NANOPARTICULATE DRUG DELIVERY SYSTEM OF CYCLOSPORINE

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ABSTRACT

Recent research on idealizing drug delivery systems which is progressing at a prodigious rate, aims at the development of drug delivery systems with maximum therapeutic benefits of drug delivery thus resulting in safe and effective management of diseases. In the present study Nanoparticlulate drug delivery system for Cyclosporine was developed, that would overcome the therapeutic risks of conventional formulations and was evaluated with respect to particle size, drug content, in vitro release and in vivo targeting. Cyclosporine is a first line immunosuppressive drug used to prevent transplant rejection and in treatment of autoimmune diseases. The commercially available formulations of Cyclosporine are associated with acute hemodynamic changes that result in nephrotoxicity and also have low bioavailability.

Cyclosporine (CYA) loaded Eudragit RL100 nanoparticles were prepared using solvent evaporation technique, with 2% PVA as stabilizer. Four batches of nanoparticles with varying drug concentrations (CYN-1, CYN-2, CYN-3 and CYN-4) were prepared. Cumulative % drug release of formulations CYN-1, CYN-2, CYN-3 and CYN-4 was 94.35%, 93.89%, 88.28% and 85.36% respectively. Formulation CYN-2, which proved to be the best showed a mean particle size of 236 nm and entrapment efficiency of 58.27%. The in vivo result of formulation CYN-2 revealed that the drug loaded nanoparticles showed preferential drug targeting to liver followed by spleen, lungs and kidneys. Stability studies showed that maximum drug content and closest in vitro release to initial data was found in the sample (formulation CYN-2) stored at 4°C. So, in the present study Cyclosporine loaded Eudragit Nanoparticles were prepared and targeted to various organs to a satisfactory level and the prepared nanoparticles were stable at 4°C.

Keywords: Nanoparticles; Cyclosporine; Eudragit nanoparticles; PVA (polyvinyl alcohol); Solvent evaporation method.

INTRODUCTION

Drug delivery to the body can be divided into two broad groups (1) Local and (2) Systemic. The local delivery of drugs is available only for the external sites of the body while drug delivery to the internal sites is usually systemic. An essential requirement modern drug therapy is the controlled delivery of a drug or an active substance to the site of action in the body in an optimal concentration-versus- time profile. There are two potential directions that can be taken to improve (or optimize) a drugs action; first is the design of new biologically active molecules, which are both potent and selective for a particular receptor (magic bullet approach of Ehrlich). The second option, which may be termed the magic gun as missile approach, purposes to use carrier systems that will target active molecules to specific sites in the body[1]. Targeted drug delivery system or site-specific drug delivery systems are systems that can deliver the drug selectively to the diseased site, in a specified steady concentration, for a prescribed time. This site specific or targeted delivery combined with delivery at an optimal rate would not only improve the efficacy of a drug but would also reduce the possibility of unwanted toxic side effects of the drug, thus improving the therapeutic index[2].

Three order of site-specific delivery are suggested:

1) First order targeting – describes specific distribution of dosage form over a particular organ (including endothelial passage).

2) Second order targeting – relates to selective targeting to a specific (diseased) part of the organ.

3) Third order targeting – refers to selective intracellular delivery of drugs.

Numerous ideal characteristics have been proposed for carrier systems intended for site-specific delivery of drug molecules. These characteristics are restricted drug distribution to target, prolonged control, ready access to tissue parenchyma, uniform TDS target capillary distribution, controllable and predictable rate of drug release, high capacity for drug and drug type, drug release does not affect drug action, therapeutic amounts of drug released, minimal drug leakage during TDS transit to target, drug protected, biocompatible surface properties, host protected from agent's allergic properties, biodegradable TDS, no carrier induced modulation of disease state, easy to prepare. Different types of particulate targeted drug delivery systems: Microspheres, Nanoparticles, Liposomes, Niosomes, Resealed erythrocytes, etc[3].

One response is the use of colloidal drug carriers that can provide site specific or targeted drug delivery combined with optimal drug release profile. Among these carriers liposomes and nanoparticles have been the most extensively investigated. Nanoparticles are solid colloidal particles ranging in size from 10-1000 nm in which the active principle (drug or biologically active material) is dissolved, entrapped or encapsulated and/or to which the active principle is adsorbed or attached[4]. The macromolecular material from which they are made, can be of synthetic or natural origin. Some Macromolecular Materials Employed as Nanoparticular Carriers:-

Natural – Serum albumin (Human, Bovine, Rabbit, Egg), Gelatin, Lecithin, Collagen, Iron oxide, Casein, etc.

Synthetic – Polymethyl metha acrylate (PMMA), Poly alkyl cyano acrylate (PACA), Poly methyl cyano acrylate (PMCA), Poly (D, L-Lactide), Polyarylamide, Ethyl cellulose, Eudragit, etc[5].

Organ transplantation is the surgical replacement of diseased organs with healthy organs (grafts) from live or cadaver donors. Transplantation occurs because the recipient's organ has failed or has been damaged through illness or injury. Organs used in clinical transplantation are: *Isografts, allografts* and *xenografts*. An isograft is an organ transplanted from a donor who is genetically identical to the recepient (i.e. identical twin). An allograft is an organ transplanted from a donor to the recipient of the same species who is not genetically identical. A xenograft is an organ transplanted from a donor to recipient of a different species. His major physiological barrier in transplantation is the potential for rejection of transplanted organs as a result of normal, protective host immune responses. Put another way, tissue transplanted from one individual to another will be rejected if the recipient's immune system recognizes the transplanted organ or tissue as foreign. When immune cells attack new organ, we call it rejection. There are

three general forms of rejection: hyper-acute, acute and chronic.[6]

Techniques for manipulation of immune responses:

1) **Non-specific manipulation of immune response:-** The various approaches used for manipulation of immune responses are as follows:

- Drugs
- Irradiation → Using variety of radioactive sources like cesium, cobalt, strontium. This approach includes whole body irradiation, extracorporeal and total lymphatic irradiation.
- Lymphocyte depletion → A direct attack on lymphocytes and lymphoid tissue can be accomplished by chronic thoracic duct drainage, xenogeneic antilymphocyte globulin, thymectomy and splenectomy.

2) **Immunologically specific manipulation of the immune response:-** This includes

- Tolerance
- Enhancement.

Tolerance in its strict sense involves clonal deletion of antigen recognition lymphocytes and total absence of any type of immune response to the alloantigens in question. Immunological *enhancement* is a less rigorous form of specific immunosuppression which does not require deletion of clones of lymphocytes able to react to donor alloantigens. Recepients retain their ability to recognize donor antigens but a combination of blocking antibodies, anti-idiotypic antibodies and specific suppressor T-lymphocytes evolve, which modulate or inhibit the effector phase of the rejection response.

3) **Hybridomas and monoclonal antibodies:-** Using hybridoma techniques, monoclonal antibodies against T-lymphocyte differentiation markers (i.e. T1, T3, T4, T5) may be developed which act as lymphocytotoxic agents. Monoclonal antibodies against donor histocompatibility antigens might serve as "enhancing antibody" and suppress specifically the rejection response to those donor antigens[7].

Successful transplantation between two individuals who are not genetically identical requires diligent assessment of those differences before transplantation and individualized immunosuppressive therapy after transplantation, to minimize recognition and subsequent rejection of the foreign graft by the recipients immune system[6]. Cyclosporine is most active when administered before antigen exposure, but can suppress the responses of primed helper T-cells also, hence useful in autoimmune diseases as well. Cyclosporine selectively suppresses cell-mediated immunity, prevents graft rejection and yet leaves the recipient with enough immune activity to combat bacterial infection which is a selective immunosuppressant. Whereas specific T-cell inhibitors and antibodies are non-selective immunosuppressants which acts on the total body immunity and with the glucocorticoids the long term complications are the greatest limitations of steroid use.[8]

Cyclosporine is a cyclic polypeptide produced as a metabolite of the fungus species *Beauveria nivea* and is commonly used to prevent graft rejection. Cyclosporine selectively suppresses cell mediated immunity, prevents graft rejection and yet leaves the recipient with enough immune

system to combat bacterial infection. Recently Cyclosporine has shown to be effective in treatment of systemic and local auto-immune disorders in which T-cells plays a major role[8].

Targeting of drug to the organ transplanted, helps in achieving greater concentration at the site of rejection so that it effectively reduces the dose required and also reduces the toxicity to non-target organs. Oral administration of Cyclosporine, as an oily solution, results in incomplete absorption and bioavailability ranges from 6 to 60%. Nephrotoxicity is the most common side effect and effects 80% of transplant recipients. To overcome these inherent drawbacks associated with oral administration of Cyclosporine, an attempt is being made to design an alternative drug delivery for Cyclosporine in the form of nanoparticles which will have the following advantages: Decrease in toxicity and occurrence of adverse reaction, Better drug utilization, Enhancement in overall therapeutic effectiveness of the drug.

MATERIALS AND METHODS

Cyclosporine was supplied by Cipla Ltd. (Mumbai, India) and Eudragit (RL 100) was purchased from Rohm GmbH & Co. KG (Darmstadt, India) and Poly vinyl alcohol was purchased from West Coast Laboratories (Mumbai).

Formulation of Cyclosporine Nanoparticles:

Biodegradable nanoparticles containing Cyclosporine were prepared by solvent evaporation method. Five hundred mg of polymer Eudragit RL100 was dissolved in solvent mixture of 2 ml alcohol and 8 ml of dichloromethane very slowly on a magnetic stirrer and twenty five mg of drug Cyclosporine was added to it and the contents were allowed to stand at room temperature for 30 to 45 minutes with occasional vortexing to allow complete solubilization of drug and polymer. This solution was poured into 5 ml of an aqueous 2% polyvinyl alcohol (PVA) solution and the resulting solution was homogenized using high pressure homogenizer for 3 minutes to from w/o emulsion. This w/o emulsion was immediately added dropwise to 125 ml of aqueous PVA (2%) solution. The contents were stirred for 6 hours at room temperature with a magnetic stirrer to evaporate the methylene chloride, allowing the formation of a turbid Nanoparticle suspension. The suspension was filtered through 0.45 μ Millipore filter. The filtrate was centrifuged (1,000 rpm for 10 minutes) followed by ultra centrifugation (35,000 rpm, for 1 hr) of supernatants. Following ultracentrifugation, the pellet was washed two times with deionized water to remove adsorbed drug and was suspended in deionized water to prevent clumping on storage. Four batches of nanoparticles were prepared by above mentioned method and label as CYN-1, CYN-2, CYN-3 and CYN-4[9, 10] (Table 1).

Table 1 : Formulation design, drug entrapment efficiency and size distribution of different nanoparticles formulation

Formulation	Drug : Polymer	Entrapment efficiency (%)	Average particle size
CYN-1	1:05	59.46	224 nm
CYN-2	1:10	58.27	236 nm
CYN-3	1:15	36.54	372 nm
CYN-4	1:20	28.88	516 nm

Characterization of nanoparticles:

Particle size analysis

Particle size of the formulations was observed under a scanning electron microscope (JSM-T330A, JEOL). A drop of nanoparticle suspension was placed onto a piece of conductive paper mounted with adhesive on a cuprum stub and was allowed to air dry. Then the gold coating was done by using Polaron SC 7640 sputter coater and observed under scanning electron microscopy at different magnifications.[11, 12, 13]

Drug entrapment efficiency

10 mg nanoparticles were suspended in water and kept in an ultrasonic bath for 5 minutes and then centrifuged. To determine surface drug (unentrapped drug). the supernatant was diluted suitably with methanolic PBS and was assayed spectrophotometrically at 204 nm. The settled nanoparticles were completely dissolved in methanol, by digesting the particles in methanol for 24 hours. After 24 hrs the solution was filtered through 0.45μm filter and was diluted suitably with PBS and the drug content (entrapped drug) was measured spectrophotometrically at 204 nm[14, 15].

***In vitro* drug release studies**

Nanoparticles equivalent to 10 milligrams of drug from each batch were taken into a 250 ml conical flask and 100 ml of pH 7.4 phosphate buffer saline was added to it, then the flask was kept in a metabolic shaker and the shaker was adjusted to 80 horizontal shakes per minute at 37° C $\pm$ 0.5ºC. Ten ml of the medium was withdrawn at various time intervals of 15 min, 1, 2, 4, 8, 16, 24 hrs and replaced by the same volume of phosphate buffer saline. The samples were diluted suitably with methanolic PBS and the drug released was estimated in each batch by UV spectrophotometer at 204 nm[15, 16].

***In vivo* drug targeting studies**

This study was carried out to compare the targeting efficiency of drug loaded nanoparticles with that of free drug in terms of percentage increase in targeting to various organs of reticuloendothelial system like liver, lungs, spleen and kidneys.

Nine healthy adult mice weighing 35 - 40gms were selected, a constant day and night cycle was maintained and they were fasted for 12 hrs. The animals were divided into 3 groups, each containing 3 mice. Group I received nanoparticles equivalent to 955.5 mcg of Cyclosporine intravenously in the tail vein after redispersing them in sterile phosphate buffer saline solution. Nanparticles from CYN-3 batch were selected for the study. Group-II received 955.5 mcg of pure Cyclosporine intravenously. Group-III mice were treated as solvent control and were injected intravenously with sterile phosphate buffer saline solution.

After 3 hrs the mice were sacrificed and their liver, lungs, spleen and kidneys were isolated. The individual organs of each mice were homogenized separately by using a tissue homogenizer with 5ml of methanol and the homogenate was centrifuged at 15,000 rpm for 30 minutes. The supernatant was collected and filtered through 0.45 μm filter and analysed spectrophotometrically after dilution with phosphate buffer saline at 204 nm[16, 17].

Stability studies

All the four batches of cyclosporine nanoparticles were tested for stability. All the formulations were divided into three sets and

stored at 4ºC, ambient temperature, and at 40ºC room temperature respectively, in thermostatic oven. After 15, 30, 60 and 90 days drug content of all the samples were determined by the method discussed previously in entrapment efficiency section. In vitro release study was also carried out of the best one formulation   CYN-3[18, 19].

RESULTS

Percentage practical yield

Percentage practical yield increased as the amount of drug added to each formulation increased, although it may not be dependent upon drug concentration in the formulation. Maximum yield was found to be 37.22% in CYN-1.

Drug entrapment efficiency

Drug entrapment efficiency was calculated from the drug content. The drug content in four batches of Cyclosporine loaded Eudragit RL100 nanoparticles was studied. The amount of drug bound per 10mg of nanoparticles was determined in each batch.

Table 1 shows the results of the drug entrapment efficiency in each of these formulations. It was observed that the entrapment efficiency increased with the increase in concentration of drug in the formulations.

The maximum entrapment was found in CYN-1 (59.46 %) and lowest entrapment in CYN-4 (28.88 %) and surface drug was found to be 235.5 μg/10mg, 156.5 μg/10 mg, 84.25 μg/10 mg and 31.75 μg/10 mg for the formulations CYN-1, CYN-2, CYN-3 & CYN-4 respectively.

Particle size analysis

Scanning electron photomicrographs of all the four formulations are shown in Figure 1. Average particle size of Cyclosporine nanoparticles were found to be in range of 224nm, 236nm, 372nm and 516nm for CYN-1, CYN-2, CYN-3 and CYN-4, respectively. Particles of all the formulations were found to be spherical, smooth and discrete.

Fig. 1 : Scanning electron microscopy of prepared nanoparticles
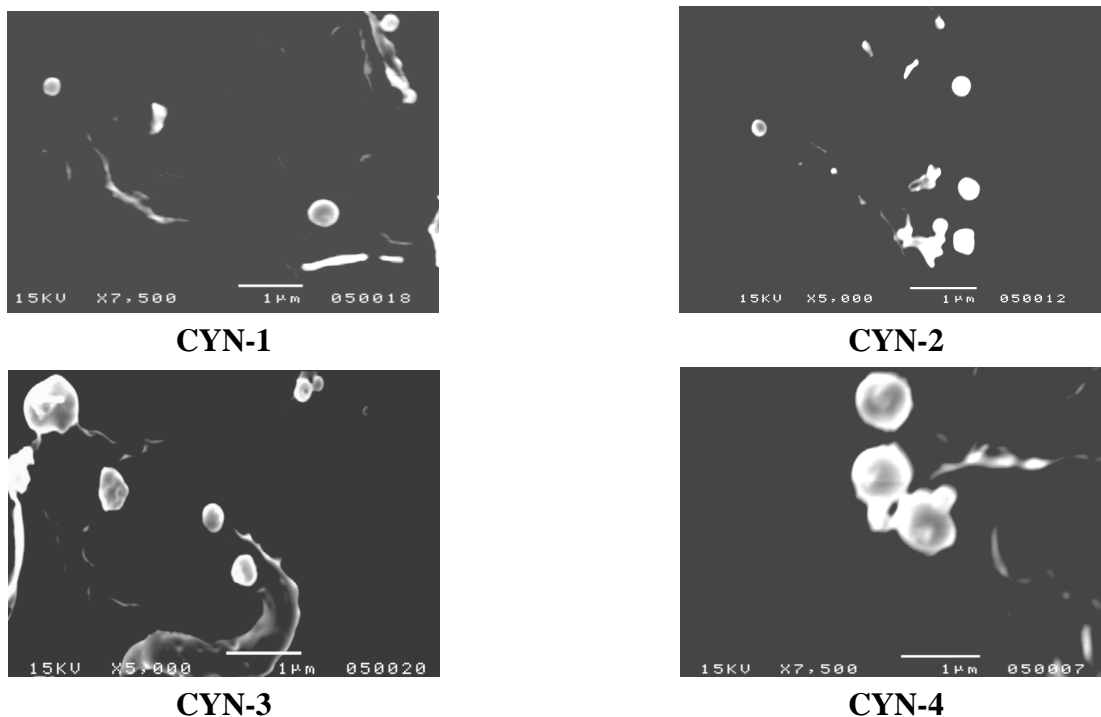
CYN-1

CYN-2

CYN-3

CYN-4

In vitro release kinetic studies

Cumulative percent drug released after 24 hours was 94.35%, 93.89%, 88.28% and 85.36% for CYN-1, CYN-2, CYN-3 and CYN-4 respectively and was 91.86% in 3 hrs for pure drug Cyclosporine by UV spectroscopy. It was that the drug release from the formulations increased with decreased in amount of polymer added in each formulation. (Table 2)

Table 2 : *in vitro* release studies of different formulations

Time (T) in hrs	Cum. % drug released			
	CYN-1	CYN-2	CYN-3	CYN-4
0.25	26.15	24.55	23.18	21.45
1	35.10	33.45	31.74	30.12
2	46.86	45.23	44.32	41.76
4	57.64	56.18	53.40	49.34
8	69.05	65.61	64.83	59.70
16	81.22	80.08	77.62	70.22
24	94.35	93.89	88.28	85.36

The in-vitro release of all the four batches of nanoparticles showed an interesting bi-phasic release with an initial burst effect. In the first hour, drug released was 26.15%, 24.55%, 23.18% and 21.45% for CYN-1, CYN-4, CYN-3 and CYN-4 respectively (Figure 2). Afterwards the drug release followed a steady pattern approximating zero order release. The burst release in the first hour can be attributed to the drug loaded on the nanoparticles surface, which was found to be 235.5 μg, 156.5 μg, 84.25 μg and 31.75 μg per 10mg for formulations CYN-1, CYN-2, CYN-3 and CYN-4 respectively.

The regression co-efficients for formulations CYN-1 to CYN-4 of zero order plots were found to be 0.9392, 0.9464, 0.9350 and 0.9862 respectively. The regression co-efficients for formulations CYN-1 to CYN-4 of first order plot were found to be – 0.9879, -0.9875, -0.9919 and -0.9861. These results indicate that zero order plots were not linear for all formulations and the first order plots were almost linear for all formulations except CYN-4, although after the initial burst effect the release appears to approximate zero order.

Fig. 2: Plots of cumulative % drug released vs. root time for different formulations of cyclosporine nanoparticles (*in vitro* release studies) [Higuchi matrix]

CYN-1 [— ◆ —], CYN-2 [—■—], CYN-3 [—▲—] and CYN-4 [—●—]
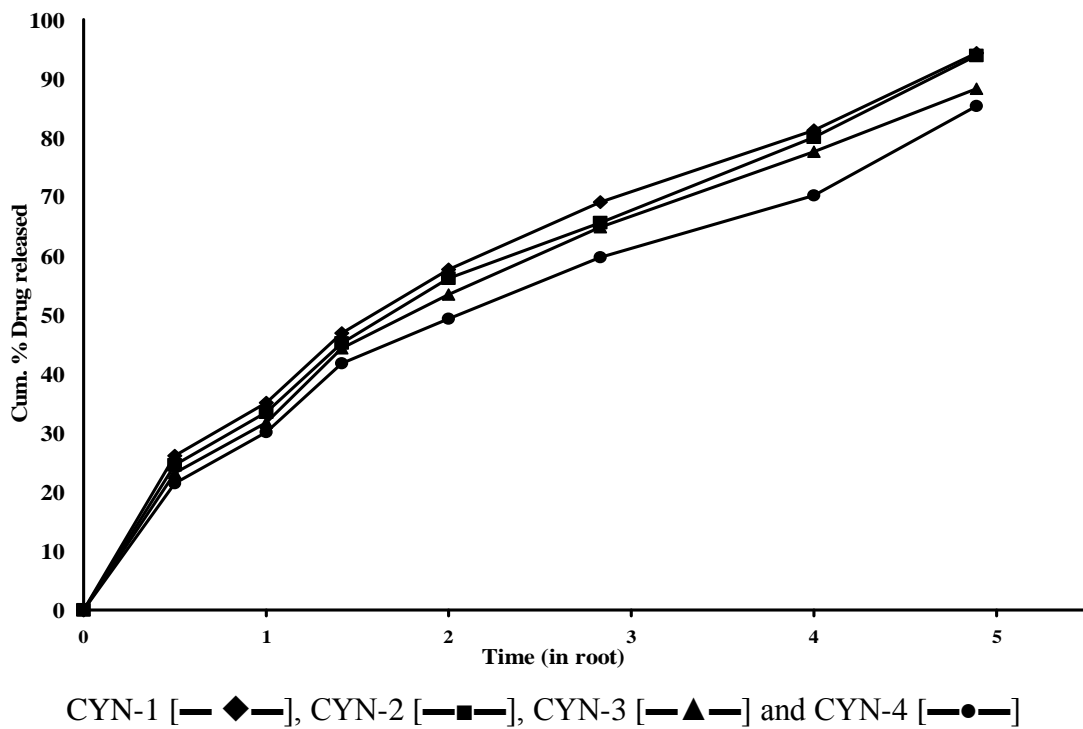
This Higuchi's plot was almost linear with regression co-efficient values of 0.9895, 0.9917, 0.9883 and 0.9879 for formulations CYN-1 to CYN-4. The linearity suggests that the release of Cyclosporine from Eudragit nanoparticles was diffusion controlled. The 'n' values for CYN-1 to CYN-4 were 0.2854, 0.2963, 0.2992 and 0.2994 respectively, which is less than 0.43. This indicates that the release approximates Fickian diffusion mechanism. Hixson Crowell plot regressions co-efficient of formulations CYN-1 to CYN-4 were found to be –0.9866, –0.9885, –0.9804 and –0.9803. These results indicate that the release rate was limited by the drug particles dissolution rate and erosion of the polymer matrix. (Fig. 3).

Fig. 3 : Scanning electron microscopy of before, after 30 minutes and after 24 hr. *in vitro* release nanoparticles of CYN-2. Before erosion (A), after 30 minutes erosion (B) and after 24 hr. erosion (C)


(1)   (2)   (3)
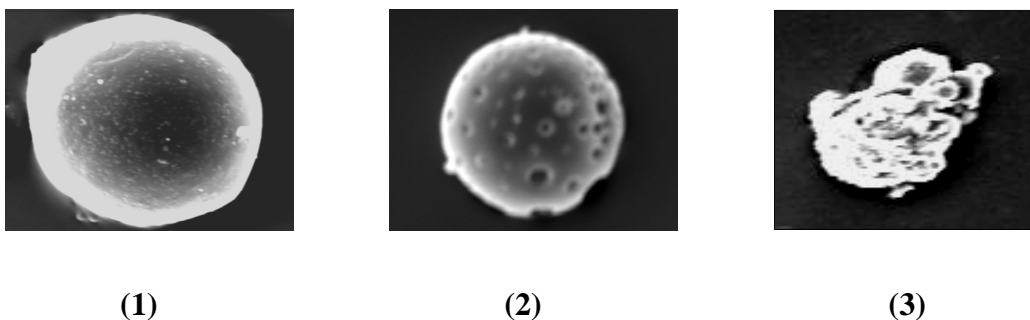
In vivo tissue distribution studies

The average targeting efficiency of drug loaded nanoparticles was found to be 45.09% of the injected dose in liver, 11.78% in lungs, 26.55% in spleen and 3.71% in kidney, whereas accumulation of pure drug was 20.08% in liver, 5.80% in lungs, 10.11%, in spleen and 1.05% in kidneys of the injected dose after 3h shown in Figure 4.

Fig. 4: Comparison between amount of drug targeted from nanoparticles (CYN-2) and free drug in various organs [*In vivo* drug targeting studies]
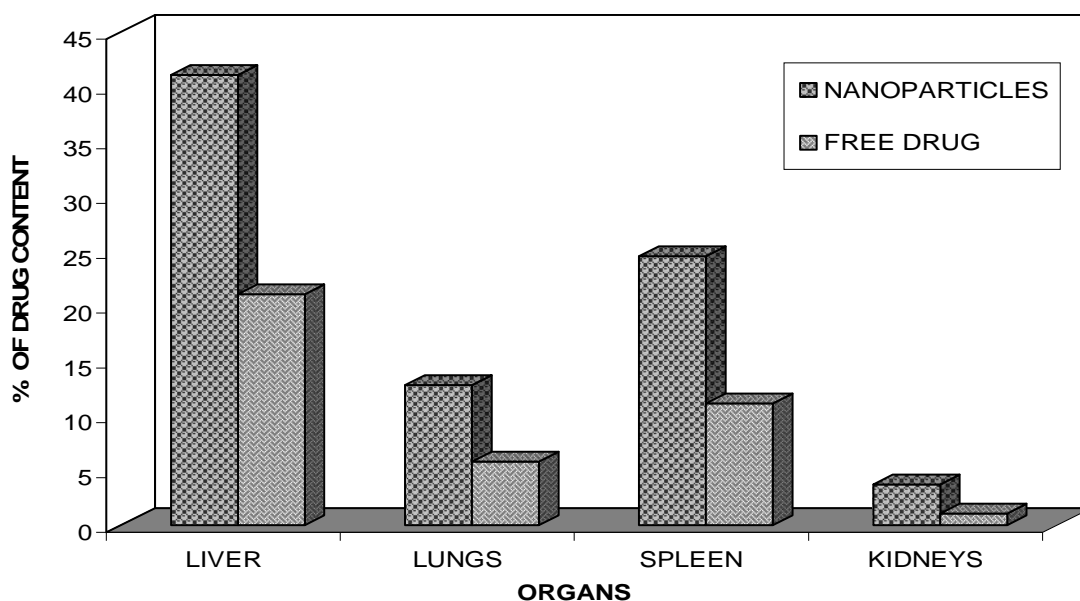
Stability studies

After storage of the formulation CYN-2 at 4°C, ambient temperature and 40°C for 90 days drug content was 58.21%, 54.45% and 51.16% respectively. The cumulative percent drug released for the formulation CYN-2 after 90 days storage was 94.897% at 4°C, 96.34% at ambient temperature and 97.52% at 40°C after 24 hours.

These results indicate that the drug release from the formulation stored at 4°C was highest followed by formulation stored at ambient temperature and 40°C.

DISCUSSION

Cyclosporine a potent immunosuppressant, has contributed greatly to the success of organ transplantation and also in treatment of autoimmune diseases. However Cyclosporine associated side effects like nephrotoxicity and also the low bioavailability of the drug have tempered these benefits. The ideal dosage form for Cyclosporine is the one that provides specific delivery to the site of rejection i.e., organ transplanted for a longer period of time to provide local immunosuppression, thereby eliminating the effects of nephrotoxicity associated with conventional dosage forms.

Among various carriers for targeting the drug to a specific site, Nanoparticulation is perhaps, a useful strategy towards targeted drug delivery. Most of the nanoparticles used as drug carriers are rapidly taken up from blood by macrophages of reticuloendothelial system (RES), therefore nanoparticles can provide an effective strategy in drug targeting for concentration of drug substances in liver, spleen, lungs and bone marrow. Considering

this property of RES, an attempt was made in designing and studying the targeting efficiency of Cyclosporine nanoparticles prepared by using Eudragit RL100 as a carrier for the drug. Cyclosporine nanoparticles were prepared by solvent evaporation method. Four formulations (CYN-1, CYN-2, CYN-3 and CYN-4) were prepared varying the concentration of drug in these formulations.

Preformulation studies revealed that the drug Cyclosporine and Eudragit RL100 were satisfactorily compatible, without any significant changes in the chemical nature of the drug.

Percentage practical yield was found to be maximum in formulation CYN-1. Particle size of the drug-loaded nanoparticles revealed that the particles were found in nanometer range between 224-516nm. Drug entrapment efficiency was found to be maximum in CYN-1. It was observed that drug entrapment efficiency increased with increase in concentration of drug added in consecutive formulations.

In vitro release study was analyzed using various mathematical models. Cumulative % drug release with respect to time was found to be highest for formulation CYN-1 and lowest for formulation CYN-4. The regression coefficient data of Zero order release indicated non-linearity in case of all the formulation, but regression values of first order release showed non-linearity for formulation CYN-4 and almost linearity for other formulations. This shows that the formulation F3 did not follow zero or first order release kinetics and for other formulations the release must have approximated first order release.

The regression co-efficient of Higuchi matrix suggests linearity and thus diffusion controlled release.

The 'n' values of Peppas suggest Fickian release and Hixson Crowell regression data shows that formulations also appear to release drug by erosion mechanism and the release is drug dissolution limited.

Overall the curve fitting into various mathematical models was found to be average and the in vitro release of formulations best fitted into the Peppas model followed by Higuchi's and Hixson-Crowell model. This indicates that the nanoparticles followed Fickian controlled release mechanism and in addition the release appears to be also by erosion mechanism and is drug dissolution limited.

On the basis of particle size morphology, drug content, in vitro release and satisfactory release kinetics, formulation CYN-2 was selected as an optimum formulation for in vivo and stability studies. Present study shows that the targeting efficiency of drug-loaded nanoparticles over a free drug was higher, which may provide increased therapeutic efficiency. Moreover, higher concentration of drug targeted to various organs may help in reduction of dose required for the therapy and there by dose related side effect could be minimized. The in vivo drug targeting (tissue distribution) studies revealed the following order of targeting – Liver > Spleen > Lungs > Kidneys.

Drug content data of stability studies indicate that maximum drug was retained by formulation CYN-2 when stored at 4°C. Drug content of the formulation CYN-2 at various temperature conditions reduced in the following order, 4°C > ambient temperature > 40°C. In

vitro release data of stability studies indicate that very less variation in release was found at 4°C followed by ambient temperature and 40°C. From the above studies it can be concluded that 4°C is the most suitable temperature for storage of Cyclosporine nanoparticles.

REFERENCES

1. Khar RK, and Diwan M, "Targeted delivery of drugs", In; Jain, N.K., Ed., "Advances in Controlled and Novel Drug Delivery", First Edition 2001, CBS Publishers and Distributors, New Delhi, 2001; 452.

2. Jain S, Jain NK. Resealed erythrocytes as drug carriers. In: Jain NK, editor. Controlled and Novel Drug Delivery. New Delhi: CBS Publishers and Distributors; 2002. p. 256-7.

3. Tomilson E, "Site specific drug delivery using particulate systems", In; Banker, GS, Ed., "Modern Pharmaceutics", Second Edition, Revised and Expanded, Marcel Dekker, 1996, Inc., USA; 673.

4. Kreuter J, (1983), "Evaluation of nanoparticles as drug delivery systems, I. Preparation method", Pharm. Acta. Helv., 58; 156.

5. De Jaeghere F, Doelker E, and Gurny R, "Nanoparticles", In; Mathiowitz, E, Ed., "Encyclopaedia of controlled drug delivery", Vol.2, John Wiley and Sons, Inc., New York, USA, 1999, 641.

6. Susansmith MN. Immunological Aspects of Organ Transplantation: Isograft, Allograft, or Xenograft, 2002.

7. Ketel BL, Stuart FP. Immunobiology of Transplantation. In: Chatterjee SN, editor. Organ Transplantation. Bristol: John Wright and Sons (Printing) Ltd; 1982. p. 15-42.

8. Tripathi KD, editor. Essentials of Medical Pharmacology. 5th ed. New Delhi: Jaypee Brothers Medical Publishers(P) Ltd; 2003. p. 785-7.

9. Kompella UB, Bandi N, Ayalasomayajula SP. Poly(lactic acid) Nanoparticles for Sustained release of Budesonide. Drug Deliv Tech, 2001 Oct, 1(1):227-235.

10. Kim Y, Fluckiger L, Hoffman M, Idjouadiene IC, Atkinson J, Maincent P. The antihypertensive effect of orally administered nifedipine loaded nanoparticles in spontaneously hypertensive rats. Br J Pharmacol 1997;120:399-404.

11. Zengshuan MA, Yeoh H, Lim LY. Formulation PH modulates the interaction of insulin with chitosan nanoparticles. J Pharm Sci 2002;91:1404-11.

12. Pednekak DV, Singh KK. Particle size analysis- newer developments. Indian J Pharm Educ 2004;38(3):130-3.

13. Lee J. Drug nano- and microparticles proceed into solid dosage forms: Physical properties. J Pharm Sci. 2003;92 (10):2057-67.

14. Tiyaboanchal W, Woiszwillo J, Middaugh CR. Formulation and charactrization of Amphotericin B-Polyethylenimine-Dextran sulfate nanoparticles. J Pharm Sci 2002;91:1396-403.

15. Li LK, Wang N, Wu S. A novel biodegradable system based on gelatin nanoparticles and poly (lactic-co-glycolic acid) microspheres for protein and peptide drug delivery. J Pharm Sci. 1997; 86(8):891-5.

16. Santhi K, Dhanraj SA, Ponnusankar S, Suresh B. Study on Formulation and Targeting Efficiency of Amphotericin B Nanospheres. Indian J Pharm Sci 2000(6):421-3.

17. Gosh MN, editor. Fundamentals of Experimental Pharmacology. 2nd ed. Calcutta: Scientific Book Agency; 1984. p. 154-5.

18. Kulkarni GT, Gowthamarajan K, Suresh B. Stability testing of pharmaceutical products: An overview. Indian J Pharm Educ. 2004;38(4):194-202.

19. Oliva A, Santovena A, Llabrews M, Farina JB. Stability study of human serum albumin pharmaceutical preparations. J Pharm Pharmacol. 1999;51:385-92.