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Research Article

PULLULAN ACETATE CONTROLLED-RELEASE BIODEGRADABLE MICROSPHERE CONTAINING A BIOLOGICALLY ACTIVE AGENT: PREPARATION, CHARACTERIZATION AND *IN VITRO* EXPERIMENTS

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ABSTRACT

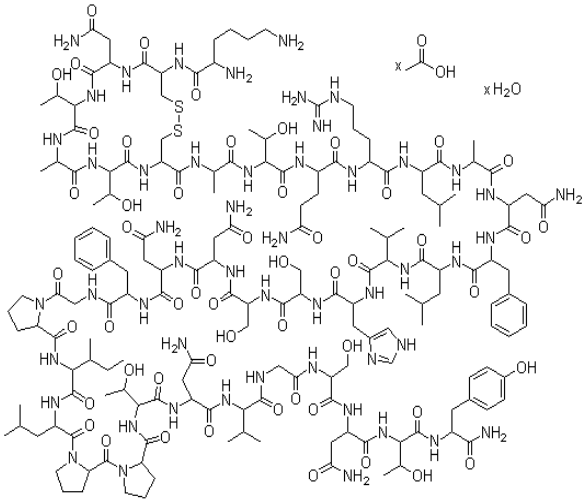
The purpose of our present study was to investigate the feasibility of prolonged delivery of a synthetic peptide, Pramlintide, with a biodegradable polymer microsphere depot formulation. Pramlintide loaded microspheres were prepared by the W/O/W double emulsion method and the *in vitro* drug release profiles from microspheres were investigated. Pramlintide loaded microspheres with a suitable particle size (mean diameter about 45–100 μm) and high entrapment efficiency (>90%) were prepared. All the prepared microspheres were subjected to various physicochemical studies, such as drug-polymer compatibility by Thin Layer Chromatography (TLC), surface morphology by Scanning Electron Microscopy (SEM), frequency distribution and encapsulation efficiency by High Performance Layer Chromatography. The *in vitro* release study showed sustained drug release over 14 d with obvious "burst release" in the first 24 h. Pramlintide microspheres can be uniformly suspended in aqueous medium for subcutaneous injection, and may be used for sustained delivery of Pramlintide to Diabetic patients.

Keywords: Controlled release, Biodegradable polymers, Peptide delivery, Microspheres.

INTRODUCTION

According to *World Health Organization* published in 1994, the Diabetes mellitus is the only non-infectious disease designated as an epidemic. The prevalence of all types of diabetes is estimated to be 2.3 % of the world's population, with the number of diabetic increasing by 4 to 5% per annum. It is projected that as many as 40 to 45% of people aged 65 or greater have either type 2 diabetes or its precursor state, impaired glucose tolerance (IGT). Diabetes can treat by a combination of lifestyle and dietary changes medication. The United Kingdom Prospective Diabetes Study (UKPDS), a long-term study of type 2 diabetics, showed that rigorous management of blood pressure substantially reduced the incidence of complications such as peripheral nerve damage, kidney damage, impaired blood circulation and damage to the retina[1,2].

Pramlintide (25,28,29-pro-h-amylin) is the synthetic analogue of human amylin, which is a 37-amino acid peptide related to calcitonin gene-related peptide (CGRP) and calcitonin, and is co-secreted with insulin in response to elevated plasma glucose concentrations from pancreatic β-cells (Fig. 1).Pramlintide is an injectable drug that lowers the level of sugar (glucose) in blood. It is used for treating type 1 and insulin-using type 2-diabetes[3-8].

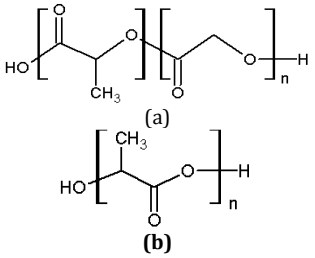


Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Pro-Ile-Leu-Pro-Pro-Thr-Asn-Val-Ser-Asn-Thr-Tyr-NH_2

Figure 1: Chemical structures of Pramlintide acetate.

An injectable, multi-dose liquid formulation for Pramlintide drug product has been marketed to permit chronic self-administration by the anticipated patient population[8]. However, the complexity of the Pramlintide treatment regimen, including the frequency of administration and duration of treatment, negatively affects patient compliance. Reduction of the required frequency of administration is one strategy that might significantly enhance compliance [9-12].

Encapsulation with biodegradable polymers has been considered as one possibility to overcome various obstacles associated with the systemic delivery of peptide drugs[13-19]. Biodegradable polymer microspheres using poly (lactide-co-glycolide) (PLGA) and poly(lactic acid) (PLA) as wall materials containing water-soluble bioactive substances (such as proteins, peptides) have received a great deal of interest in recent years due to their many advantages, such as improved patient compliance, increased bioavailability and reduced immunogenicity[20-22]. PLGA microspheres have been widely investigated and used as injectable depot drug carriers[23-27]. (Fig. 2)



(a)

(b)

Figure 2: Chemical structures of poly (lactic acid – co –glycolic acid) (PLGA) (a) and poly (lactic acid) (PLA) (b).

Apart from PLGA, Pullulan acetate (PA) with different degree of substitution with acetyl groups has also been selected as a polymer for the preparation of microspheres, because it has a biocompatible and biodegradable polymer, which is degraded into non-toxic oligomers or monomers. Thus, it has been investigated for use in biomedical and biomaterial applications [28-34]. (Fig. 3)

The aim of our present study was to investigate the feasibility of developing an injectable depot formulation for chronic delivery of Pramlintide, as well as improving patient compliance and achieving better therapeutic efficiency by reducing the frequency of injections and decreasing the fluctuations in plasma drug levels. In this study, Pramlintide loaded microspheres were prepared by the W/O/W double emulsion method and the *in vitro* release profiles were investigated.

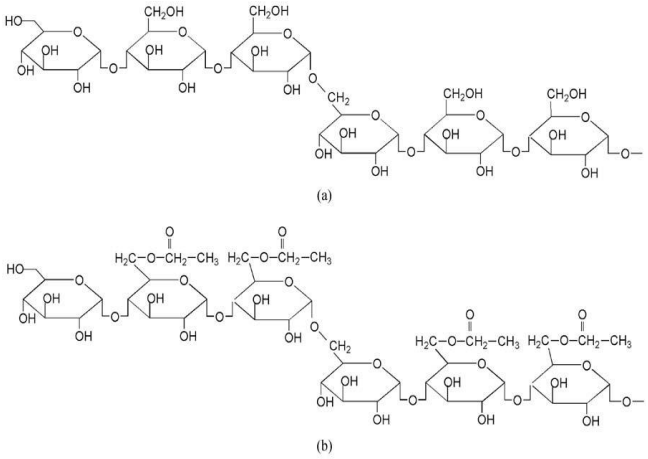


Figure 3: Chemical structure of pullulan (a) and PA (b).

MATERIALS AND METHODS

Materials

In this study, Pramlintide (purity > 99%), PLGA5050 (LA/GA = 50: 50, IV = 0.78 dL/g) and Pullulan acetate with different degree of substitution (D.S.) with acetyl groups (D.S. =1.8, 2.4 and 2.8) were provided by Sun Pharmaceutical Ltd. Co (India). Polyvinyl alcohol (PVA, Mw: 30-70 kDa) was procured from Merck (Germany). All other chemicals and reagents were used for formulation development were of analytical grade from commercial sources.

Preparation of microspheres

Pramlintide microspheres were prepared using water-in-oil-in-water (W/O/W) double emulsion method. Briefly, 50 mg of Pramlintide were dissolved in 1.0 mL of water for injection. 950 mg of polymer was dissolved in 4.5 ml of dichloromethane. The Aqueous phase was added to oil phase and sonicated to prepare a primary W/O emulsion. The obtained emulsion was emulsified in 300 ml continuous phase (W: 0.5% w/v PVA in water for injection) by homogenization at 5000 rpm at 37^{o}C for 2.5 hour (Lab Mixer, silverson) thereby removing dichloromethane and hardening the polymer. The resultant microspheres were collected by centrifugation, washed three times with water for injection, and freeze dried. In preparing formulations by the same method as above, the suspension for injecting the primary emulsion was suspended in 1.5 M lysine aqueous solution + 3 % w/v PVA, 1.5 M histidine aqueous solution + 3 % w/v PVA and 1.5 M arginine aqueous solution + 3 % w/v PVA, 1.5 M urea aqueous solution + 3 % w/v PVA. As a control, PLGA microspheres were also prepared by same method. (Fig. 4)

In vitro drug release

A sample of accurately weighed microspheres (10 mg) was dispersed in 1 ml of a release test solution (10 mM HEPES, pH 7.5, 100 mMNaCl), and incubated at 37^{o}C under mild stirring at 5 rpm. At intervals, the tubes were taken out and centrifuged at 5,000 r/min for 10 min, and the supernatants were removed and stored in a refrigerator until HPLC analysis, and 1 ml fresh release media was added to each tube. The microspheres were redispersed uniformly by vigorous vortexing before further release studies. Each experiment was performed in triplicate. The shape and surface morphology of the microspheres were observed after different periods in the *in vitro* release experiments.

Analysis of Pramlintide

Reversed phase high performance liquid chromatography (RP-HPLC) was used to determine the concentration of Pramlintide. The liquid chromatograph was equipped with a 215 nm detector and an YMC Pack Pro C18 column (250 × 4.0) mm, 3 µm. A mixture of phosphate buffer and acetonitrile (70: 30) was used as the mobile phase at a flow rate of 0.8 ml per minute.

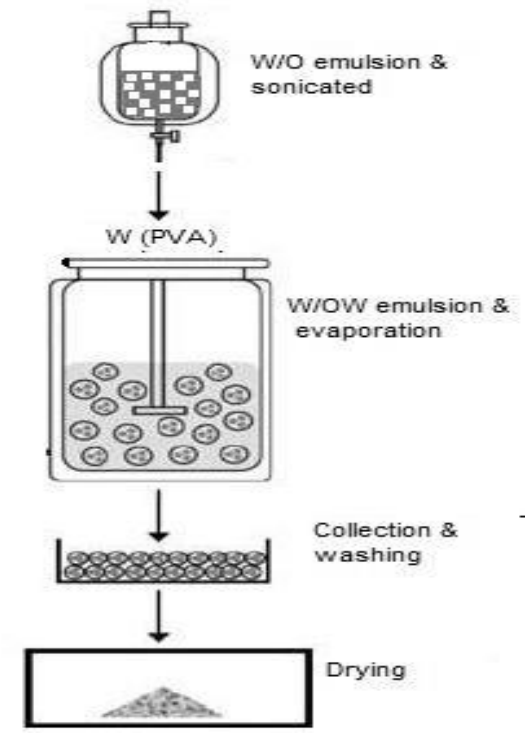


Figure 4: Schematic illustration of the water-in-oil-in-water (W/O/W) double emulsion method used to prepare the Pramlintide microspheres.

Thin Layer Chromatography (TLC)

Thin Layer Chromatography was carried out in TLC chamber. The sample solution of pure drug and prepared microspheres were prepared by dissolving in methanol: water (80:20) and applied to silica gel G plates. The plates were then developed in the following solvent systems.

Solvent system 1: n-butanol: water: methanol: ammonia (20%) (14:0.2:0.2:2 %v/v/v/v)

Solvent system 2: Concentrated ammonia: alcohol (20:80 % v/v)

The $R_f$value of the pure drug as well as prepared microspheres were determined by placing the plates in an iodine chamber and the $R_f$valueof pure drug was compared with the $R_f$value of prepared microspheres.

Scanning Electron Microscopy (SEM)

Scanning electron microscope (LEO, 430 surface controlled digital SEM) was performed to characterize the surface of formed microspheres. A small amount of microspheres were spread on glass stub. Gold palladium coating on the prepared stub was carried out by using sputter coater. Afterwards, the stub containing the sample was placed in the electron microscope. The scanning electron photomicrograph (Plate-1, Plate-2) was taken at acceleration voltage of 15 kV, chamber pressure of 0.3 Torr.

Viscosity measurement

A Brookfield rotational digital viscometer DVLV-II was used to measure the viscosity (cPs) of the internal and external phases at 25 ºC. The spindle number 1was rotated at 100 rpm.

Frequency distribution analysis

Samples of microspheres were analyzed for frequency distribution with calibrated optical microscope fitted with a stage and an ocular micrometer. Small quantities of microspheres were spread on a clean glass slide and the average size of 200 particles, frequency distribution as determined in each batch using the calibration factor.

Determination of Percent yield and Drug Entrapment (PDE)

Efficiency of drug entrapment for each batch was calculated in terms of percentage drug entrapment (PDE) as per the following formula;

% Yield = Weight of microspheres*100 / Theoretical weight of drug and polymer

% PDE = Practical drug loading*100 /Theoretical drug loading

Theoretical drug loading

Theoretical drug loading was determined by the calculation assuming that the entire drug present in the polymer solution used gets entrapped in microspheres, and no loss occurs at any stage of preparation of microspheres[35].

Practical drug loading

Practical drug loading was analysed as follows:

20 mg of microsphere were added to 100 ml of acetonitrile and methanol in ratio of 3:2 and occasionally shaken for 30 min. The solution was centrifuge and 1 ml of clear supernatant was diluted to 10 ml of water, the supernatant liquid was filter through Watt mann filter paper and analysed for Pramlintide by High Performance Liquid Chromatography.

RESULTS AND DISCUSSION

Preparation and characterization of microspheres

In order to improve the conventional method of a peptide delivery system, PAs with different degrees of substitution (D.S.) with acetyl groups (1.8, 2.4 & 2.8). Their solubility tests on a variety of organic solvents were initially conducted in order to determine the optimum method for preparation of microspheres. The polymers were well dissolved in volatile solvents such as methylene chloride, Acetonitrile, Ethyl acetate and tetra-hydrofuran, but were not soluble in water and ethanol (Table 1). These results help for preparation of microsphere. Microspheres prepared by the water-in-oil-in-water (W/O/W) double emulsion method normally have a wide particle size distribution and a mean diameter that varies from batch to batch. The mean diameters of the microspheres made from different types of Polymer were between 44-100 µm. Normally, the higher acetylation of the polymer used, the lower the mean diameter obtained (Table 2). In general, as one of the major issue with the peptide delivery of PLGA microspheres is the profound initial release but short period of release. Pullulan acetate is found decrease in initial release with long period of overall release. The scanning electron micrographs showed that the Pramlintide microspheres were spherical in shape with a smooth surface. The typical entrapment efficiencies were more than 90% as shown in Table 2. Improvement of loading efficiency was observed with higher level of acetylation. In order to obtain a high trapping efficiency with decrease in initial release with long period of overall release, a W/O/W double emulsion method procedure was adopted.

In vitro drug release

The typical drug release profiles of Pramlintide PLGA microspheres exhibited significant "burst" release followed by slow drug release for over 7 days (Fig. 5). Although, the microspheres with the Pullulan acetate showed even the lowest "burst" release, the drug release rate was also slow for over 14 days. As shown in Figure 5 Sometimes, the burst release was caused by the rapid diffusion release of drug from the surface of the microspheres. We believe that the initial rapid release observed in present studies may due to some of the Pramlintide molecules migrating from the inner phase to the surface of the microspheres during the drying process. Microspheres coated with the coating materials exhibit considerably decreased initial burst compared with microsphere that is not coated with the coating materials. Although the decreased amount of initial burst slightly varies depending on the kind of coating material used. Hence, various polymer and coating materials were used as materials for microspheres in further investigations in order to lowest "burst release". As shown in Table 3 the decrease of initial burst release (not more than 1% in the first 24 h) slightly varies depending on the kind of coating material used. By using blends of different degree of substitution (D.S.) with acetyl groups (D.S. =1.8, 2.4 and 2.8) of Pullulan as matrix material for the microspheres, the burst release in the first 24 h could be limited to less than 1% of the total drug loaded as using different kind of coating materials, According to our present results, the Pullulan acetate of the matrix materials had a significant effect on loading efficiency and *in vitro* drug release, but further experiments should be carried out to obtain ideal release profiles.

Drug release from microspheres was generally considered as a polymer degradation-controlled process. The SEM observations of microspheres after different periods of *in vitro* drug release testing showed a gradual degradation of the microsphere matrix. As shown in Figure 6, with the degradation of Pullulan acetate, the microsphere morphology changed from an original smooth surface to a porous surface after 7days and, finally, the microspheres disruption was observed after 14 days.

Compatibility studies

Chemical interaction between drug and the polymeric material, if any, during the preparation of microspheres was studied by using a TLC. The comparable Rf values of microspheres in the TLC study indicated the compatibility of drug with polymer and other excipients used in the preparation of Pramlintide microspheres[36].

Table 1: Solubility of Pullulan acetate in various solvents.

PA with different D.S with acetyl groups	water	EA	MDC	Ethyl alcohol	Methanol	Acetone	Ether	Acetone	Acetonitrile	THF	Heptane
1.8	-	±	+	-	±	±	-	+	+	+	-
2.4	-	+	++	-	±	±	-	++	++	++	-
2.8	-	++	+++	-	±	+	-	+++	+++	+++	-

Note : The solubility of Pas was determined by the measuring of doluble rate (1mg/ml).(+++ :- 4-10s;++10-20s:+:-40s±:- swells ; - :- no solubility).MDC :- Methlene chloride ; THF :- Tetra-hydro-furan ;EA :- Ethyla acetate.

Table 2: Efect of different degree of substitution (D.S) with acetyl groups.

Sr.No	Formulation code	PA with different D.S with acetyl groups/PLGA	Particle size (µm) Means±S.D	Drug entrapment efficiency (%) means ± S.D
1	Q	1.8	60.70±3.0	85.11±1.2
2	R	2.4	55.83±2.4	91.32±2.2
3	S	2.8	45.90±1.5	96.12±1.3
4	T	PLGA5050 , 0.78dL/g	98.70±3.0	95.11±1.2

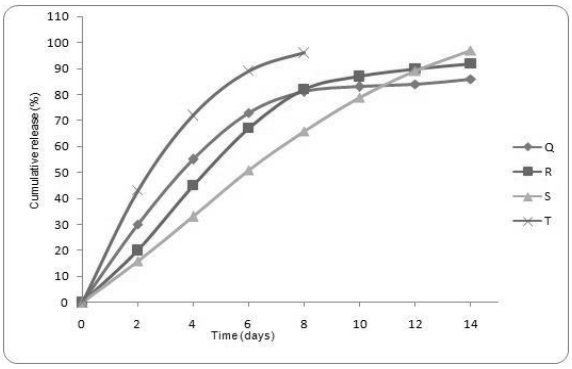


Figure 5. *In vitro* release of Pramlintide Microsphers.

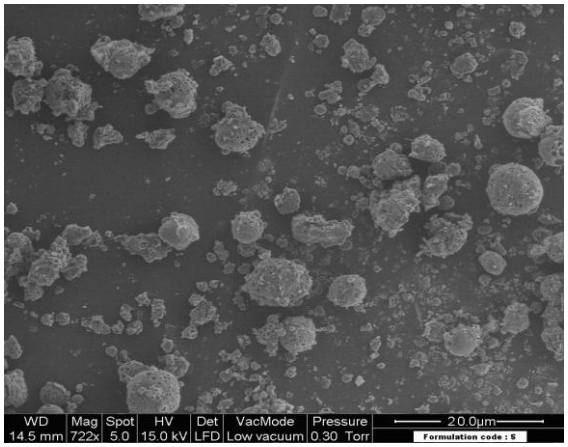


Figure 6: PA microspheres showing pore on surface during in-vitro release.

Morphological characteristics (SEM)

The surface morphology of Pramlintide loaded microspheres were studied by scanning electron microscopy (Figure 7). Surface smoothness of microsphere was increased by increasing the degree of substitution (D.S.) with acetyl group up to certain limits, which was confirmed by SEM.

Particle size distribution

The results of accuracy and precision of frequency distribution studies showed the normal frequency distribution of microspheres.

Under conditions of high stabiliser concentration, shear induced disruption of the secondary emulsion occurs forming smaller microspheres up to certain concentration of PVA (Table 4).

And also as the degree of substitution (D.S.) with acetyl groups of PA was increased, the mean particle size of Pramlintide PA microspheres was also decreased (Table 2) [35, 36].

Drugentrapment efficiency

The drug loading efficiency of Pramlintide microspheres was determined by HPLC method. A more than 90% of drug entrapment efficiency was obtained by the method employed (Figure 8). And also the advantage was that an improvement in loading efficiency was inevitably increased at the same time as degree of substitution (D.S.) with acetyl groups increase (Table 2).

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Table 3; Effect of coating material on the Initial Burst of Pramlintide loaded microsphere.

Sr.No	Formulation code	PVA suspension	1h release %	24 release %
1	U	3% PVA + 1.5 M lysine	0.67	1.87
2	V	3% PVA + 1.5 M histidine	0.57	1.74
3	W	3% PVA + 1.5 M Arginine	0.39	1.23
4	X	3% PVA + 1.5 M Urea	0.33	1.09

Table 4:Effect of PVA stabilizer concentration on the characteristics of microspheres.

Sr.No	Formulation code	% w/vPVA suspension	Particle size (µm) Mean±S.D	Entrapment efficiency (%) Mean±S.D	1h release %	24h release %
1	M	0.5	98.70±3.0	80.12±1.3	1.9	3.6
2	N	1.5	85.83±2.4	81.32±2.2	1.55	2.9
3	O	3	69.00±1.5	83.01±1.2	1.65	3.3
4	P	5	67.93±3.2	79.33±3.2	1.82	4

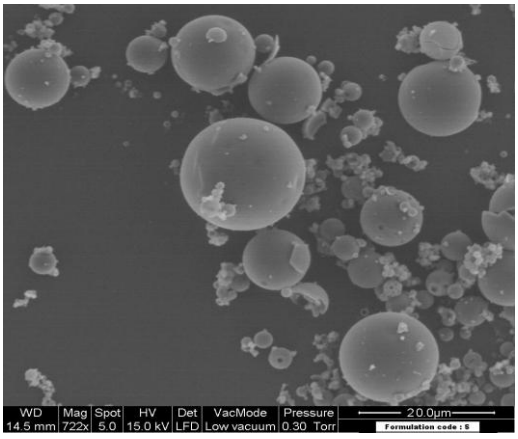

Figure 7. PA microspheres showing spherical in shape with a smooth surface.

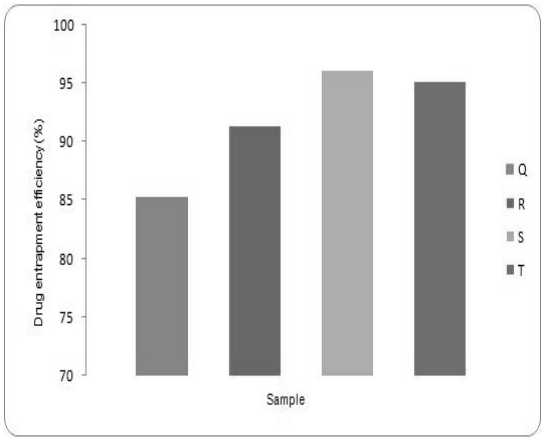

Figure 8.Drug entrapmentefficienies in various PA microspheres.

REFERENCES

1. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes., Lancet 1998; 352: 837-852.
2. Turner RC, Cull CA, Frighi V, Holman RR. Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Groups. J Am Med Assoc 1999; 281: 2005-2012.
3. Young A, Pittner R, Gedulin B, Vine W, Rink T. Amylin Regulation of carbohydrate metabolism. Biochem Soc Trans 1995; 23: 325-331.
4. Scherbaum WA. The role ofamylin in the physiology of glycemic control. Experimental and Clinical Endocrinol and Diabetes 1998; 106(2): 97-102.
5. Amiel S. Amylin and diabetes. LANCET 1993; 341: 1249-1250.
6. Thompson RG, Gottlieb A, Organ K, Kolterman OG. Pramlintide, a human amylin analog reduced post-prandial plasma glucose, insulin and c-peptide concentrations in patients with type II diabetes. Diabetic Medicine 1997; 14(7): 547-555.
7. Thompson RG, Peterson J, Gottlie A, Mullane J. Effects of pramlintide, an analog of human amylin, on plasma glucose profiles in patients with IDDM: results of a multi-center trial. Diabetes 1997: 46(4): 632-636.
8. Brower V. Amylin's pramlintide best of bad bunch of diabetes drugs. Nature Biotechnol 1997; 15(10): 935.
9. Heller J. Controlled release of biologically active compounds from bioerodible polymers. Biomaterials 1980; 1: 51-57.
10. Lewis DH. Controlled release of bioactive agents from lactide/glycolide polymers. In: Chasin M, Langer R, editors. Biodegrable polymers as drug delivery systems. New York, US: Marcel Dekker 1990; 1-14.
11. Tice TR, TabibiEs. Parentral drug delivery: injectables. In: kydoieusA, editor. Treatise on controlled drug delivery: fundamentals optimization, applications. New York, US: Marcel Dekker 1991; 315-339.
12. Ogawa Y, Yamamoto M, Okada H, Yashiki T, Shimamoto T. A new technique to efficiently entrap leuprolide Acetate into microcapsules of polylactic acid or co poly (lactic/glycolic) acid. Chem Pharm Bull 1988; 36: 1095—1103.
13. Esposito E, Cortesi R, Bortolotti F, Menegatti E, Nastruzzi C. Production 7 characterization of biodegradable microparticles for the controlled delivery. Int J Pharm 1996;129: 263—273.
14. Tice TR, Cowsar DR. Biodegradable controlled-release parenteral systems. Pharm Technol 1984; 11: 26-35.
15. Arshady R. Preparation of biodegradable microspheres and microcapsules: 2. Polylactides and related polyesters. J Control Rel 1995: 17: 1-22.
16. Brannon-Peppas L. Recent advances on the use of biodegradable microparticles and nanoparticles in controlled drug delivery. Int J Pharm 1995; 116: 1-9.
17. Cohen S, Alonso MJ, Langer R. Novel approaches to controlled release antigen delivery. Int J Technol assessment Health Care 1994; 10(1): 121-131.
18. Zhao Z, Leong KW. Controlled delivery of antigens and adjuvants in vaccine development. J Pharm Sci 1996; 85(12): 1261-1270.
19. Eldridge JE, Staas JK, Chen D, Marx PA, Tice TR, Gilley RM. New advances in vaccine delivery systems. SemHematol 1993; 30(4): 16-24.
20. Heller J. Biodegradable polymers in controlled drug delivery. Crit Rev Therap Drug Carrier System 1984; 1(1): 39-90.
21. Kitchell JP, Wise DL. Poly(lactic/glycolic acid) biodegradable drug-polymer matrix systems. Methods Enzymol 1985; 112: 436-448.
22. Vert M. The complexity of PLGA-based drug delivery systems. Proceedings of the International Conference on Advances in Controlled Delivery, Balimore, MD 1996; 32-36.
23. Jalil R, Nixon JR. Biodegradable poly(lactic acid) and Poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties. J microencapsulation 1990; 7: 297-325.
24. Tice T, Gilley , Mason D, Ferrell T, Staas J, Love D, McRae A, Dahlstrom A, Ling E, Jacob E, Setterstrom J. Site-directed drug delivery with biodegradable microspheres. Proceeding of the International Conference on Advanes in Controlled Delivery, Baltimore, MD 1996, 30-31.
25. Wu XS. Preparatation, cheracterization, and drug delivery application of microspheres based on biodegradable lactic/glycolic acid polymers. In: Wise et al., editors. Encyclopaedic handbook of biomaterials and Bioengineering. New York, US: Marcel Dekker 1995; 1115-1200.
26. Wu XS. Synthesis and properties of biodegradable lactic/ glycolic acid polymers. In: Wise et al., editors. Encyclopedic Handbook of biomaterials and Bioengineering. New York, US: Marcel Dekker 1995; 1015-1054.
27. Rajesh RD, Rajesh HP. Two-stage optimization process for formulation of chitosan microspheres, AAPS Pharm Sci Tech 2003; 5: 1-9.
28. Xi K, Tabata Y, Uno K, Yoshimoto M, Sokawa Y, Ikada Y. Liver targeting of interferon through Pullulan conjugation. Pharm Res 1996; 13: 1846-1850.
29. Sinha VR, Bansal K, Kaushik R, Kumria R, Trehan A. Poly- ε-caprolactone microspheres and nanospheres: an overview. Int J Pharm 2004; 273: 1-23.
30. Yi-Yan Y, Tai-Shung C, Ngee Ping Ng. Morphology, drug distribution, and *in vitro* release profiles of biodegrradeble polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 2001; 22:231-241.
31. Lager R, Siegel R, Brown L, Leong K, Kost J, Edelman E. Controlled release three mechanism. Chemtech 1986; 2: 108-10.
32. Shah SS, Cha Y, Pitt CG. Poly (glycolic acid-co-DL-Lactic acid): diffusion or degradation controlled drug delivery. J Control Rel 1992; 18:261-270.
33. Hombreiro-pérez M, Siepmann J, Zinutti C, Lamprecht A, Ubrich N, Hoffman M, *et al*. Non-degradable microparticles containing a hydrophilic and/or a lipophilic drug: preparation, characterization and drug release modeling. J Control Rel 2003; 88:413-428.
34. Hoffart V, Ubrich N, Simonin C, Babak V, Vigneron C, Hoffman M, *et al.* Low molecular weight heparinloaded polymeric nanoparticles: formulation, characterization, and release characteristics. Drug DevInd Pharm 2002; 28:1091-2000.
35. Manna AK, Ray S, Gupta BK, Ghosh LK. Product development studies on controlled release delivery system of Nitrofurantoin. J Pharm Res 2005; 4: 16-20.
36. Dash AK. Determination of the physical state of drug in microcapsule and microsphere formulations. J Microcapsulation 1997; 4: 567-576.