency in different samples may be because of formation of smaller particles at higher speed which could only entrap a smaller amount of ovalbumin containing solvent (Table 1). Further, negatively charged surface of the PLGA microspheres might be due to ionization of surface group, i.e., the ionization of carboxyl group in PLGA.

The protein release from biodegradable microspheres is governed by many factors (Smith *et al.*, 1990). These include the degradation rate of PLGA copolymer, which largely depends on the physical properties of polymer such as molecular weight, hydrophilicity and the ratio of lactide to glycolide (Smith *et al.*, 1990). All these properties influence the release of drug from the delivery system. Composition and morphology of microspheres plays key role in modulating protein release. *In vitro* release profile of ovalbumin from PLGA microspheres was bi-phasic with an initial burst release of loosely bound ovalbumin near to the microsphere surface. This generally occurs by simple diffusion. This was followed by release of the protein from the polymer matrix (in the core) as it eventually erodes. The release profile of the various samples were similar. The differences in sustained release of protein from the various samples might also be due to the different particle sizes existed in a formulation with the usually varied surface areas of microspheres of the various samples prepared (Fig. 8). Protein release study showed that the protein from all the formulations released in a sustained manner for the entire period of study in a similar trend.

As the stability study was concerned, the experimental formulations were found to be stable in the experimental range of temperatures.

CONCLUSIONS

In conclusion, poly (D, L lactide-co-glycolic acid) (PLGA) may be used to develop microspheres containing ovalbumin. These PLGA based formulations were capable of providing a controlled and sustained release of the protein from the microspheres. Various physico-chemical parameters obtained from this study, such as shape, size, surface morphology, loading, % loading, loading efficiency, zeta potential were also found to be favorable for the development of the formulation. Some drug-excipient interactions, although, present between amino group ($-NH_2$) of drug and the keto group (C = O) of PLGA polymers, seem to contribute the slower and sustained release of ovalbumin from PLGA microspheres. Furthermore, varying speeds of homogenization during primary emulsification varied formulation characterization.

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