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Preparation and *in vitro* evaluation of ethyl cellulose microspheres containing stavudine by the double emulsion method

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Received April 27, 2006, accepted May 11, 2006

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Pharmazie 62: 117–121 (2007) *doi: 10.1691/ph.2007.2.6076*

The aim of this study was to formulate and evaluate microspheres of stavudine by water-in-oil-in-oil (w/o/o) double emulsion solvent diffusion method using ethyl cellulose and ethyl cellulose in combination with polyvinyl pyrrolidone. A mixed solvent system consisting of acetonitrile and dichloromethane in an 1 : 1 ratio and light liquid paraffin was chosen as primary and secondary oil phase, respectively. Span 80 was used as surfactant for stabilizing the secondary oil phase. The influence of formulation factors like stirring speed, surfactant concentration on particle size and polymer : drug ratio and combination of polymers on drug release characteristics of the microspheres was investigated. The prepared microspheres characterized by micrometric properties, drug loading, Fourier transform infrared spectroscopy, X-ray powder difractometry and scanning electron microscopy. The prepared microspheres were white, free flowing and spherical in shape, stable in nature, with 41–65% of drug entrapment efficiency. The best-fit release kinetics was achieved with Higuchi plot followed by first order and zero order. The release of stavudine was influenced by the drug to polymer ratio, particle size and polymer combination.

1. Introduction

Stavudine (D4T, thymidine) is an FDA approved drug for clinical use for treatment of HIV infection, AIDS and AIDS related conditions either alone or in combination with other antiviral agents. Stavudine is typically administered orally as capsule and oral solution. The drug has a very short half life (1.30 h). Patients receiving stavudine develop neuropathy and lactic acidosis. The side effects of stavudine are dose dependent and a reduction of the total administered dose reduces the severity of the toxicity (Goodman 2001).

Microspheres are a multiparticulate delivery system and are prepared to obtain prolonged or controlled drug delivery, to improve bioavailability or stability and to target to specific sites. Microspheres can also offer advantages like limiting fluctuation within the therapeutic range, reducing side effects, decreasing dosing frequency and improving patient compliance (Davis et al. 1989). Ethyl cellulose (EC), a biocompatible, non-biodegradable and one of the most extensively studied encapsulating materials for the controlled release of pharmaceuticals (Chowdary et al. 2004), was used as the retardant material. Polyvinyl pyrrolidone (PVP) was included in the formulation as a pore forming agent, in order to modify the drug release pattern. The objective of the present investigation was to prepare ethyl cellulose microspheres containing stavudine to achieve a controlled drug release profile for p.o. administration.

2. Investigations, results and discussion

2.1. Effect of experimental variables on mean particle size

Various manufacturing parameters like drug to polymer ratio, stirring speed, stabilizer concentration affect the particle size of the microspheres (Lee et al. 2000). The drug to polymer ratio appeared to influence on the particle size distribution of microspheres (Fig. 1). When the drug to polymer ratio was increased from 1 : 1 to 1 : 2 and 1 : 3, the proportion of larger particles formed became higher, because the viscosity of the primary emulsion was increased with increase of polymer-to drug ratio. Due to this increased viscosity, large emulsion droplets were formed and it was difficult to break them and, hence, they are

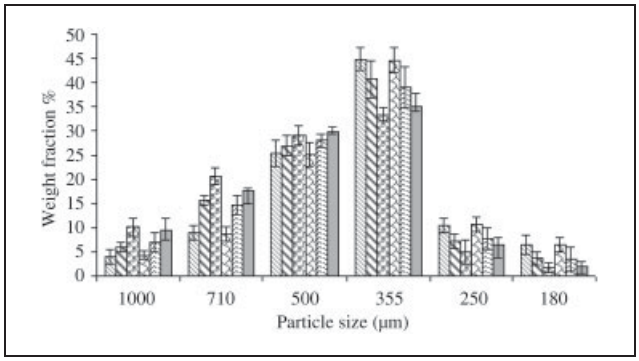

Fig. 1: Particle size distribution of the microspheres of formulations F1 (▨), F2 (▨), F3 (▨), F4 (▨), F5 (▨), F6 (▨)

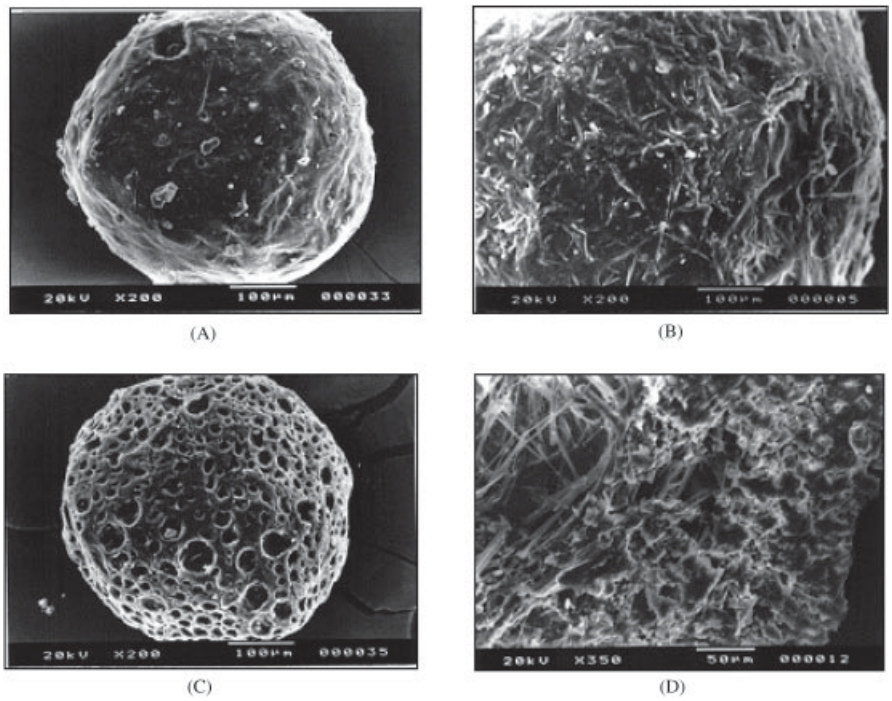

Fig. 2:
SEM Photographs indicate blank microspheres (A). Microspheres (F6) before dissolution (B) and after dissolution (C). Cross-section of microspheres (D)

precipitated as such leading to an increase in the mean particle size, as shown in Table 1. The minimum concentration of Span 80 required to form a stable emulsion was found to be 0.5%. As the concentration of Span 80 increased from 0.5% to 1% and 2%, the mean particle size reduced from 517 to 368 µm. This is in accordance with the theory of effect of surfactant concentration on particle size. Changing the stirring speed of the second emulsification process also seems to influence the mean particle size of the microspheres. When the stirring speed was decreased from 1000 to 500 rpm, the mean particle size of the microspheres was increased and they became large and aggregated. When the speed was increased above 1000 rpm the size of the microspheres was found to decrease. Compressibility index, the packing factor of each formulation was excellent compared to that of pure drug (Martin 1991). The compressibility index lies in the range of 5–15 and packing factor lies in the range of 0 to 1.2 (Table 2). This indicates that microspheres were free flowing in nature. The improvements of flow properties suggest that the microspheres can be easily handled.

Table 1: % Yield, mean particle size and entrapment efficiency of various formulation

Batch	Yield (%)	Mean Particle Size (µm)	Entrapment efficiency
F1	81.91 ± 2.49	425 ± 13.10	65.89 ± 0.708
F2	80.90 ± 2.30	474 ± 15.94	52.53 ± 1.19
F3	76 ± 3.26	527 ± 19.67	43.67 ± 0.999
F4	81.93 ± 3.38	420 ± 17.03	54.15 ± 0.377
F5	79.09 ± 1.74	477 ± 20.57	44.06 ± 0.426
F6	73.7 ± 2.07	512 ± 21.07	41.09 ± 0.875

* Each observation is the mean (±SD) of three determinations

2.2. *Entrapment efficiency*

The entrapment efficiency of various formulations was found to be optimum in the range of 41–65% (Table 1). The entrapment efficiency less than expected is due to high solubility of the drug in the mixed solvent system. Because of its high solubility, the drug may be migrated to the processing medium during extraction and evaporation processes of dichloromethane and acetonitrile, respectively. This was supported by SEM analysis, which showed the presence of drug particles on the surface of the microspheres, revealing the migration phenomena of the drug to the processing medium.

2.3. *Scanning electron microscopy*

From scanning electron microscopy (SEM) study, it was found that the microspheres were spherical. The surface of the microspheres was rough. The study of drug-loaded microspheres shows the presence of drug particles on the surface, which was responsible for an initial burst release

Table 2: Micromeritic properties of drug loaded microspheres

Batch Code	Bulk density (g/ml)	Tapped density (g/ml)	Carr's index	Packing Factor
Drug	0.204 ± 0.0015	0.282 ± 0.0015	27.62 ± 0.615	1.37 ± 0.15
F1	0.263 ± 0.0152	0.276 ± 0.0151	4.85 ± 1.98	1.04 ± 0.20
F2	0.070 ± 0.0021	0.296 ± 0.0115	8.7 ± 4.34	1.09 ± 0.55
F3	0.316 ± 0.0152	0.350 ± 0.0012	9.49 ± 4.31	1.12 ± 0.035
F4	0.326 ± 0.0231	0.373 ± 0.0208	12.54 ± 1.89	1.15 ± 0.015
F5	0.330 ± 0.0034	0.383 ± 0.0025	14 ± 2.35	1.16 ± 0.030
F6	0.373 ± 0.0153	0.436 ± 0.0152	14.54 ± 1.84	1.16 ± 0.015

*Each observation is the mean (± SD) of three determinations

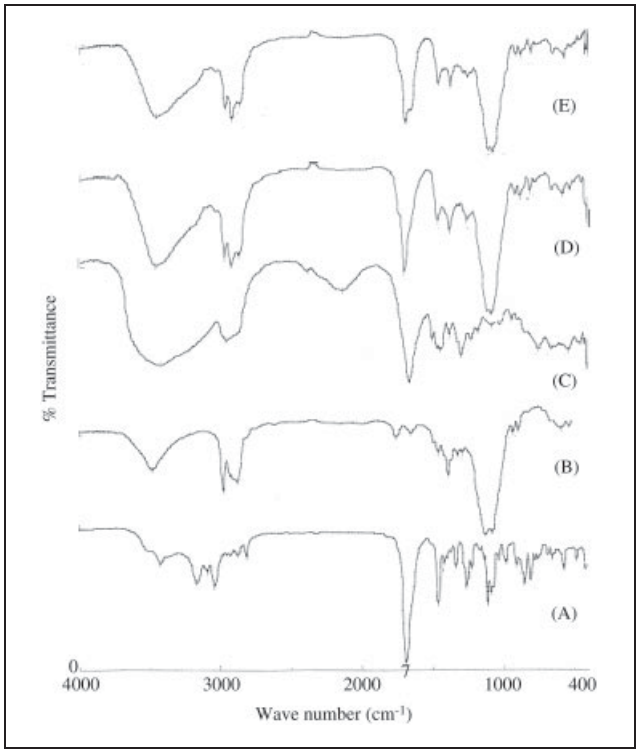

Fig. 3: IR Spectra of pure stavudine (A), EC blank microspheres (B), EC: PVP blank microspheres (C), formulation F2 (D), formulation F6 (E)

of the drug during dissolution. Surface study of the microspheres after dissolution shows bigger pores. Cross-section of the microspheres shows that they are spongy in appearance (Fig. 2).

2.4. Infrared spectroscopy

The IR spectra of pure stavudine, blank EC microspheres and EC : PVP combination microspheres, EC loaded microspheres and EC : PVP loaded microspheres of stavudine are shown in Fig. 3. Drug spectrum showed prominent peaks at 3424.9 cm^{-1}, 3170.1 cm^{-1}, 3093.2 cm^{-1}, 3043.1 cm^{-1}, 2881.1 cm^{-1}, 2820.3 cm^{-1} and 1690.3 cm^{-1} corresponding to OH stretching (symmetric), NH stretching of secondary amine, C−H stretching (–C=CH), C−H stretching (CH_3), C−H stretching (asymmetric), C−H stretching (symmetric) and C=O stretching. In IR spectra of EC loaded microspheres and EC : PVP loaded microspheres of stavudine, there was widening of peak occurred at OH stretching and NH stretching. This was due to intermolecular hydrogen bonding. The characteristic peaks of C−H stretching (−C=CH), C−H stretching (CH_3), C−H stretching (asymmetric), C−H stretching (symmetric) correspond to drug peaks. This indicates the stable nature of the drug during the encapsulation process.

2.5. X-ray powder diffraction

The X-ray powder diffraction patterns of pure drug, EC loaded microspheres and EC : PVP loaded microspheres are shown in Fig. 4. X-RD study suggests that when pure stavudine was incorporated in the EC and EC : PVP, the intensity of drug peaks was decreased due to decreasing crystallinity of the drug.

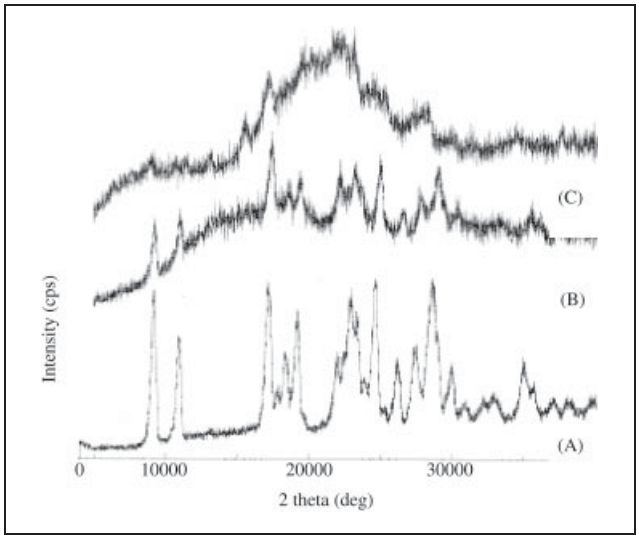

Fig. 4: X–RD Spectra of pure stavudine (A), F2 (B) and F6 (C)

2.6. In vitro release

In order to keep the total surface area of the microspheres constant and thus to get compatible result, the release studies were carried out with 355 μm fractions. The *In vitro* release of stavudine from EC and EC : PVP microspheres was biphasic with an initial burst effect. The initial burst effect was due to the presence of drug particles on the surface of the microspheres, which was revealed by SEM analysis studies. The initial burst effect may be attributed as a desired effect to ensure initial plasma concentration.

The rate of release of drug from the EC micro spheres was slow and found to further decrease with increase in EC ratio, but when PVP was used along with EC, the release rate becomes faster (Fig. 5). It is due to pore forming nature of PVP in aqueous medium, which facilities faster drug release.

According to Davis the gastrointestinal transit time is 6–8 h for young healthy men. Considering the gastrointestinal transit time, release profile from F_1, F_4 and F_5 seems to be too fast for a controlled release and incomplete absorption in case of F_3 in which drug release is sustained for a period of 14 h. Formulation F_2 and F_6 were found to release more than 85% of drug in 7 h.

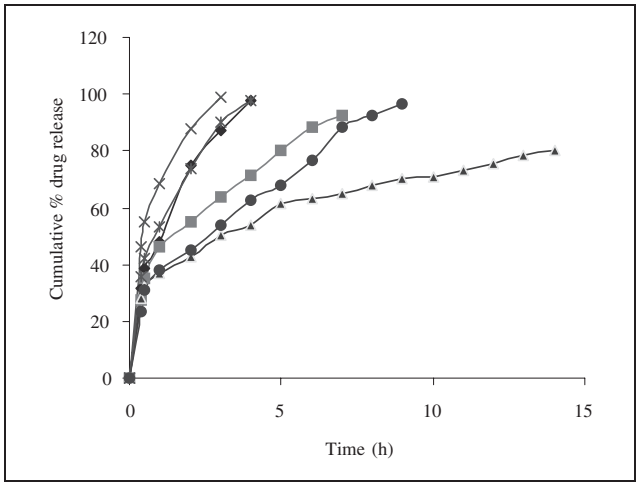

Fig. 5: Cumulative percent release of stavudine (n = 3) from different microspheres prepared with different drug: polymer ratio. F1 (—◆—), F2 (—■—), F3 (—▲—), F4 (—✕—), F5 (—✱—), F6 (—●—)

Table 3: Various parameters of the model equations on the *in vitro* release kinetics

Batch Code	Higuchi model		Zero order		First order		Korsemeyer peppes model	
	r^2	K_h	r^2	K_0	r^2	K_1	r^2	n
F1	0.994	48.33	0.984	21.53	0.960	0.851	0.988	0.350
F2	0.997	32.43	0.984	10.81	0.985	0.321	0.997	0.332
F3	0.995	18.02	0.960	4.14	0.991	0.0950	0.993	0.237
F4	0.999	54.04	0.984	26.53	0.951	1.26	0.998	0.285
F5	0.995	48.26	0.984	21.04	0.973	0.8807	0.991	0.324
F6	0.991	29.01	0.993	9.12	0.956	0.31	0.994	0.332

* r^2 is the correlation coefficient and K_0 , K_1 and K_h, are the release rate constants of the zero order, first order and Higuchi models and 'n' is the release exponent of Korsemeyer Peppas model

The effect of particle size on the release profile was also investigated. Microspheres of different size fraction i.e. 250, 355 and 500 μm were carried out for 6 h (Fig. 6). The release profile was in line with the general hypothesis of the effect of the particle size on dissolution. As the particle size decreased, the drug release was fast because of more available surface area.

The *In vitro* release profile was analyzed by various kinetic models. The best-fit order with highest correlation co-efficient was shown in Higuchi, first order, zero order and Hixion Crowell equations (Table 3.). The rate constants were calculated from the slope of the respective plots. Highest correlation was observed in the Higuchi plot rather than first order, zero order. The drug release was proportional to square root of time indicating that the drug release from EC and EC : PVP microspheres was diffusion controlled. The data obtained were also fitted to Korsemeyer Peppas model in order to find out the 'n' value, which describes the drug release mechanism. The 'n' value of all formulations lies between 0 and 0.5 indicating the mechanism of the drug release to be diffusion controlled. The release also shows high correlation with the Korsemeyer-Peppas model.

Stavudine microspheres were prepared successfully using the double emulsion solvent diffusion method. Polymer: drug ratio, stirring speed and the content of surfactant influenced the mean particle size of microspheres. The entrapment efficiency was in the range of 41–65%. Stavudine release rate from EC microspheres (F_3) was very slow where chances of incomplete drug absorption lies after p.o. administration, and too fast for F_1,F_4 and F_5. The drug release profile aimed for p.o. administration could be obtained by using formulation F_2 and F_6.

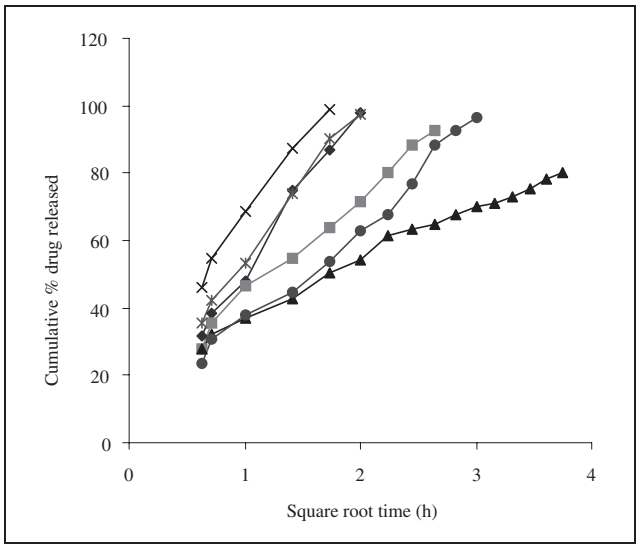

Fig. 6: Higuchi plot of stavudine release (n = 3) from different microspheres prepared with different drug: polymer ratio. F1 (—◆—), F2 (—■—), F3 (—▲—), F4 (—✕—), F5 (—✱—), F6 (—●—)

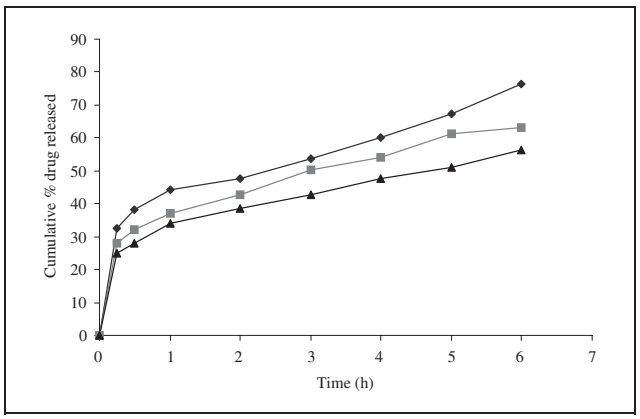

Fig. 7: Effect of particle size on release profile of stavudine loaded microspheres of F3.
Release of drug from particle size fraction 250 μm (—◆—), 355 μm (—■—) and 500 μm (—▲—)

3. Experimental

3.1. Materials

Stavudine was obtained as a gift sample from Hetero Labs Ltd. (Hyderabad, India). Ethyl cellulose (EC) and poly vinyl pyrrolidone (PVP) were purchased from Central Drug House (Mumbai, India). All other reagents and solvents used were of pharmaceutical or analytical grade.

3.2. Preparation and characterization of microspheres

Microspheres were prepared using the water-in-oil-in-oil (w/o/o) double emulsion solvent diffusion method (Ramarao et al. 2005; Badri Viswanathan et al. 1999), using different ratios of polymer to stavudine. EC alone or in combination with PVP was dissolved in 10 ml of the mixed solvent system consisting of acetonitrile and dichloromethane in an 1 : 1 ratio. The initial w/o emulsion was prepared by adding aqueous solution of drug (2 ml) to the polymeric solution while stirring by a mechanical stirrer (lab stirrer, Remi motors, India) at 500 rpm for 10 min. This w/o primary emulsion was slowly added to 100 ml of light liquid paraffin, the second oil phase containing 0.5% Span 80 (HLB value of 4.3) as a surfactant while stirring at 1000 rpm. After 2 h, 10 ml of n-hexane (non solvent) was added to harden the microspheres (Sprockel 1990 et al.). Stirring was continued for a further 1 h and the hardened microspheres were collected by filtration and washed with three portions of 50 ml of n-hexane and air dried for 12 h. Batches were prepared in triplicate to obtain reproducible results. The composition of various formulations is shown in Table 4.

Microspheres dried at room temperature were then weighed and the yield of microspheres preparation was calculated using the following formula.

$$\text{Percent yield} = \frac{\text{Amount of microspheres obtained (g)}}{\text{Theoretical amount (g)}} \times 100$$

Microspheres were separated into different size fractions by sieving for 10 minutes using a mechanical sieve shaker (Cuprit Electrical Co. India)

Table 4: Composition of microsphere formulations

Batch	Drug (g)	EC (g)	PVP (g)	Acetonitrile and dichloromethane (1 : 1) (ml)	Span 80 (%)	n-Hexane (ml)	Liquid paraffin light (ml)
F1	0.500	0.500	–	10	0.5	10	100
F2	0.500	1	–	10	0.5	10	100
F3	0.500	1.500	–	10	0.5	10	100
F4	0.500	0.300	0.200	10	0.5	10	100
F5	0.500	0.800	0.200	10	0.5	10	100
F6	0.500	1.300	0.200	10	0.5	10	100

containing standard sieves having apertures of 1000, 710, 500, 355, 250 and 180 μm (Indian Pharmacopoeia, 1996). The particle size distribution of the microspheres for all the formulations was determined and mean particle size of microspheres was calculated by using the following formula.

$$\text{Mean particle size} = \frac{\sum (\text{Mean particle size of the fraction} \times \text{weight fraction})}{\sum \text{weight fraction}}$$

The flow properties of prepared microspheres were investigated by measuring the bulk density, tapped density, carr's index and packing factor. The bulk and tapped densities were measured in a 10 ml graduated measuring cylinder as a measure of packability of the microspheres. The sample contained in the measuring cylinder was tapped mechanically by means of constant velocity rotating cam with the change in its initial bulk density to a final tapped density when it has attained its most stable from (i.e. unchanging arrangement). Each experiment was carried out in triplicate.

3.3. Drug entrapment efficiency

About 50 mg of accurately weighed drug loaded microspheres were added to 50 ml of phosphate buffer, pH 6.8. The resulting mixture was kept shaking on a mechanical shaker for 24 h. Then, after the solution was filtered (0.45 μm pore size) and 1 ml of this solution was appropriately diluted to 25 ml with phosphate buffer, pH 6.8 and analyzed spectophotometrically at 266 nm using a Systronic 2101 UV-Visible Spectphotometer.

3.4. Scanning electron microscopy (SEM)

A JEOL JSM-5200 scanning electron microscope was used to characterize the surface topography of the microspheres. The microspheres were placed on a metallic support with a thin adhesive tape and microspheres were coated with gold under vacuum (fine coat, ion sputter JFC-1110). The surface was scanned and photomicrographs were taken at 20 kV accelerating voltage for the drug-loaded microspheres before and after dissolution.

3.5. Fourier transform infrared spectroscopy (FTIR)

The spectra were recorded for pure drug and drug loaded microspheres using FTIR JASIO (Model No. 410). Samples were prepared in KBr disks (2 mg sample in 200 mg KBr). The scanning range was 400–4000 cm^{-1} and the resolution was 2 cm^{-1}.

3.6. X-ray powder diffractometry (X-RD)

X-ray powder diffractometry was carried out to investigate the effect of the microencapsulation process on crystallinity of drug. Powder X-RD patterns were recorded on Rigaku, Japan (Model-Meniflex) using Kb filtered Cu k α radiation, a voltage of 30 kV and a current of 25 mA. The scanning rate employed was 2.00 degree/min, over the 4 °–40 ° diffraction angle (2θ) range. The X-RD patterns of drug powder and drug-loaded microspheres were recorded.

3.7. In vitro release

The *in vitro* release studies were carried out at 37 °C and 100 rpm using phosphate buffer pH 6.8 (500 ml) in a USP basket type dissolution test apparatus (LABINDIA, disso-2000, Mumbai, India) (US Pharmacopoea XX III, 1995) under sink conditions. Accurately weighed samples of microspheres (size fraction 355 μm) were added to dissolution medium and at preset time intervals 2 ml aliquots were withdrawn and replaced by an equal volume of fresh dissolution medium. After suitable dilution, the samples were analyzed spectophotometrically at 266 nm. The concentration of stavudine in test samples was corrected and calculated using a regression equation of the calibration curve (Narishetty et al. 2004).

Data obtained from *in vitro* release studies were fitted to various kinetics equations to find out the mechanism of drug release from microspheres. The kinetics models used were zero order equation, first order equation and Higuchi model. The following plot were made Q_t vs. t (zero order model), log (Q_0–Q_t) vs. t (first order model) and Q_t vs. $\sqrt{t}$ (Higuchi model) where Q_t is the drug release at time t and Q_0 is the initial amount of drug present in the microspheres. The rate constants were also calculated for the respective models.

Further to confirm the mechanism of drug release, the first 60% of drug release was fitted in Korsmeyer Peppas model (Korsmeyer et al. 1983)

$$M_t / M\alpha = Kt^n$$

where $M_t/M\alpha$ is the fraction of drug released at time t and k is the release rate constant and 'n' is the release exponent. The 'n' value is used to characterize different release mechanisms and is calculated from the slope of the plot of log of fraction of drug released vs. log of time.

Acknowledgement: The authors greatly acknowledge Hetero Labs Ltd, Hyderabad, India, for supply of stavudine as a gift sample. The authors are grateful to Indian Institute of Technology (IIT), Karagpur, India, University Science of Instrumental Centre (USIC), Jadavpur, Kolkata, India and Indian Institute of Chemical Biology (IICB), Kolkata, India, for help in performing characterization studies.

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