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Stavudine Loaded Biodegradable Polymeric Microspheres as a Depot System for Parenteral Delivery

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ABSTRACT

Injectable biodegradable polymeric microspheres of Stavudine were prepared using different viscosity grades of PLGA 50:50 (RESOMER® 504H and RESOMER® 502H) by solvent evaporation technique with different drug/polymer ratios. Tailored release of Stavudine facilitates reduction in symptoms of HIV infection and delay AIDS progression by reducing viral load to undetectable levels for extended period of time. The influence of formulation variables on microparticle characteristics like polymer type and concentration, vehicle type, polymer solution/vehicle volume ratio, surfactant concentration and drug to polymer ratios were evaluated. Microspheres were evaluated for yield, entrapment efficiency, particle size and *in-vitro* release behavior as well as characterized by scanning electron microscopy (SEM), differential scanning calorimetry (DSC), X-ray diffraction (XRD), residual solvent analysis and confocal laser scanning microscopy (CLSM). Microspheres showed excellent surface topography with uniform distribution and structural integrity of the drug having the entrapment efficiency of 90.96 ± 1.09 and mean particle diameter below 65µm. Drug release kinetics data were obtained from various kinetic models and best explained by "Korsmeyer-Peppas equation" for both the polymers which depicted that drug release mechanism is anomalous transport, i.e. diffusion as well as polymer relaxation. Drug release from microspheres exhibited the characteristic release pattern of a monolithic matrix system with 90-100% drug release in 6-8 weeks demonstrating the feasibility of prolonged delivery of Stavudine using biodegradable microspheres for parenteral depot system.

Keywords: HIV, Microspheres, Stavudine, Biodegradable Polymer, Prolonged Release, Parenteral delivery.

INTRODUCTION

Acquired Immuno Deficiency Syndrome (AIDS) is a chronic immunodeficiency disease that damages, and ultimately destroys, the immune system through Human immunodeficiency virus (HIV) capturing 1500 people everyday throughout the world. HIV infection results in a chronic, progressive illness which assails the immune system and attacks CD4 cells, which are necessary to fight off illnesses. Eventually, the virus overwhelms the CD4 cells and depletes the count below normal levels (500-1500 cells/mm^3) to <200 cells/mm^3 of blood, resulting in opportunistic infection taking hold of weakened immune system; the association with complications is the rationale for this CD4 cell threshold to be used to define AIDS. [1-2]

After infection with HIV, there is usually a seroconversion illness followed by an asymptomatic stage which lasts months to years. This is followed by symptomatic phases which correlate with progressive immunodeficiency which is dependent on the stage of the infection. The progression of HIV disease varies from person to person and depends on a number of factors including genetics and mode of transmission. Viral load is an important surrogate marker which measures the quantity of virus in plasma and predicts the rate of progression. It is a measured in RNA copies/ml. It is also used to assess response to drug therapy and may predict the development of drug resistance. After seroconversion, patient develops a viral load set point. The lower the viral load the slower the progression of HIV disease and eventually clinical symptoms and opportunistic conditions. [3-5]

HIV infection progressing towards AIDS, constitute one of the most serious infectious disease challenges to public health making 33.2 million people living with HIV/AIDS globally. Although the current efforts including AIDS counseling, educational tools and antiretroviral drug therapy have contributed to transforming HIV infection from a fatal to a manageable chronic infectious disease. [6] Looking in to above scenario, approach of present research is focused to provide a novel solution to this global pandemic. Current strategy involves treatment of HIV infections using anti-HIV drugs. Antiretroviral (ARV) therapy refers treating viral infections like HIV with drugs. The drugs do not kill the

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virus; however they slow down the growth of virusas well as the HIV disease. [7-8]

Currently highly active antiretroviral therapy (HAART) is the most common treatment regimen for HIV including the combination therapy but failure of the treatment is also competing to the effectiveness as well; due to the development of resistance mutations to both the specific drug in question and cross-resistance to other available treatment options. A randomized trial for "Salvage therapy" of Stavudine has shown that in heavily pre-treated patients, the recycling of antiretroviral agents that have been utilized previously may, however, be associated with antiviral efficacy. In a recently completed phase II trial, Stavudine treatment resulted in delayed progression of clinical disease in patients who had previously been treated with other antiretrovirals. Salvage therapy with Stavudine can decrease viral loads to undetectable levels whereas Stavudine monotherapy can stabilize CD4 counts and weight loss in heavily pre-treated individuals. Apparently potential benefits were observed in all CD4+ cell strata and clinical stages of HIV disease due to well tolerated status of Stavudine and has been proved to delay the progression of AIDS. [9-10]

In recent trends of developments; In spite of the availability of a large number of controlled release dosage forms with their specific routes of administration, demand for prolonged release parenteral drug delivery systems has grown exponentially in recent years with a rise of 9.5% annually. Parenteral route not only provides dose reduction resulting from the avoidance of peaks and valleys, as well as the enhancement of patient compliance by reducing the dosing frequency but also lead to direct entry of the drug molecule into the systemic circulation providing quicker therapeutic benefits. Short duration of action obtained by IV/IM/SC injection of drug may be accounted for by developing prolonged release depot formulation. So a long acting parenteral dosage form that is safe and efficacious for a long period of time (days/months) is more beneficial because it ensures that patient is receiving complete medication. [11-12]

In a case of chronic treatment, it again becomes difficult in practice to do so on long term routine basis i.e. to take several doses in a day for several days or months. This problem is circumvented in case of controlled release parenteral dosage forms where once a week or once a month injection is required or it can be tailored according to patient needs. Generally, parenteral depot systems could minimize side effects by achieving constant, 'infusion-like' plasma level time profiles. Parenteral drug administration, especially intravenous infusion, leads to easy and complete absorption of drug in systemic circulation, eliciting a prompt drug response. But this requires direct medical supervision and hospitalization of the patient. Parenteral drug administration through intramuscular route has a fairly rapid onset of action followed by a rapid decline in the blood-drug level leading to relatively short duration of therapeutic response. Thus considering these problems associated with different modes of drug administration, injectable depot formulation seems to provide the solution. [13-14] Parenteral depot formulations can overcome these problems by controlling the drug release over a predetermined time span. They can deliver a sustained dose in vivo for extended periods of time, usually in order of days to weeks to months primarily due to low degradation rates of the biodegradable polymers. [15]

Biodegradable polymeric parenteral microspheres are an emerging trend of formulations that facilitates prolonged residence of medication in the body with significant therapeutic adherence and convenience benefits to patients. Biodegradable polymers were first used for sustained release parenteral drug delivery in early 1970s. These polymers have been approved for human use as implantable devices, surgical sutures and drug delivery systems by the US Food and Drug Administration (FDA). The application of parenteral microspheres formulated using biodegradable polymers such as polylactide (PLA) and poly (lactide-co-glycolide) (PLGA) to deliver small molecules, proteins, and macromolecules using multiple routes of administration have been studied and successfully used for the treatment of a variety of disease states. Some of the advantages of microspheres as drug delivery devices include enhanced stability of protein therapeutics, continuous and controlled drug release, reduced dosage, decrease in systemic side effects, reduced possibility of dose dumping, reduced frequency of administration; therefore increased patient compliance. [16-17]

At present Stavudine is available in both immediate as well as extended release (Zerit XR) dosage forms for oral delivery in a dose of 60-80 mg/day. Presenting the drug in the form of parenteral depot formulation in accordance with the previous discussion will not only provide the patient compliance by reducing the dosing frequency and providing the maximum therapeutic benefits but also avoid the plasma level fluctuations of the drug. Parenteral biodegradable microspheres will provide an edge in the treatment of HIV disease in case of neonatal infections from HIV positive mother, unconscious patients unable to take oral medications and moreover the patients with damaged hepatic or gastric systems due to complications associated with HIV infections; which are the major indications associated with the disease.

MATERIALS AND METHODS

Materials

Stavudine was obtained as gift sample from Panacea Biotech, Lalru, Chandigarh. The polymers used were Poly (D, L-lactide-co-glycolide) 50:50 (RESOMER® 504H and RESOMER® 502H) from "Boehringer Ingelheim" Germany. Organic solvents (chloroform, dichloromethane, acetone & acetonitrile), were purchased from Central Drug House New Delhi. Other chemicals used for microsphere preparation were purchased from commercial suppliers and used without further purification.

Preparation of microspheres

Stavudine loaded biodegradable polymeric microspheres were prepared by PLGA 50:50 (RESOMER® 504H and RESOMER® 502H) for the prolonged release of the drug. Emulsification followed by solvent evaporation technique was used for the preparation of an o/o emulsion, using acetonitrile as organic solvent and sesame oil as liquid manufacturing vehicle with 0.5, 1.0 and 2% of span 80. Preparation variables like different drug/polymer ratio (1:10, 1:20, 1:50, 1:100) and a polymer solution/vehicle volume ratio of 1:2 were optimized for best results. The resulting microspheres were filtered, washed with excess of n-hexane and distilled water to remove the oil and unentrapped drug and finally dried. [18-19]

Characterization Studies

The dried delicate microspheres were characterized through various physicochemical methods such as determination of

yield; entrapment efficiency, particle size, scanning electron microscopy (SEM), differential scanning calorimetry (DSC), X-ray diffraction technique (XRD) and Fourier transform infrared (FTIR) Spectroscopy, Residual solvent analysis and confocal laser scanning microscopy (CLSM). *In-vitro* release studies were performed in pH 7.4 phosphate buffered saline. [20-25]

Determination of entrapment efficiency and percentage yield

Stavudine loaded microspheres (10 mg) were mixed with 1.0 ml of dichloromethane and then 5.0 ml of water was added into above solution for the extraction of Stavudine. The solution was mixed by vortex mixer, clarified by standing for few minutes and then upper layer (water) was analyzed for Stavudine spectrophotometrically at 267 nm. The entrapment efficiency and percent yield were calculated using the Equations (1) and (2), respectively. [21, 23]

$$\% \text{ EE} = \frac{\text{Mass of Incorporated drug}}{\text{Mass of drug used in formulation}} \times 100 \quad [1]$$

$$\% \text{ Yield} = \frac{\text{Weight of microspheres prepared}}{\text{Total weight of drug and polymer}} \times 100 \quad [2]$$

***In-vitro* release profile studies**

The release from the microspheres is dependent both on diffusion through the polymer matrix and on polymer degradation. Microspheres (15 mg) were suspended in 1.5 ml of PBS (phosphate buffered saline; pH 7.4) in eppendorf tubes and placed in incubator shaker (37°C) at 50 strokes per minute. At predetermined time intervals, samples were centrifuged at 7000 rpm at room temperature. [18] Supernatants were withdrawn and replaced with fresh PBS. The process was continued until the completion of the dissolution study. Concentration of Stavudine in supernatants was analyzed spectrophotometrically at 267 nm. [19, 22]

Scanning electron microscopy (SEM)

The surface morphology of the microspheres was investigated using scanning electron microscopy. The dry samples of microspheres were mounted onto metal stubs using double-sided adhesive tape. The stubs were then vacuum coated with gold using fine coat ion sputter (Hitachi-E-1010) under reduced pressure to render them electrically conductive. Then the microspheres were examined with SEM (Hitachi, S-3400N). The accelerating voltage was kept constant at 15 KV under an argon atmosphere. [17-18, 20, 23]

To observe the influence of release medium on the microspheres with time, experiments were carried out for 60 days and any changes in morphology as well as surface characteristics of microspheres were observed. For the study, the microspheres were placed in the release medium (PBS, pH 7.4) and samples of microspheres were collected at different time intervals of 7, 15, 30 and 60 days and scanned by SEM.

Size distribution analysis

The mean diameter and particle size distribution of the microsphere samples were performed by laser diffractometry using Malvern Mastersizer 2000. For laser diffraction measurements, microspheres were suspended in distilled water with 0.1 % tween 80, which was continuously homogenized at 10,000 rpm and sonicated for 60 seconds prior to particle size determination.

Differential scanning calorimetric studies (DSC)

A small amount of drug, microspheres and polymer samples were placed in hermetically sealed aluminium pans and heated from 0°C to 400°C at a heat flow rate of 10°C min^{-1} under nitrogen spurge of 70 cm^3 min^{-1} (DSC-Q20, TA Instruments, Waters LLC, USA). The glass transition temperature (Tg) and degradation temperatures of the samples were recorded as endotherms.

Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectra were recorded for the drug loaded microspheres, Stavudine and the RESOMER® using 60 MHz Varian EM 360 Perkin Elmer, using potassium bromide pellets in the scanning region of 4000 to 500 cm^{-1}.

X-ray diffraction studies (XRD)

Powder X-ray diffraction pattern of microspheres, Stavudine and RESOMER® were recorded employing XPERT-PRO diffractometer system, using Cu Kα and Kβ radiations, at 45 kV, 40 mA and a temperature of 25°C. The sample was analyzed between 2θ angles of over 5-50°.

Residual solvent analysis

Residual solvent analysis was carried out through gas chromatography. The gas chromatograph (GC-2014, Shimadzu) using GC Solution was calibrated by the internal standards provided by the concerned firm. To determine the amount of acetonitrile in microspheres, known weight of microspheres were directly taken in head space vial and injected in to a gas chromatograph. The column used for the determination was DB-624, 30 m × 0.25 mm (fused silica, 1.0µ film).

Confocal laser scanning microscopy (CLSM)

A rational method based on fluorescent imaging with a Carl Zeiss LSM 510 confocal laser scanning microscope (CLSM, Carl Zeiss Microimaging, Inc., Thornwood, NY) was used to provide a unique approach to explore the internal structure of the microspheres and drug distribution pattern as a nondestructive visualization technique. The microspheres were prepared using coumarine (Fluorescence spectrum 458-540 nm) labeled Stavudine in the drug/polymer ratio of 1:10. Permanent slide of dry fluorescently labeled microspheres using coumarine; was prepared and visualized with the instrument equipped with an Enterprise UV laser and a Carl Zeiss inverted Axiovert 100 M microscope. The fluorescent dye was excited by UV laser at 450 nm and required were used in conjunction with a Plan-Neofluar 100X oil immersion objective lens with numerical aperture of 1.2 to build images. The laser power was set at 150µW, and the detection gain was set at 710. The pinhole was 170µm, which resulted in an optical slice of less than 2.5µm. The images were scanned by 16 bit plane mode at a scan speed of 6.40µs/ pixel and the image size was 512×512 pixels. [26-27]

RESULTS AND DISCUSSION

Stavudine loaded biodegradable microspheres of RESOMER® 504H and RESOMER® 502H with different drug to polymer ratios have undergone scanning electron microscopy (Fig. 1, 2). Microspheres were observed in well spherical and homogeneous in shape for all sizes. The surface topography of the microspheres of RESOMER® 504H was smooth with very few minute pores whereas comparatively rough surface, more pits and small pores were observed on the surface of the microspheres of RESOMER® 502H. Increment of polymeric concentration by increasing the drug/polymer ratio resulted in microspheres of increased mean particle size with very smooth surface topography free

Table 1: Batch specification, Surfactant concentration, Percentage yield, Entrapment efficiency and Mean particle size of Stavudine loaded biodegradable polymeric microspheres made of RESOMER® 504 H with different drug to polymer ratios

S. No.	Batch specification	D:P Ratio	Surfactant Conc. (%)	% Yield* (% ± SD)	% E.E.* (% ± SD)	Mean Particle Size# (µm) (% ± SD)
1	RESO 504H, D1P10	1:10	0.5	36.29 ± 1.59	9.82 ± 0.98	47.17 ± 1.51
2	RESO 504H, D1P10	1:10	1.0	38.07 ± 1.65	12.44 ± 0.57	37.57 ± 1.45
3	RESO 504H, D1P10	1:10	2.0	41.95 ±1.67	13.65 ± 0.40	35.73 ± 1.36
4	RESO 504H, D1P20	1:20	0.5	52.60 ± 2.15	14.46 ± 1.04	48.39 ± 0.95
5	RESO 504H, D1P20	1:20	1.0	54.22 ± 1.22	17.12 ± 1.50	46.75 ± 0.66
6	RESO 504H, D1P20	1:20	2.0	55.66 ± 1.89	18.89 ± 0.96	44.84 ± 1.43
7	RESO 504H, D1P50	1:50	0.5	74.03 ± 1.13	38.26 ± 1.32	56.59 ± 2.07
8	RESO 504H, D1P50	1:50	1.0	77.75 ± 1.14	41.37 ± 1.63	53.24 ± 1.17
19	RESO 504H, D1P50	1:50	2.0	80.32 ± 2.36	43.03 ± 0.87	51.23 ± 1.45
10	RESO 504H, D1P100	1:100	0.5	80.73 ± 2.15	62.36 ± 1.38	64.32 ± 1.59
11	RESO 504H, D1P100	1:100	1.0	85.09 ± 1.42	65.53 ± 1.44	61.06 ± 1.30
12	RESO 504H, D1P100	1:100	2.0	88.25 ± 1.34	70.39 ± 1.78	58.49 ± 2.16

*# Value expressed as Mean ± SD, n = 3.

Table 2: Batch specification, Surfactant concentration, Percentage yield, Entrapment efficiency and Mean particle size of Stavudine loaded biodegradable polymeric microspheres made of RESOMER® 502 H with different drug to polymer ratios

S. No.	Batch specification	D:P Ratio	Surfactant Conc. (%)	% Yield* (% ± SD)	% E.E.* (% ± SD)	Mean Particle Size# (µm) (% ± SD)
1	RESO 502H, D1P10	1:10	0.5	36.12 ± 1.46	21.76 ± 1.76	46.46 ± 1.61
2	RESO 502H, D1P10	1:10	1.0	41.07 ± 2.49	23.88 ± 1.04	44.34 ± 1.36
3	RESO 502H, D1P10	1:10	2.0	43.63 ± 2.23	25.91 ± 1.62	33.03 ± 2.06
4	RESO 502H, D1P20	1:20	0.5	63.61 ± 1.12	36.95 ± 1.04	47.77 ± 1.36
5	RESO 502H, D1P20	1:20	1.0	68.76 ± 2.06	41.44 ± 0.98	46.14 ± 1.39
6	RESO 502H, D1P20	1:20	2.0	74.62 ± 0.55	43.48 ± 1.32	45.78 ± 1.00
7	RESO 502H, D1P50	1:50	0.5	70.38 ± 2.26	71.82 ± 0.95	54.10 ± 0.90
8	RESO 502H, D1P50	1:50	1.0	74.77 ± 1.35	74.85 ± 1.07	52.14 ± 0.62
19	RESO 502H, D1P50	1:50	2.0	77.64 ± 1.49	78.49 ± 0.73	51.11 ± 1.01
10	RESO 502H, D1P100	1:100	0.5	84.22 ± 0.38	82.24 ± 0.66	63.82 ± 0.72
11	RESO 502H, D1P100	1:100	1.0	87.02 ± 0.67	85.65 ± 1.27	62.33 ± 0.57
12	RESO 502H, D1P100	1:100	2.0	88.04 ± 2.33	90.96 ± 1.09	60.39 ± 1.53

*# Value expressed as Mean ± SD, n = 3

Table 3: Model fitting and the release mechanism for the microspheres prepared from RESOMER® 504 H using five different kinetic models

Formulation code	Kinetic Models						Mechanism
	Zero order	First order	Hixson crowell law	Higuchi matrix	Korsemeyer Peppas		
					r^2	n	
D1P10	0.766	0.822	0.612	0.907	0.960	0.812	Anomalous
D1P20	0.899	0.937	0.761	0.978	0.984	0.853	Anomalous
D1P50	0.883	0.933	0.741	0.980	0.990	0.819	Anomalous
D1P100	0.780	0.827	0.575	0.906	0.955	0.881	Anomalous

Table 4: Model fitting and the release mechanism for the microspheres prepared from RESOMER® 502 H using five different kinetic models

Formulation code	Kinetic Models						Mechanism
	Zero order	First order	Hixson crowell law	Higuchi matrix	Korsemeyer Peppas		
					r^2	n	
D1P10	0.402	0.832	0.346	0.610	0.852	0.201	Anomalous
D1P20	0.498	0.845	0.411	0.716	0.901	0.335	Anomalous
D1P50	0.638	0.922	0.534	0.804	0.928	0.229	Anomalous
D1P100	0.740	0.864	0.628	0.891	0.967	0.314	Anomalous

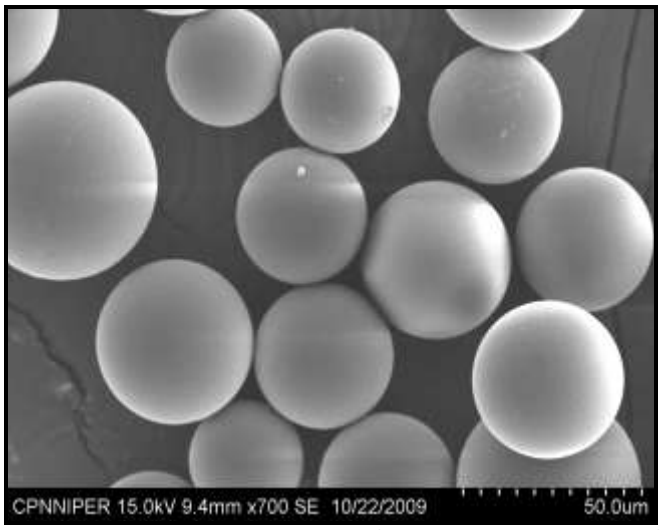


Fig. 1: Scanning electron micrograph of drug loaded microspheres of RESOMER® 504 H

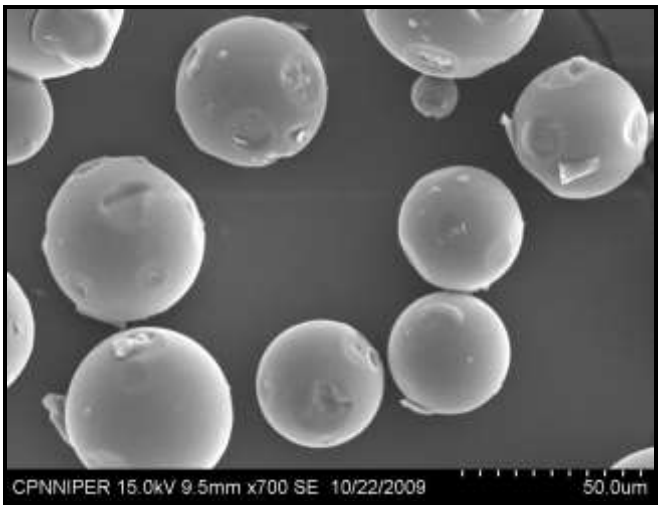


Fig. 2: Scanning electron micrograph of drug loaded microspheres of RESOMER® 502 H

from pits and pores attaining the maximum encapsulation efficiency. [21, 24]

Percent yield, entrapment efficiency and mean particle size

The results of percent yield, percent entrapment efficiency and particle size analysis for the microspheres of RESOMER® 504H and RESOMER® 502H are listed in Table 1 and Table 2 respectively. Higher percentage yield, entrapment efficiency and optimum mean particle size were obtained in the resultant batches of microspheres by using the drug/polymer ratios in increasing order (1:10, 1:20, 1:50, 1:100) with acetonitrile as organic solvent and sesame oil as liquid manufacturing vehicle. Microspheres of RESOMER® 504H has shown significant increment in maximum percentage yield (36.29 ±1.59 to 88.25 ± 1.34 %), entrapment efficiency (9.82 ± 0.98 to 70.39 ± 1.78 %) with their mean particle diameter from 47.17 ± 1.51µm to 58.49 ± 2.16µm. Similar observations were obtained for the microspheres of RESOMER® 502H with reference to maximum percentage yield (36.12 ± 1.46 to 88.04 ± 2.33 %), entrapment efficiency (21.76 ± 1.76 to 90.96 ± 1.09 %) with their mean particle diameter ranging between 46.46 ± 1.61µm to 60.39 ± 1.53µm. The observed results might be rationally attributed due to increased polymer concentration which led to the enhanced polymer viscosity of the internal phase resulting in enhanced percentage yield, entrapment efficiency as well as mean particle diameter of the prepared microspheres. [22, 24-25]

Effect of polymer concentration and viscosity

Polymer concentration also plays an influential role in microsphere formulation. Detailed data presented in Table 1 and 2 depicted that the microsphere size and Stavudine encapsulation efficiency were significantly increased when the concentrations of RESOMER® 504H and RESOMER® 502H were increased in internal phase of acetonitrile. It was demonstrated that the size of emulsion droplet depends on the balance between stirring shear force and droplet cohesion. It was considered that the higher concentration of polymers at a fixed stirring shear force, results in a higher viscosity of the oil phase which leads to higher percentage yield and entrapment efficiency with enhanced mean particle size. [18, 21, 23]

Effect of surfactant concentration in external oil phase

The stability of oil in oil emulsion with their small droplet size is an important factor affecting the drug-encapsulation efficiency. Increment in surfactant concentration (0.5 to of 2.0 %) leads to decrease in droplet coalescence resulting in more surface area attributed by decreased particle size with enhanced percentage yield and encapsulation efficiency (Table 1 and 2). It was observed that increase in surfactant concentration keeping the constant drug to polymer ratio significantly decrease the mean particle size with increased encapsulation efficiency; whereas moving towards higher polymer concentration leads to increased mean particle size which might be attributed due to dominance of polymer concentration rather than concentration of surfactant used. [28-29]

Effect of release medium

The influence of the release medium on changes in the microsphere morphology and surface topography with time was shown through scanning electron microscopy (Fig. 3-10). It was observed that the microspheres retain their morphology till the end of the study (60 days). Scanning electron micrograph of the microspheres taken after seven days showed negligible changes in surface morphology whereas samples after 15 days showed some rupture as well as some minor hairline demarcations at the surface leading to wrinkled surface of the microspheres for both the polymers. Surface topography and characteristics of the 30 day sample of the microspheres showed some remarkable changes as the minor pits and prominent rupturing of the microsphere surface is prominently visible for both the polymers. The scanning electron micrograph of the sample after 60 days has shown evident changes with reference to appearance of pores and wrinkled surface with the appendages of polymeric fractions attached to the surface of the microspheres of both the polymers. The observed phenomenal behaviour of the sampled microspheres may be explained due to the slow degradation characteristics of the polymers. [18-22]

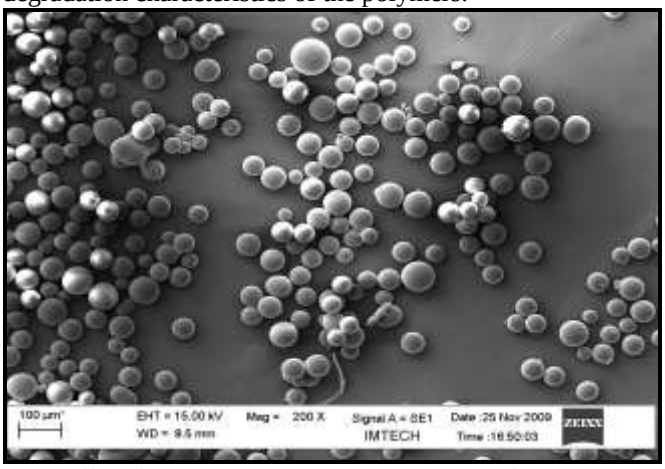


Fig. 3: Effect of release medium on morphology and surface topography of RESOMER® 504 H microspheres with time (After 07 days)

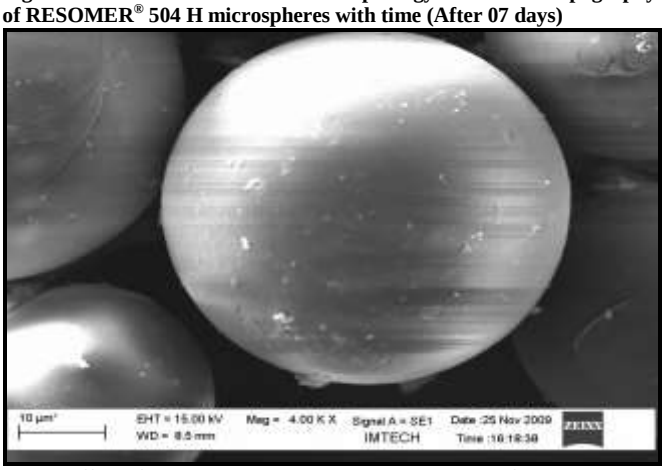


Fig. 4: Effect of release medium on morphology and surface topography of RESOMER® 504 H microspheres with time (After 15 days)

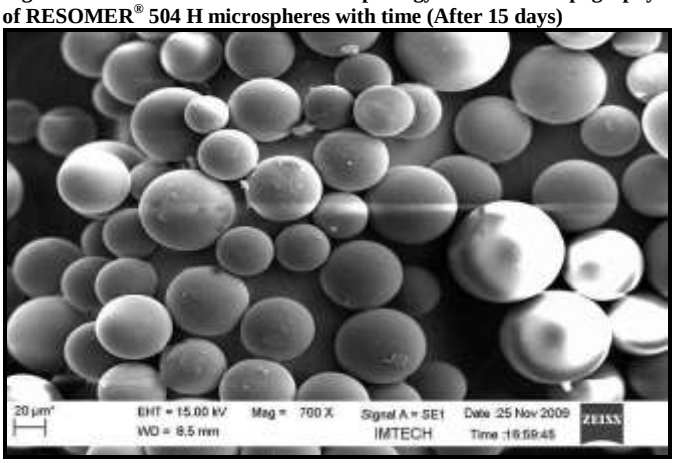


Fig. 5: Effect of release medium on morphology and surface topography of RESOMER® 504 H microspheres with time (After 30 days)

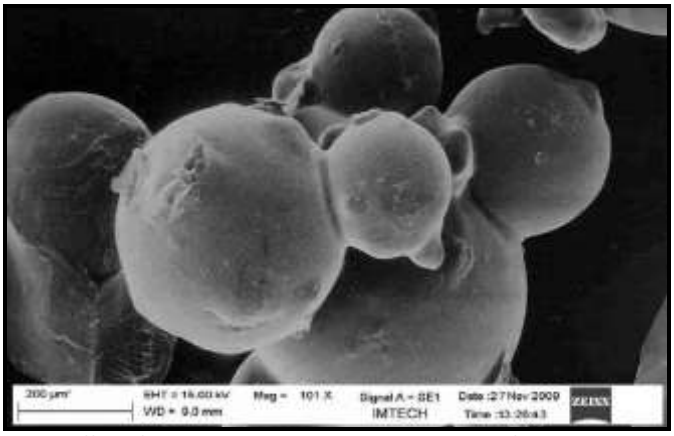


Fig. 6: Effect of release medium on morphology and surface topography of RESOMER® 504 H microspheres with time (After 60 days)

Fig. 7: Effect of release medium on morphology and surface topography of RESOMER® 502 H microspheres with time (After 07 days)

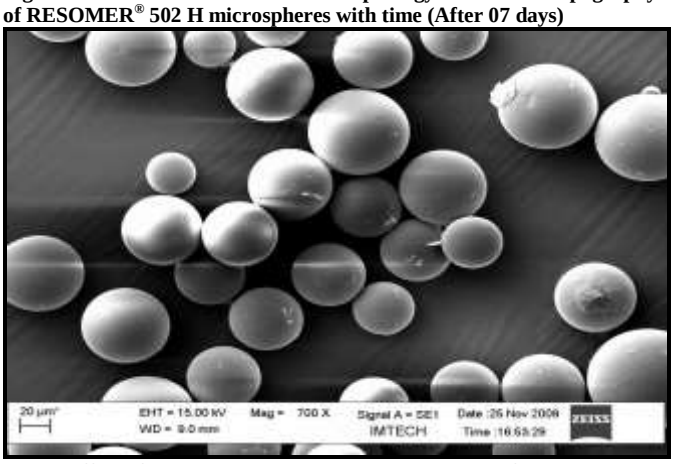


Fig. 8: Effect of release medium on morphology and surface topography of RESOMER® 502 H microspheres with time (After 15 days)

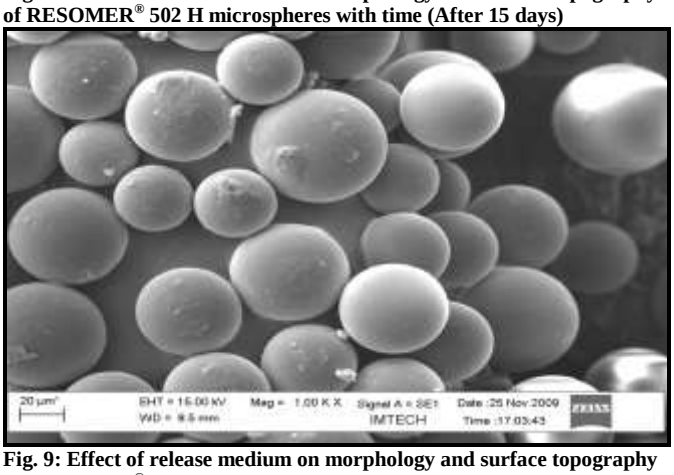


Fig. 9: Effect of release medium on morphology and surface topography of RESOMER® 502 H microspheres with time (After 30 days)

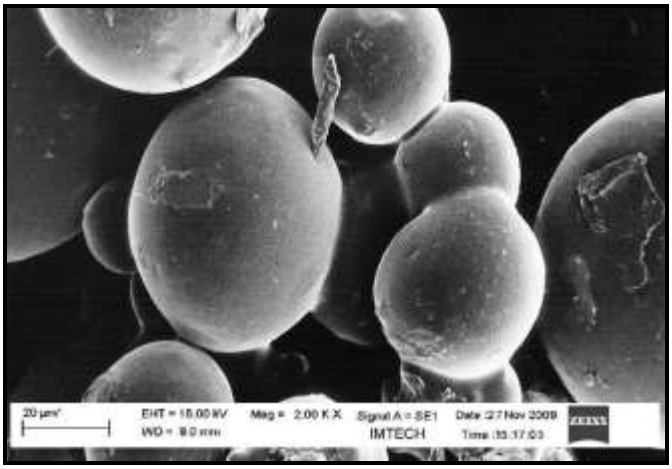


Fig. 10: Effect of release medium on morphology and surface topography of RESOMER® 502 H microspheres with time (After 60 days)

Differential scanning calorimetric studies

DSC studies were carried out on polymers, drug and microspheres to investigate physical state of drug in these carriers and possibility of interaction between the drug and polymer within the network of polymers in microspheres.

DSC thermogram of Stavudine was obtained in the temperature range of 160 to 325°C (Fig. 11). The DSC data of sample showed the presence of three polymorphic forms characterized by sharp peaks at 170.49, 176.42 and at 184.75°C showing the purity of the drug. [30] Melting of all three forms was consistently followed by an endotherm at 321.45°C representing decomposition.

DSC thermogram of pure RESOMER® 504H and RESOMER® 502H (Fig. 12, 13) depicted the endothermic peaks at 56.60°C and 39.39°C indicative of its glass transition temperature in accordance with its literature values (50-55°C and 40-45°C respectively). The endothermic peaks at 52.65°C (D1P100 4H) and 41.06°C (D1P100 2H) were observed for drug loaded microspheres of RESOMER® 504H and RESOMER® 502H respectively (Fig. 14, 15). All peaks were observed near the reported glass transition temperatures of the polymers implying the absence of drug polymer interactions. The thermograms of microspheres did not show any endothermic peak of drug hence indicating molecular dispersion of drug in to the microspheres. [24-25, 28]

Residual solvent analysis

Residual content of acetonitrile was found to be 59.559 ppm as well as 73.661 ppm in the microspheres of RESOMER® 504H and RESOMER® 502H respectively. The analysis was performed for the batch having drug/polymer ratio of 1:100; considering the best batch on basis of surface morphology, particle size as well as entrapment efficiency. The detected concentration of the organic residual solvent was found to be well below the pharmacopoeial limits, hence indicating safe use of microspheres (Fig. 16, 17). FDA limit of acetonitrile (Class II) based on recommendations of ICH and pharmacopoeia (USP) is 410 ppm.

FTIR and X-RD analysis

FTIR spectras were recorded for Stavudine, polymers and drug loaded microparticles over the range of 500 to 4000 cm^{-1} (Fig. 18, 19). Pure Stavudine spectra showed the sharp characteristic peaks at 1114.8, 1688.2, 3168 and 3423 cm^{-1} representing the C-O, C=O, N-H and O-H stretchings respectively. All the above characteristic peaks were disappeared in the spectra of drug loaded microspheres which

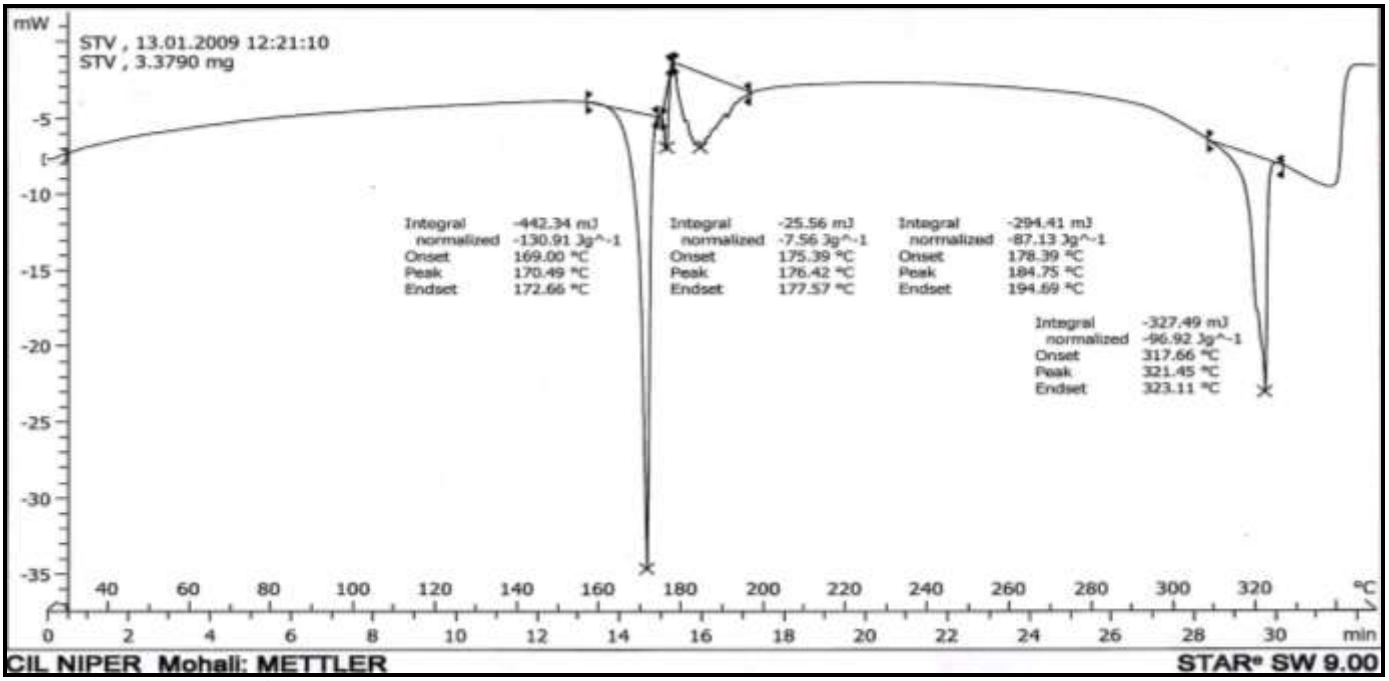


Fig. 11: DSC Thermogram of Stavudine

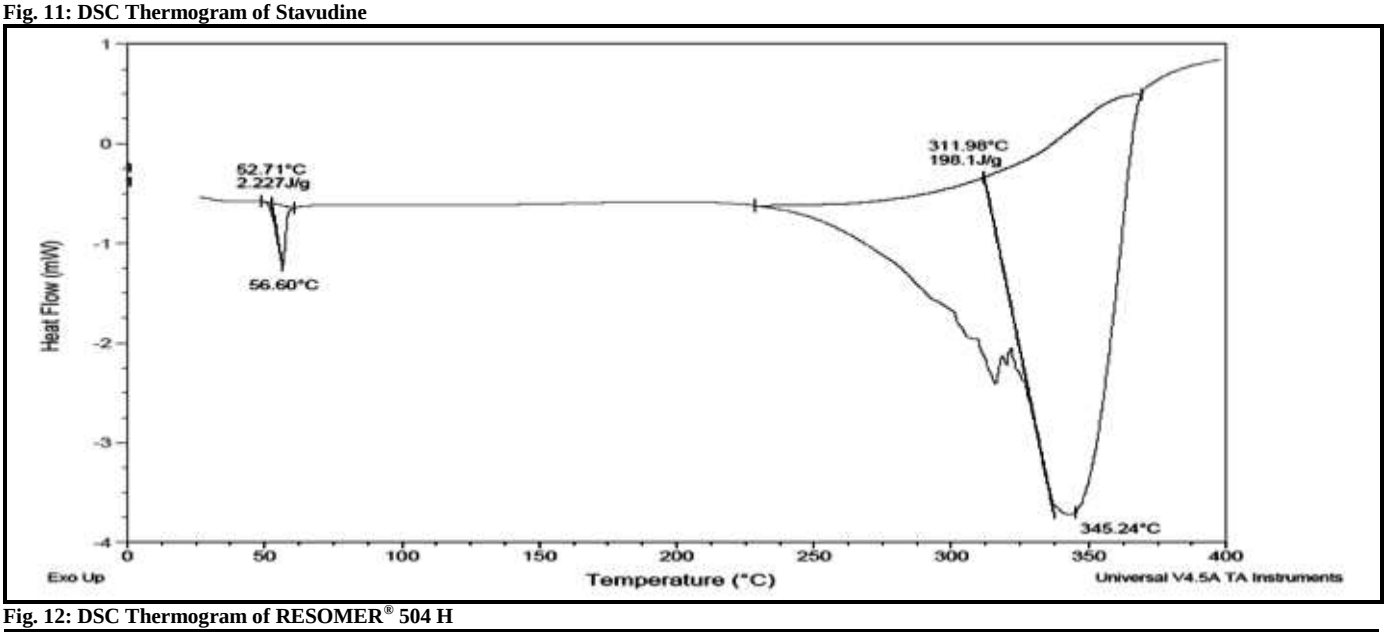


Fig. 12: DSC Thermogram of RESOMER® 504 H

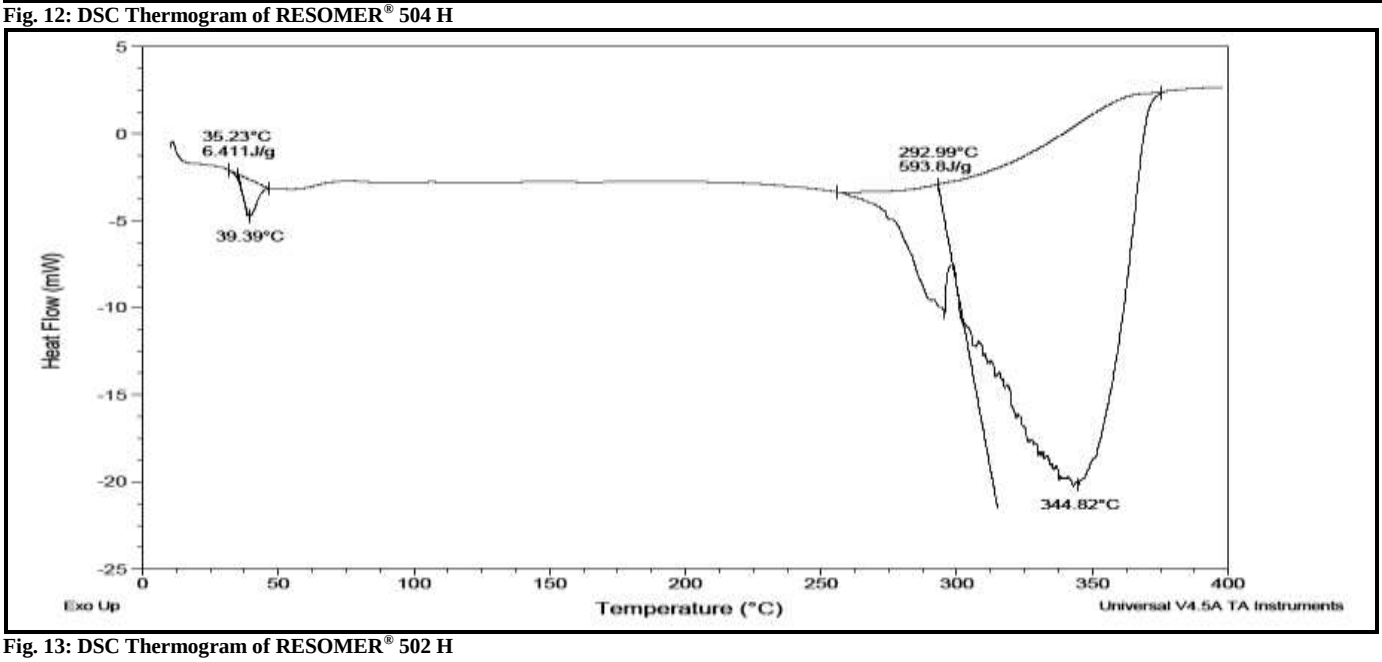


Fig. 13: DSC Thermogram of RESOMER® 502 H

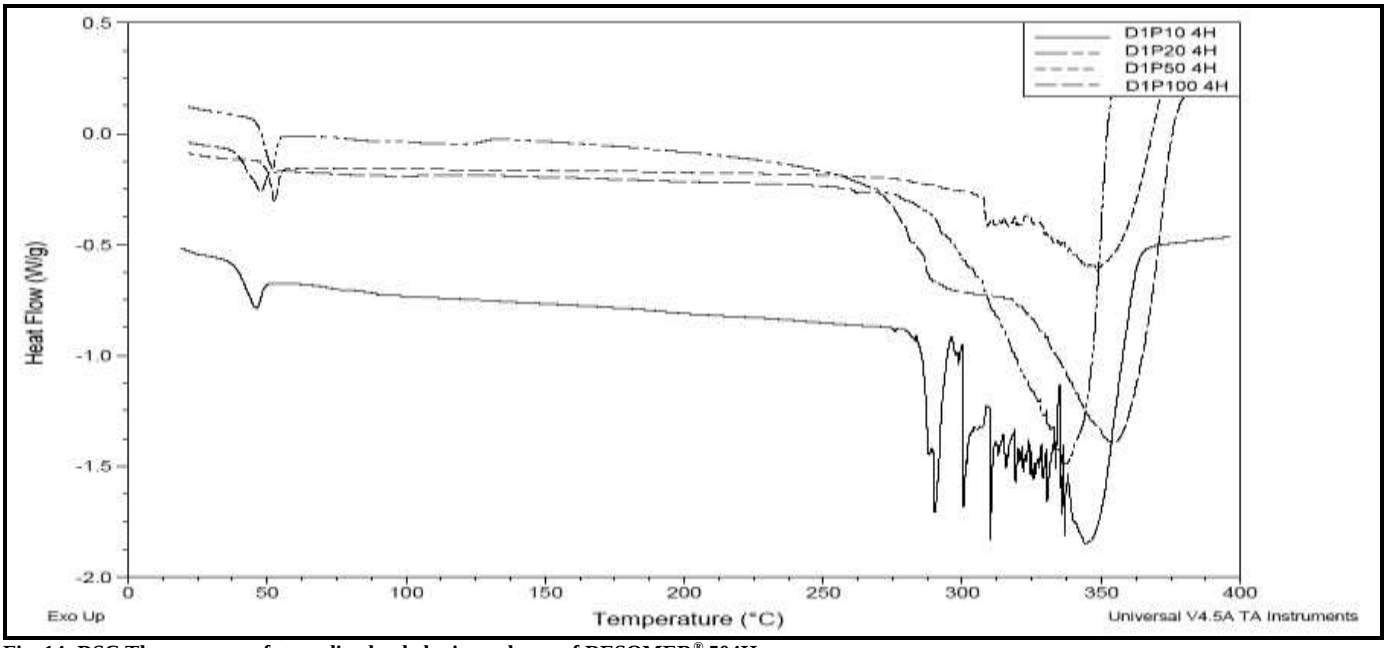


Fig. 14: DSC Thermogram of stavudine loaded microspheres of RESOMER® 504H

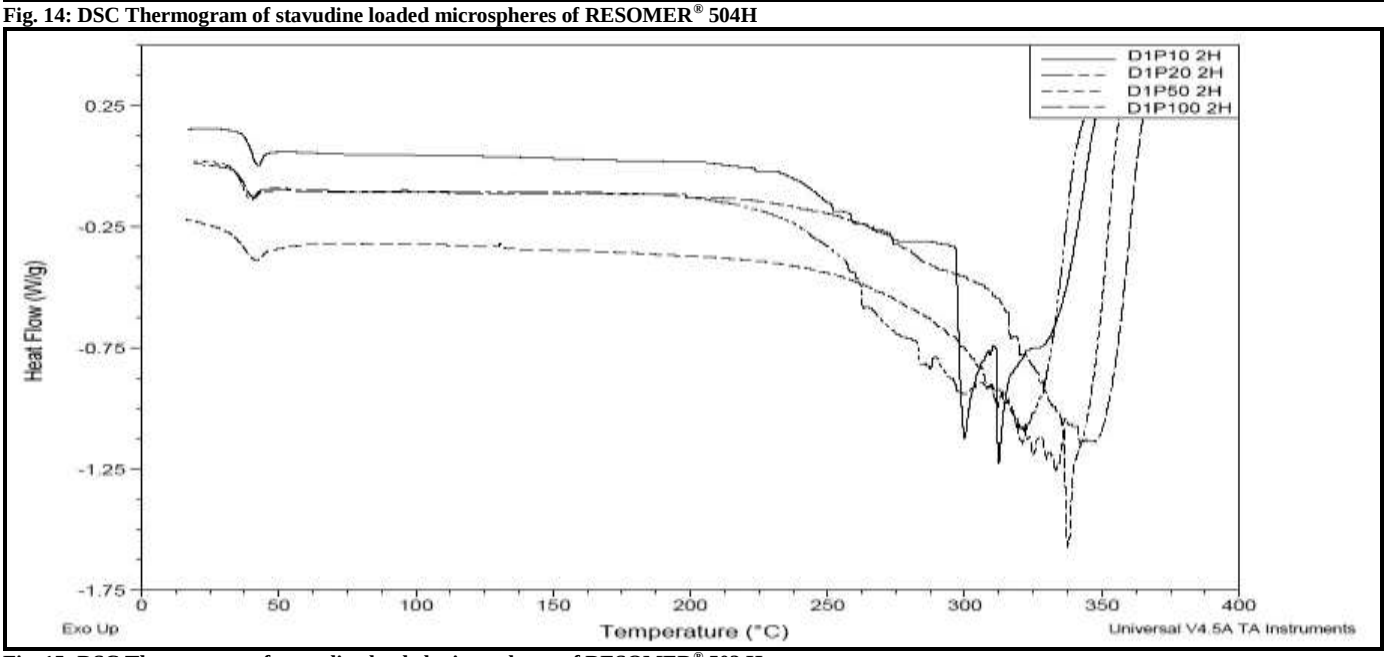


Fig. 15: DSC Thermogram of stavudine loaded microspheres of RESOMER® 502 H

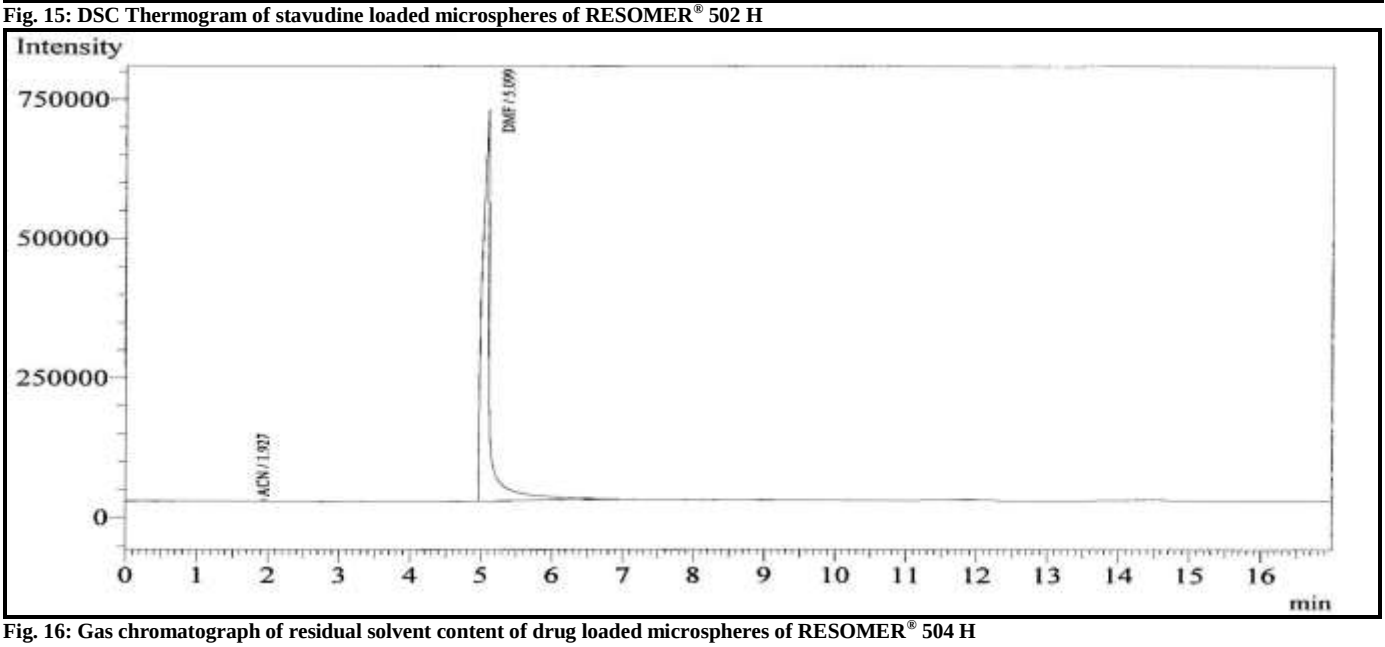


Fig. 16: Gas chromatograph of residual solvent content of drug loaded microspheres of RESOMER® 504 H

confirms the molecular dispersion of the drug in to the microspheres. [17, 19, 21]

The X-ray diffraction patterns were recorded for pure drug, polymers and drug loaded microspheres for investigating the crystallinity of the drug entrapped in the polymeric microspheres (Fig. 20, 21). It can be observed that X-ray diffraction patterns of Stavudine has shown sharp peaks representing the crystalline structure of the drug however when drug is encapsulated in polymeric microspheres the specific drug crystal peaks were disappeared. The amorphous X-ray diffractograms of drug loaded microspheres suggests that Stavudine was completely embedded and molecularly dispersed in polymeric matrix of microspheres of RESOMER® 504H and RESOMER® 502H which helps to retard the delivery of the drug. [22]

Drug distribution studies within the microspheres

Confocal laser scanning microscopy (CLSM) allows visualization and characterization of structures not only on the surface, but also inside the particles, provided the materials are fluorescently labeled. CLSM provides a unique approach to explore the internal structure of the microspheres and drug distribution as a nondestructive technique. CLSM depicted and identified the fluorescently labeled compound at light microscopical resolution. CLSM images have clearly shown the throughout and uniform distribution of coumarine labeled stavudine entrapped inside the polymeric microsphere shell. (Fig. 22); that further demonstrated the uniform drug delivery from the microspheres in a controlled manner. [26- 27]

***In-vitro* drug release studies**

The *in-vitro* release studies of all the Stavudine loaded microsphere batches of RESOMER® 504H and RESOMER® 502H were observed. The release studies were carried out in triplicate and the values expressed as mean percentage release with standard deviation (Fig. 23, 24). Two processes govern release of drug from biodegradable polymeric microspheres; first, the release is diffusion controlled. Second and later on a major part of drug, which is bound to polymeric matrix, is released by matrix erosion mechanism. [31] The results were illustrated by plotting the relative release percentages of Stavudine based on different drug to polymer ratios versus time. From the release profile of microspheres of RESOMER® 504H (Fig. 23), it can be depicted that no initial drug burst was observed indicating that there was very little surface located drug in these microspheres and the majority of the drug was embedded and uniformly distributed into the polymeric matrix, that might be due to higher inherent viscosity (0.45-0.60 dl/gm) of the polymer resulting in formation of more tightly bound polymeric network. Whereas in case of microspheres of RESOMER® 502H (Fig. 24) burst release can be significantly observed, that could be rationally explained with regard to inherent viscosity (0.16-0.24 dl/gm) of the polymer, which led to the development of less dense, weakly bounded matrix mesh in comparison to matrix structure formed by RESOMER® 504H; In spite of using the similar drug/polymer ratios used in previous polymer. [16] That ultimately releases the loosely bound drug near the periphery of the microsphere; providing an initial burst release effect followed by prolonged release of Stavudine for 6-8 weeks releasing 90-100 % of the drug entrapped within the microsphere systems.

It was observed that the encapsulation efficiency and drug release profiles have some correlation in case of microspheres using RESOMER® 504H. Higher encapsulation resulted in larger concentration gradient between the microspheres and the in vitro release medium. Since the gradient is the driving force for the drug diffusion, a high Stavudine encapsulation within the microspheres led to a rapid release rate as seen in the case of batches D1P10, D1P20 and D1P50, indicating the drug to polymer concentration of 1:10, 1:20 and 1:50 respectively. [23] Also by increasing the encapsulation efficiency a point is reached when the solid drug particles begin to form continuous pores or channels within the matrix providing the diffusion path of least resistance for drug molecules from areas where drug has previously leached out from the polymeric matrix resulting the more porous matrix and a faster drug release rate. Thus, continuous drug release behavior was observed throughout the desired time. [31] However in the case of D1P100, indicating the drug to polymer concentration of 1:100 showed the slower release rate than previous batches. That might be attributed due to vigorous increase in polymer concentration led to the development of very dense, least porous polymeric matrix resulting in the slower release rate. Another reason for the slower release might be due to enhanced mean particle size leading to decreased surface area available for diffusion. [16]

The microsphere systems of RESOMER® 502H have shown the entirely different behavior than the previous polymer batches. The microspheres developed using drug/polymer ratio of 1:10 has shown largest burst effect and fastest drug release pattern whereas on increasing the drug to polymer ratios (1:20, 1:50, 1:100) the burst release effect as well as drug release pattern has decreased in an increasing order of polymer concentration. These values can be represented in a decreasing order (D1P10 > D1P20 > D1P50 > D1P100) of burst release effect and drug release pattern. The observed results might be the outcome of well correlated role of inherent viscosity of the polymer, drug to polymer ratios as well as matrix density of microspheres. Mentioned specification have played a major and more significant role in determination of burst release effect and drug release pattern rather than entrapment efficiency.

Although on increasing the drug/polymer ratios encapsulation efficiencies were significantly enhanced but the contemporary increment in polymer solution viscosity leading to denser polymeric matrix of the developed microsphere systems were appeared to be more dominant to govern the release of the drug from the microspheres. [16, 31-32]

Drug release kinetics

Drug releasing pattern for a biodegradable system, following erosion-diffusion kinetics from polymeric matrix is very complex. The in vitro release data of all the formulations of RESOMER® 504H and RESOMER® 502H were applied to various kinetic models to predict the drug release kinetics which were described in Table 3 and 4, respectively. The release constants were calculated from the slope of the appropriate plots and the regression coefficients (r2) were determined. It was observed that the in vitro drug release mechanism of all the batches of RESOMER® 504H and RESOMER® 502H were best explained by Korsmeyer-Peppas equation. The corresponding plot of log cumulative percentage drug release vs log time of the Korsmeyer-Peppas equation indicated a good linearity of regression coefficient (r2); 0.960, 0.984, 0.990 and 0.955 for the MS of RESOMER® 504H and (r2); 0.852, 0.901, 0.928 and 0.967

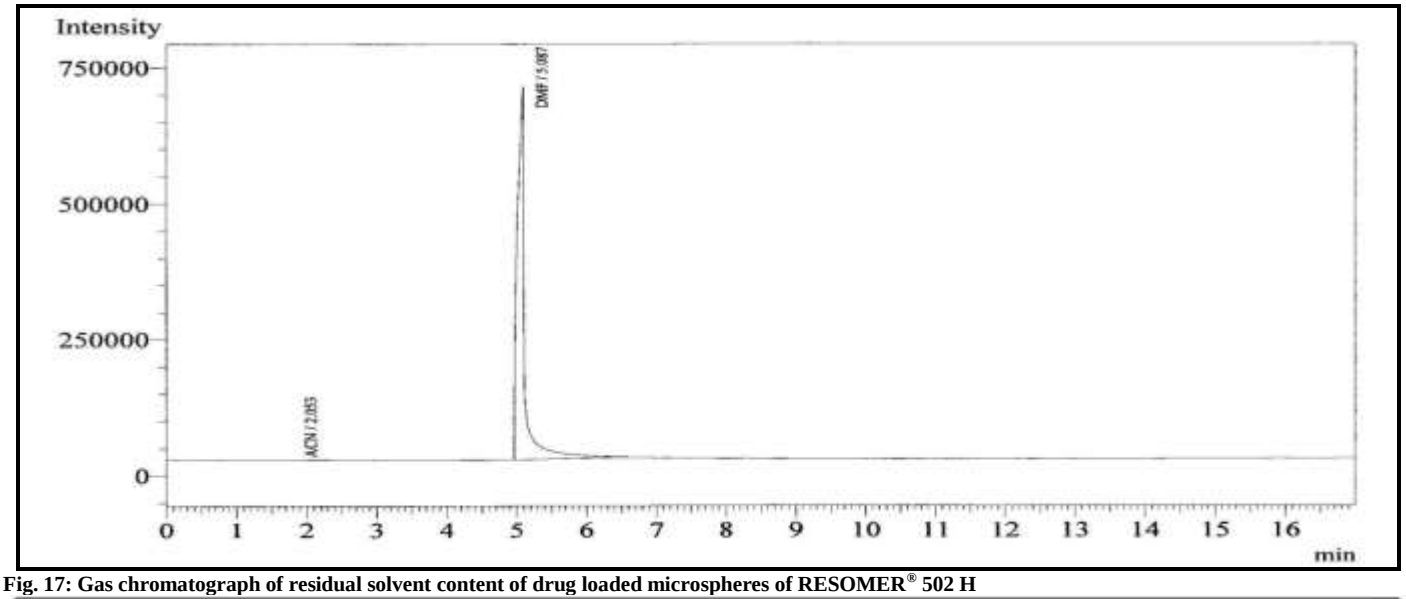


Fig. 17: Gas chromatograph of residual solvent content of drug loaded microspheres of RESOMER® 502 H

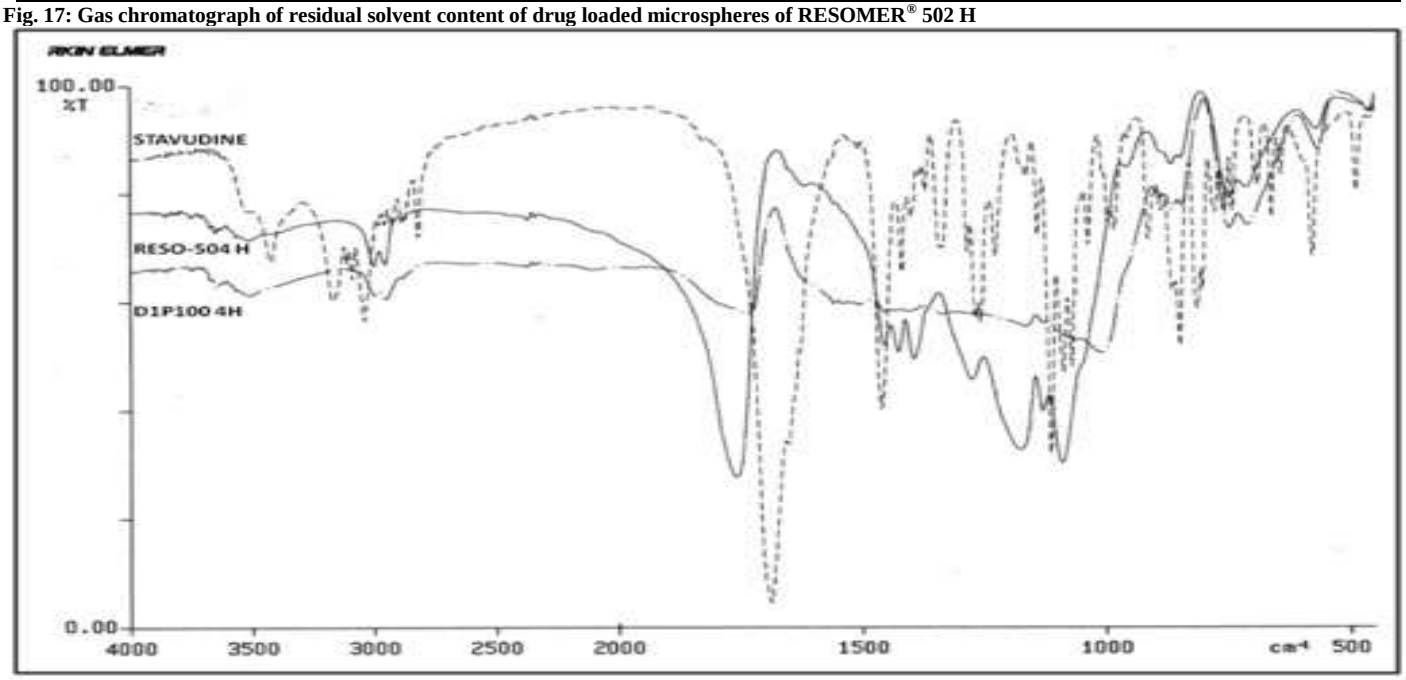


Fig. 18: FTIR Spectra of Stavudine, RESOMER® 504 H, and drug loaded microspheres

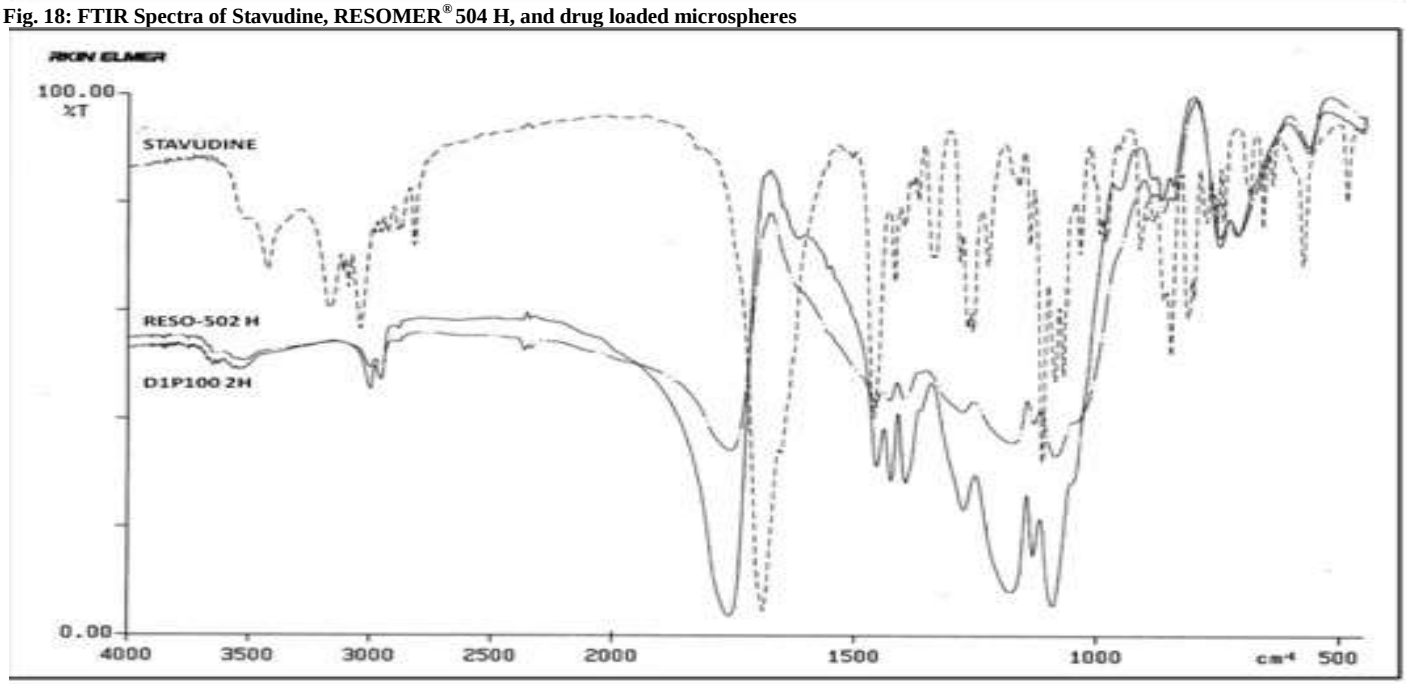


Fig. 19: FTIR Spectra of Sstavudine, RESOMER® 502 H, and drug loaded microspheres

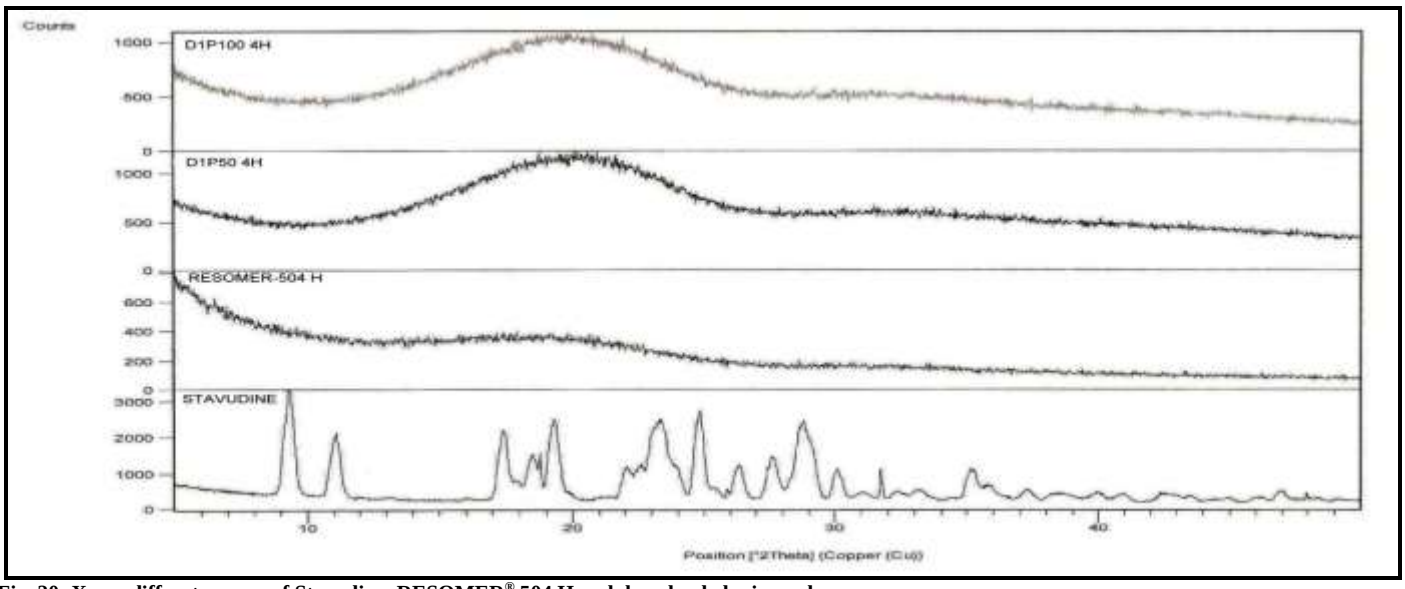


Fig. 20: X-ray diffractograms of Stavudine, RESOMER® 504 H and drug loaded microspheres

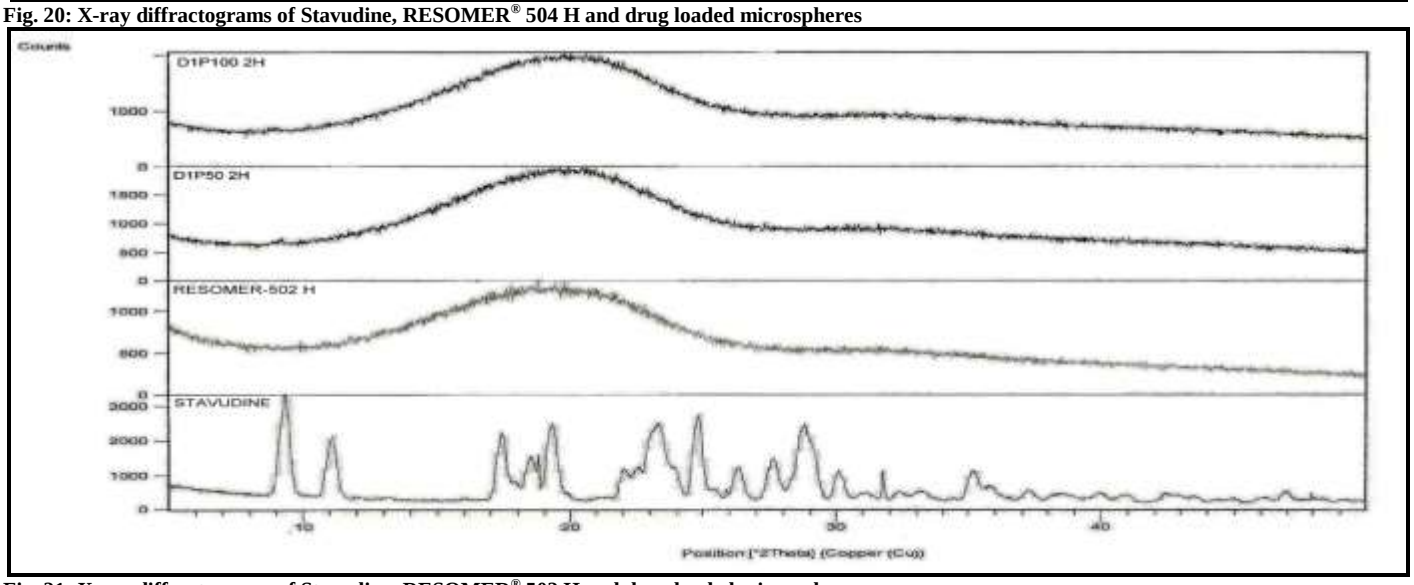
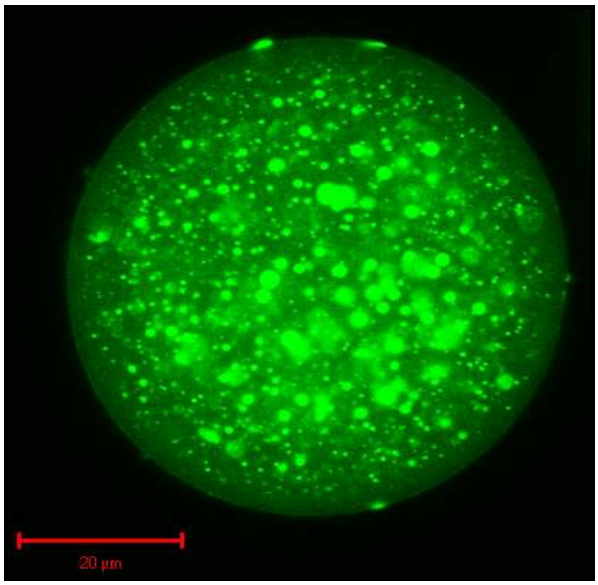
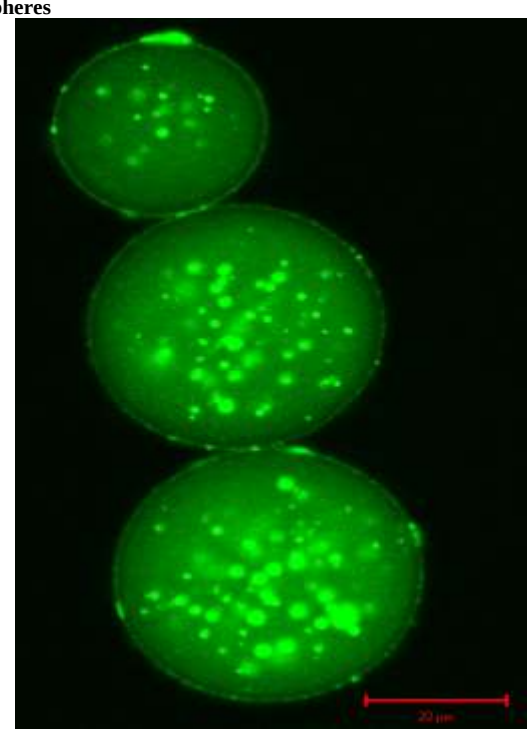


Fig. 21: X-ray diffractograms of Stavudine, RESOMER® 502 H and drug loaded microspheres



[A] - D1P100 4H



[B] - D1P100 2H

Fig. 22: Confocal scan of Stavudine loaded microsphere labeled with Coumarine

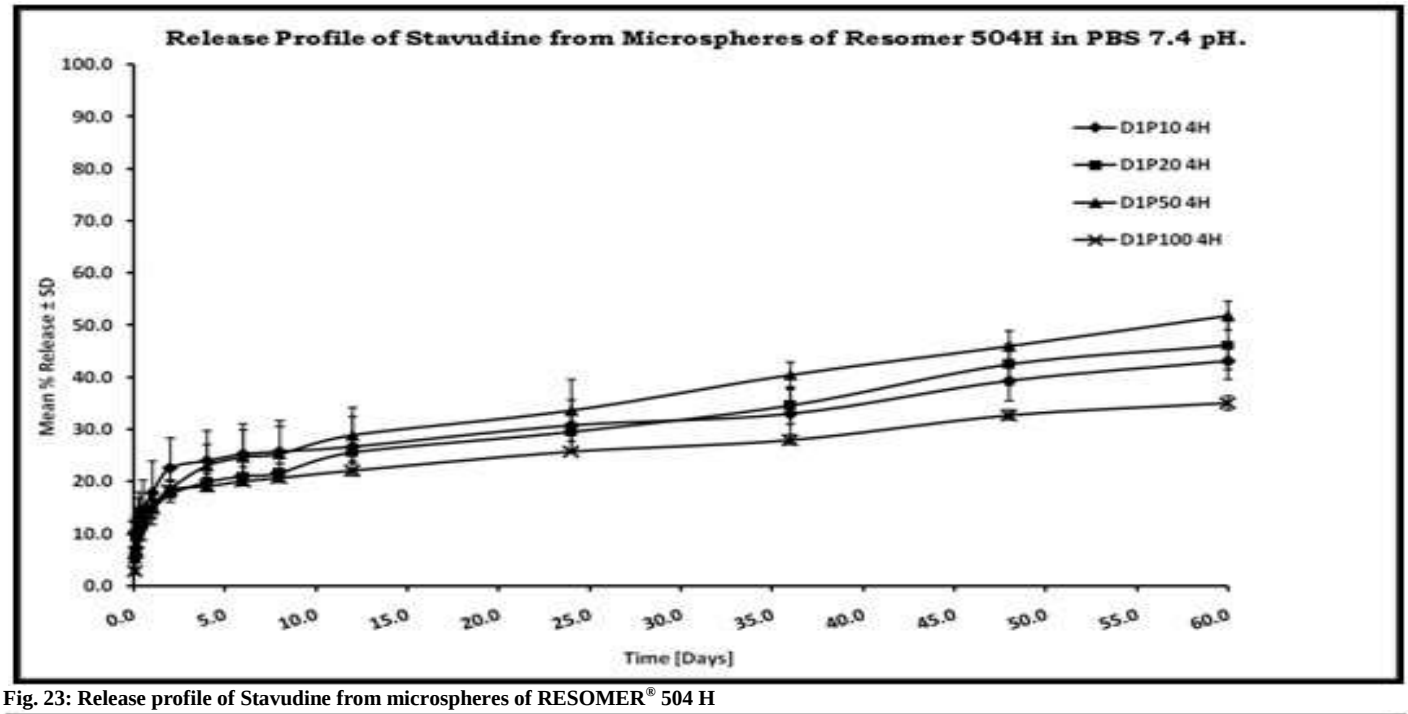


Fig. 23: Release profile of Stavudine from microspheres of RESOMER® 504 H

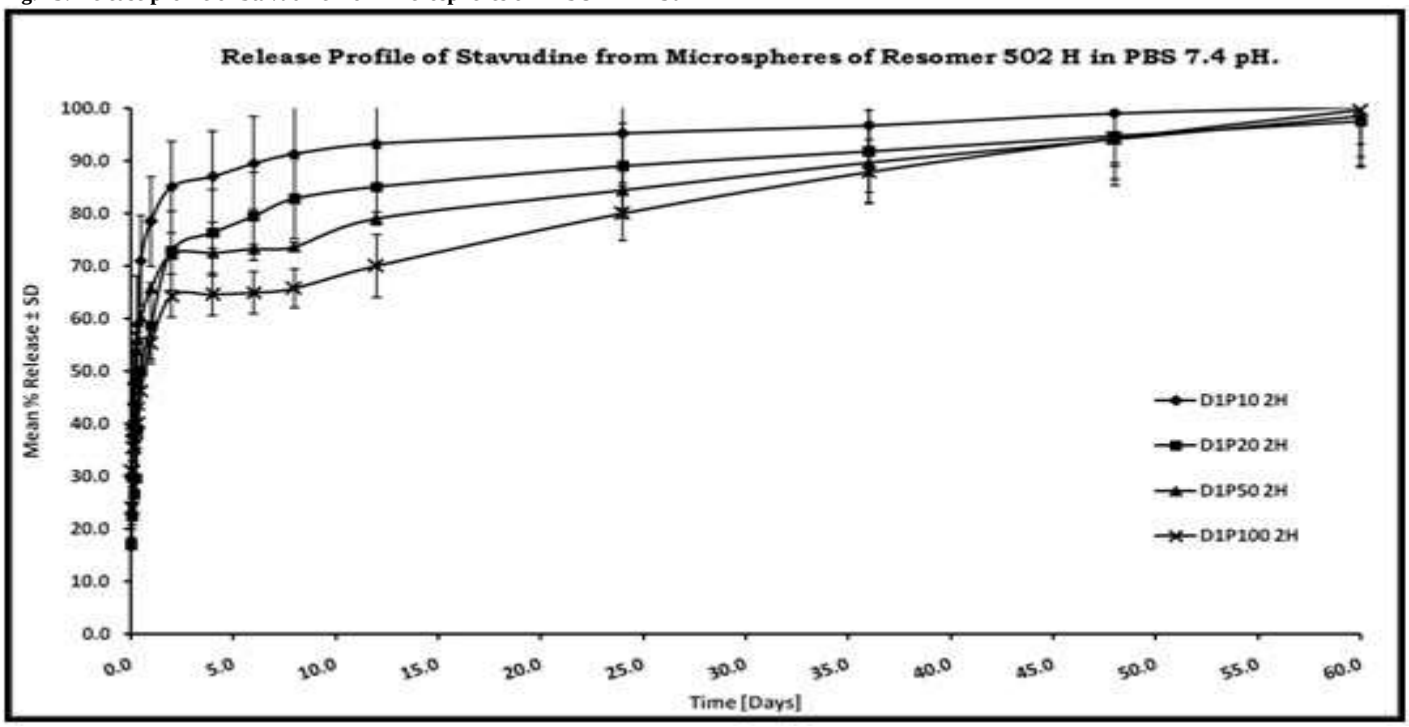


Fig. 24: Release profile of Stavudine from microspheres of RESOMER® 502 H

for the MS of RESOMER® 502H respectively. The release exponent (n) of Korsmeyer-Peppas equation were found to be in the range of 0.5-1.0 for all the batches of both the polymers depicting that drug release mechanism is anomalous transport. [16, 31-33]

The current research investigates the formulation of Stavudine loaded biodegradable polymeric microspheres of RESOMER® 504H and 502H with tailored drug release for parenteral depot system. These microparticles were prepared using solvent evaporation method exploiting the effects of variable concentrations of surfactant, inherent viscosities and concentrations of polymer solution in external phase. The preliminary investigations for the microspheres have shown that most of the microspheres are smaller than 65µm with proper sphericity and shape as well as surface topography. The different drug to polymer ratios has impacted greatly on percentage yield, entrapment efficiency and mean particle diameter of the microspheres. Different characterization studies like FTIR, XRD and CLSM has depicted the throughout, uniform distribution of the drug in to polymeric matrix. The characteristic release pattern of the microspheres has revealed 90-100% drug release in 6-8 weeks and exhibited the monolithic matrix based system indicating extended release of the drug. The study demonstrated the feasibility of prolonged delivery of Stavudine through parenteral depot system using biodegradable microspheres.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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