MATERIALS & METHODS
4. MATERIALS & METHODS

4.1 Materials

The materials employed in the present research are enlisted in Table 4.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Chemicals/Materials</th>
<th>Source/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polylactic-co-glycolic acid (PLGA 85:15)</td>
<td>Fluka, Buchs, Switzerland</td>
</tr>
<tr>
<td>2</td>
<td>Polylactic-co-glycolic acid (RESOMER® 505H)</td>
<td>Boehringer Ingelheim, Germany</td>
</tr>
<tr>
<td>3</td>
<td>Polylactic-co-glycolic acid (RESOMER® 504H)</td>
<td>Boehringer Ingelheim, Germany</td>
</tr>
<tr>
<td>4</td>
<td>Polylactic-co-glycolic acid (RESOMER® 502H)</td>
<td>Boehringer Ingelheim, Germany</td>
</tr>
<tr>
<td>5</td>
<td>Polyactic Acid (PLA)</td>
<td>Wockhardt R&amp;D, India</td>
</tr>
<tr>
<td>6</td>
<td>Poly-L-lactic acid (PLLA)</td>
<td>Wockhardt R&amp;D, India</td>
</tr>
<tr>
<td>7</td>
<td>Polycaprolactone (PCL)</td>
<td>Union carbide, India</td>
</tr>
<tr>
<td>8</td>
<td>Dichloromethane</td>
<td>Merck limited, Mumbai, India</td>
</tr>
<tr>
<td>9</td>
<td>Di-ethyl ether</td>
<td>Merck limited, Mumbai, India</td>
</tr>
<tr>
<td>10</td>
<td>Chloroform</td>
<td>Merck limited, Mumbai, India</td>
</tr>
<tr>
<td>11</td>
<td>Methanol for HPLC</td>
<td>Merck limited, Mumbai, India</td>
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<tr>
<td>12</td>
<td>Acetonitrile for HPLC</td>
<td>Merck limited, Mumbai, India</td>
</tr>
<tr>
<td>13</td>
<td>Ethanol</td>
<td>Merck limited, Mumbai, India</td>
</tr>
<tr>
<td>14</td>
<td>Phosphoric acid</td>
<td>Merck limited, Mumbai, India</td>
</tr>
<tr>
<td>15</td>
<td>Acetic acid glacial</td>
<td>Qualikems fine chemicals, New Delhi, India</td>
</tr>
<tr>
<td>16</td>
<td>Acetone</td>
<td>Loba Chemie, Pvt. Ltd., India.</td>
</tr>
<tr>
<td>17</td>
<td>Heparin</td>
<td>Gland Pharma Ltd., India.</td>
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<tr>
<td>18</td>
<td>Tween 20</td>
<td>Qualigens Fine chemicals, Mumbai, India</td>
</tr>
<tr>
<td>19</td>
<td>Tween 80</td>
<td>Qualigens Fine chemicals, Mumbai, India</td>
</tr>
<tr>
<td>20</td>
<td>Span 80</td>
<td>Qualigens Fine chemicals, Mumbai, India</td>
</tr>
<tr>
<td>21</td>
<td>Picric acid</td>
<td>S.D. Fine Chemicals Pvt. Ltd., Boisar, India</td>
</tr>
<tr>
<td>22</td>
<td>Stavudine</td>
<td>Panacea biotec, Lalru, Chandigarh</td>
</tr>
</tbody>
</table>
Materials & Methods

<table>
<thead>
<tr>
<th>No.</th>
<th>Material/Chemical</th>
<th>Manufacturer/Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Caroxymethyl cellulose CDH chemicals</td>
<td>India</td>
</tr>
<tr>
<td>24</td>
<td>Isopropyl alcohol</td>
<td>Fisher scientific, Mumbai</td>
</tr>
<tr>
<td>25</td>
<td>Water (HPLC)</td>
<td>Merck Limited, Mumbai, India</td>
</tr>
<tr>
<td>26</td>
<td>Macrophages cell line</td>
<td>J774 A1, P.G.I.M.E.R., Chandigarh</td>
</tr>
<tr>
<td>27</td>
<td>FACS buffer</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td>28</td>
<td>Triton X-100</td>
<td>Sigma chemicals Co., USA</td>
</tr>
<tr>
<td>29</td>
<td>Carbopol 971P</td>
<td>Noveon, Mumbai, India</td>
</tr>
<tr>
<td>30</td>
<td>Carbopol 71GNF</td>
<td>Noveon, Mumbai, India</td>
</tr>
<tr>
<td>31</td>
<td>Poly ethylene oxide</td>
<td>Union Carbide Corporation, Danbury, CT</td>
</tr>
<tr>
<td>32</td>
<td>Di-sodium hydrogen orthophosphate</td>
<td>S.D. Fine Chemicals Ltd., India.</td>
</tr>
<tr>
<td>33</td>
<td>Potassium di-hydrogen orthophosphate</td>
<td>S.D. Fine Chemicals Ltd., India.</td>
</tr>
<tr>
<td>34</td>
<td>Sodium hydroxide (AR) (NaOH)</td>
<td>S.D Fine Chemicals Ltd., India.</td>
</tr>
<tr>
<td>35</td>
<td>Micro crystalline cellulose</td>
<td>Ranbaxy Laboratories Ltd., Gurgaon, India</td>
</tr>
<tr>
<td>36</td>
<td>Ethyl cellulose</td>
<td>S.D. Fine chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>37</td>
<td>Magnesium stearate</td>
<td>S.D. Fine chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>38</td>
<td>Talc</td>
<td>S.D. Fine chemicals Ltd., Mumbai, India</td>
</tr>
</tbody>
</table>

4.2 Equipments

The equipments employed in the present research are enlisted in Table 5.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Equipment/Instruments</th>
<th>Manufacturer/Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Analytical Weighing Balance</td>
<td>Mettler Toledo, AG 285, Switzerland</td>
</tr>
<tr>
<td>2</td>
<td>Thermostatic water bath shaker</td>
<td>Narang Scientific Works, New Delhi</td>
</tr>
<tr>
<td>3</td>
<td>High speed mechanical stirrer</td>
<td>REMI Motors, Mumbai, India</td>
</tr>
<tr>
<td>4</td>
<td>Magnetic stirrer</td>
<td>REMI equipments, Mumbai, India</td>
</tr>
<tr>
<td>5</td>
<td>IKA Homogenizer</td>
<td>IKA-T18 Ultra Turrax, Brasil</td>
</tr>
</tbody>
</table>

*University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.*
<table>
<thead>
<tr>
<th>No.</th>
<th>Equipment Description</th>
<th>Manufacturer and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Cyclomixer</td>
<td>Remi Motors, Mumbai, India</td>
</tr>
<tr>
<td>7</td>
<td>Vacuum pump</td>
<td>Tarsons, Rockyvac 400, India</td>
</tr>
<tr>
<td>8</td>
<td>Laboratory Centrifuge(s)</td>
<td>Remi Motors, Mumbai, India</td>
</tr>
<tr>
<td>9</td>
<td>Scanning Electron Microscope</td>
<td>Hitachi, S-3400N &amp; JSM 6100 JEOL, Japan</td>
</tr>
<tr>
<td>10</td>
<td>Particle Size Analyzer</td>
<td>Master Sizer 2000, Malvern Instruments Ltd., UK</td>
</tr>
<tr>
<td>11</td>
<td>Optical Microscope</td>
<td>Olympus, CH-20i-Tr, Europe</td>
</tr>
<tr>
<td>12</td>
<td>Differential Scanning Calorimetry</td>
<td>Mettler Toledo STAR system, Switzerland &amp; Thermal Analysis Q 20, USA</td>
</tr>
<tr>
<td>13</td>
<td>Nikon Eclipse 80i with CCD camera</td>
<td>Nikon Corporation, Japan</td>
</tr>
<tr>
<td>14</td>
<td>Ultraviolet (UV) spectrophotometer</td>
<td>Thermo Spectronic, Genesys, USA</td>
</tr>
<tr>
<td>15</td>
<td>IR spectrophotometer</td>
<td>Perkin Elmer EM 360, USA</td>
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<tr>
<td>16</td>
<td>pH meter</td>
<td>L1-120 ELICO, Mumbai, India</td>
</tr>
<tr>
<td>17</td>
<td>Vortex mixer</td>
<td>Remi Equipments, Mumbai, India</td>
</tr>
<tr>
<td>18</td>
<td>Melting point apparatus</td>
<td>Veego, Mumbai, India.</td>
</tr>
<tr>
<td>19</td>
<td>NMR spectrometer</td>
<td>Bruker Avance 500 MHz DPX, Switzerland</td>
</tr>
<tr>
<td>20</td>
<td>Hot plate</td>
<td>Remi Equipments, Mumbai, India</td>
</tr>
<tr>
<td>21</td>
<td>X-Ray diffractometer</td>
<td>Panalytical-XPRT-PRO, Netherlands</td>
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<tr>
<td>22</td>
<td>Gas chromatographic system</td>
<td>GC-2014, Shimadzu, Japan</td>
</tr>
<tr>
<td>24</td>
<td>Flat-bottom tissue culture plates</td>
<td>Corning Incorporated, Corning NY, USA</td>
</tr>
<tr>
<td>25</td>
<td>CO₂ incubator</td>
<td>Forma Scientific Inc., USA</td>
</tr>
<tr>
<td>26</td>
<td>HPLC system</td>
<td>Alliance (Waters 2695) with PDA detector (Waters 2996)</td>
</tr>
<tr>
<td>27</td>
<td>Dissolution test apparatus</td>
<td>Labindia, Mumbai</td>
</tr>
<tr>
<td>28</td>
<td>Rotary tablet press</td>
<td>Cadmach, Ahmadabad, India</td>
</tr>
</tbody>
</table>
Materials & Methods

<table>
<thead>
<tr>
<th></th>
<th>Equipment</th>
<th>Manufacturer/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>Single punch tableting machine</td>
<td>Modern Engineering New Delhi, India</td>
</tr>
<tr>
<td>30</td>
<td>Roche friability tester</td>
<td>Tropical Lab Equipments, Mumbai, India</td>
</tr>
<tr>
<td>31</td>
<td>Monsanto hardness tester</td>
<td>Campbell Electronics, Mumbai, India</td>
</tr>
<tr>
<td>32</td>
<td>Extruder</td>
<td>Caleva Extruder 25, UK</td>
</tr>
<tr>
<td>33</td>
<td>Spheronizer</td>
<td>Caleva spherizer 120, UK</td>
</tr>
<tr>
<td>34</td>
<td>Particle coater</td>
<td>Pam- Glatt, Mumbai</td>
</tr>
</tbody>
</table>

4.3 Preformulation Studies

The class of studies which need to be successfully completed before actually the formulation development and optimization starts are termed as preformulation studies. These studies are important as the processes for optimizing the delivery of candidate drug through determination of physicochemical properties of drug that could influence drug performance and development of an efficacious, safe and stable dosage form [27]. These studies must resolve problems with drug analysis, stability, and pharmaceutical technology. Complete preformulation studies can decrease problems with instability of drug formulations during the shelf-life period caused by the selection of unsuitable excipients. The major parameters studied in this category are: characterization of the Active pharmaceutical ingredient (API), development of analytical methods, stability and compatibility studies of the drug with the excipients etc [28, 29]. It is through the proper completion of these preformulation studies that an optimal dosage form for desired therapeutic efficacy and utility could be designed.

Preformulation studies of stavudine were carried out, which included physicochemical characterization of drug (melting point, solubility, infra-red spectroscopy, particle size analysis, nuclear magnetic resonance spectroscopy, X-ray diffraction studies and differential scanning calorimetry). Analytical procedure was developed for estimation of the drug in microspheres and solvent system.
4.3.1 Characterization of the Drug

4.3.1.1 Ultra-Violet Spectra (UV)

One of the basic requirements of any preformulation study is the development of a simple and accurate analytical method for the quantitative estimation of the drug molecule. Ultra-violet spectroscopy involves the spectroscopy of photons in the ultraviolet region as the molecules undergo electronic transitions.

A stock solution of the drug was prepared (1 mg/ml) in water and diluted to a concentration of 10 µg/ml. The solution was scanned spectrophotometrically to determine the λ_max of the drug. The spectrum was recorded by Shimadzu-1601 spectrophotometer in the region of 200 to 400 nm. The absorption maximum was observed at 267 nm.

4.3.1.2 Infrared Spectrum (IR)

Infrared spectroscopy is widely used for identification of all types of organic and several types of inorganic compounds and determination of functional groups in organic materials. The chemical structural intactness of the pure drug was studied by recording the Fourier Transform Infrared Spectra (FTIR) of the drug using 60 MHz Varian EM 360 Perkin Elmer360 FTIR, USA. Potassium bromide pellets of the drug were scanned in the region of 4500 to 500 cm⁻¹.

4.3.1.3 Melting Point

Melting point of the drug was determined using the capillary method. Melting point was observed on a Veego’s melting point apparatus.

4.3.1.4 Solubility

The solubility of a drug molecule is an essential physicochemical parameter as it affects the rate of drug dissolution, thereby bioavailability of the drug, and thus, modulating the therapeutic efficacy of the pharmaceutical product. The solubility of stavudine was determined by the equilibrium solubility method, in 0.1 N HCl, water and phosphate buffer pH 7.4. Excess amount of the drug was added to 10 ml of the medium, and stirred continuously on a water bath shaker at 37°C for equilibration. The amount of drug was determined spectrophotometrically at absorption maxima of 267 nm, after filtering the samples through a 0.22 µm membrane filter and subsequent dilutions.
4.3.1.5 Particle Size Distribution

Particle size distribution affects various physicochemical properties of the drug substances. The effect is not only limited to physical properties of solid drugs but also in some cases on their biopharmaceutical behavior. The mean diameter and particle size distribution of stavudine was determined by the Laser diffractometry using Malvern mastersizer 2000 through dry method. The dry sample of the drug was kept in the sample holder and measured for the particle size of the same.

4.3.1.6 NMR Spectrum

Nuclear magnetic resonance (NMR) is an important preformulation tool which helps in detecting the purity of the sample. It's a property that magnetic nuclei comprise in a magnetic field and applied electromagnetic pulses, which cause the nuclei to absorb energy from the electromagnetic pulse and radiate this energy back out. NMR active nuclei (such as H or 13C) absorb at a frequency characteristic of the isotope when exposed to magnetic field. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field. Since it is a very selective technique, it is widely used in determination of the structure of several compounds. The NMR spectrum of stavudine was recorded in D2O using Bruker Avance II 400 NMR Spectrometer.

4.3.1.7 X-RAY Diffraction (XRD)

X-ray diffraction (XRD) is a multifaceted, non-destructive technique that reveals detailed information about the chemical description and crystallographic structure of natural and manufactured materials. It is based on the scattered intensity of an X-ray beam hitting the sample as a function of incident and scattered angle, polarization, and wavelength or energy. Powder X-ray diffraction pattern of stavudine was 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 20 angles of over 5-50°.

4.3.1.8 Differential Scanning Calorimetry (DSC)

The principal objective of drug/excipient compatibility studies is to quickly determine, possible interactions between the formulation excipients and the active
pharmaceutical ingredient. This is an important risk reduction exercise to be undertaken early in formulation development. Differential scanning calorimetry is the most widely used technique for compatibility studies.

As a part of preformulation studies, DSC was used to investigate the purity of the drug sample. The study was carried out using a calibrated Thermal Analysis Q20 differential scanning calorimeter. The sample was placed in a sealed aluminium pan and heated in the range of 20-400°C at an increment rate of 10°C/min, using an empty sealed pan as a reference and dry nitrogen as the purge gas.

4.3.2 Preparation of Standard Plots

4.3.2.1 Preparation of Standard Plots Spectrophotometrically

All the standard plots of the stavudine (d4T) were prepared in triplicate, unless otherwise stated. The $E_{\text{1cm}}^{1\text{%}}$ was calculated through the regressed line obtained from a plot between concentration and absorbance at selected wavelength (Literature value 265-270 nm).

(A) Standard Plot of Stavudine in Distilled Water

Known amount (10 mg) of drug was dissolved in 100 ml of distilled water to make stock solution of 100 μg/ml. Suitable dilutions were made to obtain the working standards in the range of 5-50 μg/ml and absorbance was taken at 267 nm.

(B) Standard Plot of Stavudine in Dichloromethane

Accurately weighed amount (10 mg) of drug was dissolved in 100 ml of dichloromethane to make stock solution of 100 μg/ml. Suitable dilutions were made to obtain the working standards in the range of 5-50 μg/ml and absorbance was taken at 264 nm.

(C) Standard Plot of Stavudine in PBS (pH 7.4)

Precisely weighed amount (10 mg) of drug was dissolved in 100 ml of phosphate buffered saline to make stock solution of 100 μg/ml. Suitable dilutions were made to obtain the working standards in the range of 5-50 μg/ml and absorbance was taken at 266.5 nm.
(D) Standard Plot of Stavudine in 6.8 Phosphate buffer

Known amount (10 mg) of drug was dissolved in 100 ml of 6.8 phosphate buffer to make stock solution of 100 μg/ml. Suitable dilutions were made to obtain the working standards in the range of 5-50 μg/ml and absorbance was taken at 266.5 nm.

4.3.2.2 Preparation of Standard Plot using HPLC

(A) Preparation of Analytical Standard

(i) Standard Stock Solution and Working Standards

100 mg of drug was dissolved in 100 ml of purified water. Suitable quantity (5.6 ml) of primary stock (1 mg/ml) was diluted to 250 ml with triple distilled water to make a secondary stock solution of 100μM. Suitable dilutions were made to obtain the analytical standards in the range of 0.1-100 μM. 20 μl of each concentration of analytical standard was directly injected to the chromatographic system to prepare the standard plot in distilled water.

(ii) Mobile Phase

Methanol: water: acetic acid (23:77:0.2) was used as mobile phase.

(iii) Flow Rate

The flow rate was 0.7ml/min.

(iv) Instrumentation

Samples were injected through auto-injector (Waters-SM7, C07SM7837A) with 20 μl loop to a waters quaternary gradient system manager separation modules (Waters 2695) HPLC system equipped with photodiode array detector (PDA)-Waters 2996. For chromatography, a C-18 reverse phase column (Hiber®, 250-4.6 Lichrospher® 100, RP-18e, 5μ, Merck, Germany) was used. The column was operated at ambient temperature. The wavelength of detection was set at 267 nm.
(B) Preparation of Calibration curve in Plasma

(i) Standard Stock Solution and Working Standards
100 mg of drug was dissolved in 100 ml of purified water. Suitable quantity (5.6 ml) of primary stock (1 mg/ml) was diluted to 250 ml with purified water to make a secondary stock solution of 100 μM. The desired calibration curve standards (0.1-100 μM) were prepared by spiking known amount of stavudine (working standards) to the blank plasma. 20 μl of each concentration of calibration standard was directly injected to the chromatographic system to prepare the standard plot in blood plasma.

(ii) Mobile Phase
Methanol: water: acetic acid (23:77:0.2) was used as mobile phase.

(iii) Flow Rate
The flow rate was 0.7 ml/min.

(iv) Sample Preparation
Spiked plasma (500 μl) of each concentration was mixed with 500 μl of 10% perchloric acid to precipitate the plasma proteins. The mixture was shaken for 30 seconds and centrifuged at 3000 X g for 10.0 minutes. The supernatant was transferred to microcentrifuge eppendorf tube and 20 μl was injected directly into the chromatographic system [267].

(v) Instrumentation
Samples were injected through auto-injector (Waters-SM7, C07SM7837A) with 20 μl loop to a waters quaternary gradient system manager separation modules (Waters 2695) HPLC system equipped with photodiode array detector (PDA); Waters 2996. For chromatography, a C-18 reverse phase column (Hiber®, 250-4.6 Lichrospher® 100, RP-18e, 5μ, Merck, Germany) was used. The column was operated at ambient temperature. The wavelength of detection was set at 267 nm.

4.4 Preparation of Stavudine Loaded Polymeric Microspheres
Various methods were tried for preparation of d4T loaded biodegradable polymeric microspheres. Different biodegradable polymers with their specific grade
Materials and Methods

In accordance with their peculiar inherent viscosities were used. Polycaprolactone (PCL), Poly (DL-lactide-co-glycolide) (PLGA) (85:15), Resomer® 505H, Resomer® 504H, Resomer® 502H (PLGA 50:50), Poly lactic acid (PLA), Poly (L)-Lactic Acid (PLLA) were used in order to encapsulate maximum amount of drug into the microsphere systems [147]. These polymeric systems were explored to encapsulate and tailor the release of the drug from the biodegradable matrix of microspheres to provide maximum therapeutic benefits.

4.4.1 Polycaprolactone (PCL) Microspheres

For the purpose of optimization of preparation method for biodegradable polymeric microspheres; blank microspheres of PCL were prepared by o/w emulsion method. A known amount of polymer was dissolved in sufficient quantity of dichloromethane and the solution of polymer was added drop wise in 1% polyvinyl chloride (PVA) solution (25-30 cps) [37]. PVA solution acts as stabilizer as well as external phase of the preparation system. The mixture was stirred mechanically for two hours at 1000 rpm followed by stirring over magnetic stirrer for total solvent removal [52]. The emulsion formed was observed during the progress of the process (Fig. 27). The observation revealed the presence of spherical shaped microsphere system into the external stabilizer phase. The regidized microspheres were washed thoroughly with water and collected by filtration through vacuum pump followed by air drying [150].

After optimization of the preparation method, drug loaded microspheres were prepared. They were formulated keeping all other parameters constant with a drug to polymer ratio of 1:1. The drug was dispersed in PCL solution in dichloromethane and added to the 1% PVA solution. Spherical, smooth surfaced microspheres of PCL were formed but due to excessive solubility of stavudine in water, no significant drug encapsulation was obtained [199, 201].

Keeping water solubility of d4T in consideration, preparation method of polymeric microspheres was switched to multiple emulsion method (w/o/w) to ensure the higher drug incorporation into polymeric matrix of microspheres (Fig. 10) [46]. For the initial emulsification step the drug solution was added to polymer solution in dichloromethane at high speed homogenization for 1-2 min to form w/o emulsion. The prepared primary emulsion was added to aqueous PVA solution (1%)
which exhibited the role of final external phase of multiple emulsion. The mixture was mechanically stirred at high speed for few minutes and then after attaining the desired size range of the microspheres switched to lower speed, followed by stirring over magnetic stirrer till complete evaporation of solvent [48]. But, this technique also resulted in low entrapment.

Another technique, w/o/o emulsion technique was also tried for microsphere preparation. In this method, drug was dissolved in water and added to polymeric solution of PCL into dichloromethane at high speed homogenization to form w/o emulsion [50]. This primary emulsion was added to liquid paraffin phase with 2% Span 80 (w/w) to form secondary emulsion (w/o/o) under mechanical stirring. The stirring was continued on magnetic stirrer till complete evaporation of solvent. This trial was first conducted at drug/polymer (D/P) ratio of 1:1 and later on at D/P ratios of 1:2 and 1:4. Though D/P ratio of 1:4 did result in better entrapment as compared to D/P ratio of 1:1, but the entrapment was still not good enough to further proceed with this method. This method of multiple emulsion also did not resulted in better encapsulation of the drug but certainly has given an insight that the methods involving o/o systems should be preferred for entrapment of such a highly water soluble drug as better entrapment was observed than the above methods.

Figure 10: Preparation of microspheres by w/o/w solvent evaporation technique.
Materials & Methods

O/o emulsion method for preparation of biodegradable polymeric microspheres was selected for encapsulating maximum amount of highly water soluble drug inside the polymeric matrix. In the o/o emulsion technique PCL was dissolved in sufficient quantity of dichloromethane and the drug was dispersed into polymeric solution (D/P ratio 1:1) [43]. The prepared internal phase was added drop wise to the silicon oil with 2% span 80 (w/w), which acted as external phase of the emulsion system (Fig. 11). This method did not result in the formation of microspheres but some lumps were formed [46].

Silicon oil as external phase was replaced with mixture of heavy and light liquid paraffin (with 2% span 80) as the external phase of the emulsion system. The drug was dispersed in polymeric solution (D/P ratio 1:1) in DCM and was added gradually during stirring in the paraffin phase (mixture of heavy and light paraffin; 35 ml: 15 ml) and stirring continued on mechanical followed by magnetic stirrer till complete evaporation of solvent [50].

This method did result in formation of microspheres but with low entrapment leading to further trials with change in D/P ratio. Batches prepared by using D/P ratio of 1:4 resulted in significant improvement in percent yield with reasonably good encapsulation efficiency. Higher polymer ratio led to formation of sticky mass rather than formation of microspheres due to higher viscosity of polymeric solution.

Figure 11: Preparation of microspheres by o/o solvent evaporation technique.

University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.
4.4.2 PLGA 85:15, RESOMER® 505H, RESOMER® 504H, RESOMER® 502H, PLA and PLLA Microspheres

O/o solvent evaporation technique with D/P ratio of 1:1 was employed for the preparation of PLGA 85:15 microspheres. The method which was previously used successfully in case of PCL did not result in formation of microspheres. Optimization trials for fixing the external phase were taken using several oils other than mineral oil like groundnut oil, castor oil and sesame oil. Out of which sesame oil with 2% span 80 (w/w) exhibited the best results when used as liquid manufacturing vehicle [147].

Further drug polymer ratio was varied to obtain good shape, yield and entrapment efficiency. Although the stated method was able to produce microspheres but the encapsulation efficiency was low and varied in accordance with the enhanced D/P ratio [167]. The photomicrographs of in process samples showed that even on increasing the polymer content the drug was not encapsulated and needle like drug crystals were observed outside the microsphere matrix into the manufacturing vehicle (Fig. 28).

This phenomenon might be attributed to the lesser boiling temperature of dichloromethane; which evaporates quickly leaving the drug crystals unentrapped into the external oil phase. The troubleshooting was performed by taking several trials with different organic solvents like acetone, chloroform, dichloro methane and acetonitrile. Out of which acetonitrile was found to be the most suitable organic solvent for successful and efficient preparation of biodegradable polymeric microspheres of Resomers and PLA, whereas dichloromethane was used as organic solvent for preparation of microspheres of PLLA [223].

After finalizing the external phase and organic solvent; microspheres of PLGA (85:15) were formed with proper shape and size and smooth surface topography. These microparticles have provided reasonably good percentage yield but the encapsulation of d4T was still not enough, that may be attributed to low drug to polymer ratio (D/P ratio 1:4). The polymer proportion cannot be increased more, as the drug to polymer ratio of 1:4 has given the higher mean particle size because of higher inherent viscosity of the polymer (1.3-1.7 in 0.1% chloroform at 25 °C) [147]. Increment in polymer content may further increase the particle size which was not desirable.

Stavudine loaded biodegradable polymeric microspheres were prepared using PLGA 85:15, RESOMER® 505H, RESOMER® 504H, RESOMER® 502H, PLA and PLLA. The technique of emulsification followed by solvent evaporation was used for the
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preparation of an o/o emulsion, using acetonitrile/dichloromethane as organic solvent and sesame oil as liquid manufacturing vehicle with 0.5, 1.0 or 2% of span 80 as surfactant (w/w). The emulsion was subjected to stirring for period of hours using mechanical stirrer (Remi-RQT 124A) at 7000 rpm and subsequently magnetic stirring (Remi-5MLH-DX) of period of 8 hours. Formulation variables like different drug/polymer ratio (1:4, 1:10, 1:20, 1:50, 1:100) and a polymer solution/vehicle volume ratio of 1:2 were optimized for suitable size and higher drug loading [147]. The drug/polymer concentrations were represented as w/w, keeping the constant drug concentration with variable polymer concentrations. The prepared microspheres were filtered, washed with excess of distilled water and n-hexane to remove the unentrapped drug and oil and finally dried.

4.5 Characterization Studies

The biodegradable polymeric microspheres were characterized for yield, particle size, entrapment efficiency, 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).

4.5.1 Determination of Entrapment efficiency and Percentage Yield

Stavudine loaded microspheres (10 mg) were dissolved in 1.0 ml of dichloromethane followed by addition of 5.0 ml of water. The solution was subjected to vortexing using cyclo mixer. Clarification of the resultant solution was facilitated by allowing it to stand for few minutes and then upper layer (aqueous) was analyzed for stavudine spectrophotometrically at 267 nm. The percent yield and entrapment efficiency was calculated using the Equations (1) and (2), respectively. Suitable blank was used to exclude possible interference of other microsphere components.

\[
\text{% Yield} = \frac{\text{Weight of microspheres prepared}}{\text{Total weight of drug and polymer}} \times 100 \quad [1]
\]

\[
\text{% EE} = \frac{\text{Mass of Incorporated drug}}{\text{Mass of drug used in formulation}} \times 100 \quad [2]
\]
4.5.2 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is one of the advanced techniques for the characterization of the surface morphology of a microsphere with much higher resolution. SEM allows investigation of microsphere surface topography, texture and morphology of fractured or sectioned surfaces. The SEM has a resolution of 3 nm and provides magnifications ranging from less than 30 times to 300,000 times. Scanning electron microscopy is an essential tool for physical observation of morphological features of microspheres. It provides an edge to examine microspheres shape and surface characteristics [147].

The surface morphology of the microspheres was investigated using SEM. Microspheres, in dried form, were mounted onto metal stubs using double-sided adhesive tape. Subsequently, the stubs were vacuum coated with gold using fine coat ion sputter (Hitachi-E-1010 & JFC 1100, JEOL Japan) under reduced pressure to render them electrically conductive. Then the microspheres were examined with SEM (Hitachi, S-3400N & JEOL JSM 6100, Tokyo, Japan) [50]. The accelerating voltage was kept constant initially at 15.0 KV then further reduced to 5.0 KV under an argon atmosphere. To evaluate the effect of release medium on the microspheres with time, they were placed in release medium (PBS, pH 7.4) and samples of microspheres were collected at varied time intervals of 7, 15 and 30 days and observed by SEM [51, 52].

4.5.3 Size Distribution Analysis

Particle size is a variable having significant importance in determining the parameters like rate of dissolution, rate of drug release, syringeability/injectablility and dose delivery. Laser light scattering methods for particle size analysis have shown its potential because they allow quick and absolute determination of particle size without the need for calibration. Broad particle size distributions of 0.1-1000 μ can easily be measured with instruments based on laser diffraction principles [14]. All these methods require dilute concentrations where interactions between particles and secondary scattering are minimized, although this must be balanced against the requirement for enough sample to obtain a sufficient signal to noise ratio. The mean particle size and particle size distribution were analyzed by laser diffractometry using Malvern Mastersizer 2000. For the measurements, microspheres were suspended in distilled water with 0.1 % biDCC
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tween 80, which was homogenized at 10,000 rpm and sonicated for 60 seconds prior to particle size determination [147].

4.5.4 Differential Scanning Calorimetric Studies (DSC)

Differential scanning calorimetry (DSC) is the most widely and frequently used calorimetric technique for the determination of various thermal parameters, which allow a better understanding of drug-polymer interactions as well as incompatibilities, drug excipient interactions and thermal denaturation of polymers. DSC measures the heat capacity of the system as a function of temperature and following the change in heat capacity of the sample it allows the detection of phase transitions of various orders [52-54].

DSC studies of drug, polymer and microspheres were carried out in an attempt to define physical state of drug in these carriers and possibility of interaction between the drug and polymer within the network of polymeric matrix in microspheres. Small amount of microsphere samples were placed in hermetically sealed aluminium pans and heated from 20°C to 400°C at a heat flow rate of 10°C min⁻¹ under nitrogen spurge of 50 cm³ min⁻¹ (Mettler Toledo STAR system, Switzerland). The glass transition temperature (Tg °C) and melting point of samples were recorded as endotherms.

4.5.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is a basic technique, not only used for determination of functional groups in organic compounds but also for characterization/identification of various inorganic compounds as well. The technique provides precise information regarding the structural configurations with its chemical intactness as well as the purity of the sample. The Fourier transform infrared (FTIR) spectra were recorded for the drug loaded polymeric microspheres using 60 MHz Varian EM 360 Perkin Elmer 360 FTIR, USA, using potassium bromide pellets in the scanning region of 4500 to 500 cm⁻¹. The obtained spectrum was compared with the spectra of pure d4T as well as corresponding different biodegradable polymers to conclude the structural integrity of stavudine inside the polymeric matrix mesh of the microsphere systems [43, 46].
4.5.6 X-ray Diffraction Studies (XRD)

X-ray diffraction (XRD) is an advanced and potential technique based on the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy. The versatility and non-destructive operational procedures of the technique which not only reveals the detailed information about the chemical composition but also exhibits the crystallographic structure of the raw samples with its finished products as well [43, 50].

Powder X-ray diffraction patterns of stavudine loaded microspheres, pure d4T and respective polymers were recorded using 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 20 angles of over 5-50°. The recorded diffractograms were compared with reference to their chemical composition and crystal habit.

4.5.7 Residual Solvent Analysis

Residual solvents are the volatile organic chemicals which are either used or produced during the manufacturing of drug substances or formulation development. Most of these volatile organic compounds generally cannot be removed completely through standard manufacturing procedures and traces of those chemicals are left behind within the formulation. Until the traces of residual solvents verified and quantified for their hazardous effects; the formulation could not be used for human use as high levels of residual organic solvents represent a risk to human health because of their toxicity [147]. Apart from the toxic effects; residual solvents can create foul odor as well as discoloration to the final product which not only lead to patient intolerability but also generate concerns at economic fronts.

Residual solvents could be classified in three main categories on basis of two potential characters foremore is their toxicity level and the further is the degree to which they can be considered an environmental hazard. The three main classes involve; Class I solvents which are supposed to be the most toxic and should be avoided e.g. benzene, carbon tetrachloride etc [147, 176, 177]. Class II solvents are considered at lesser risk but they should be limited in pharmaceutical products because of their inherent toxicity, for example, acetonitrile, dichloromethane etc. Class III solvents are at the lowest risk category which includes; acetic acid, pentane etc. The concentration limits for class I solvents is generally between 2-8 ppm whereas for Class II solvents limits vary from 50-
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4000 ppm. Class III solvents require only GMP based testing and are limited to 5000 ppm or 0.5% w/w [147].

For any microsphere formulation to get approved by Food and drug administration (FDA), it is mandatory to consider regulatory requirements of residual solvents content in microspheres. More or less all microsphere manufacturing processes require the use of an organic solvent such as chloroform, acetonitrile, dichloromethane, ethyl acetate etc [180, 193]. for polymer dissolution; which may pose considerable health risks on protracted exposure. At the front of regulatory authorities, they certainly require that formulator should attempt to minimize the residual solvents as much as possible.

Based on recommendations of ICH and Pharmacopoeial (USP) limits of residual solvents; authorities claim certain quantitative limits for every organic solvent for their residual content as per the classificational categories [201]. Presence of higher levels of residual solvents may not only generate clinical hazards but also affect the stability of the encapsulated drug as well. Increment in residual solvent content in many final dosage forms may alter the glass transition temperature of the polymers; causing microspheres to agglomerate under storage; ultimately leading to poor syringeability/injectablility [213, 220].

Residual solvents identification as well as quantification could be performed through various analytical methods; out of which the most popular and most appropriate, specific residual solvent analytical method is gas chromatography (GC). GC has the ability to separate component solvents, thus identifying them, and is capable of low detection limits when the appropriate detector is used. FID (flame ionization detector) is the most widely used detector for GC because of its low detection limits, wide linear dynamic range, and general reliability and utility, especially for trace organic compounds. The preferred methodology for residual solvent analysis is through head space sampling [147].

Two types of head space sampling procedures are available; one is dynamic and the other is static. Dynamic head space sampling uses a trap to concentrate volatile residual solvents before analysis by a gas chromatograph; whereas static head space sampling takes a volume of gas from the headspace above the heated sample vial directly to a gas chromatograph for analysis. Static head space analysis is probably the most extensively employed practice for analysis of residual solvent in pharmaceuticals. If the pharmaceutical samples are soluble/extractable in water, dimethylformamide (DMF),

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dimethylsulfoxide (DMSO), benzy! alcohol the satatic method is more preferred rather than dynamic technique [176, 177, 180].

Residual solvent analysis was performed using gas chromatography. The gas chromatograph using GC Solution was calibrated by the internal standards and both LOD and LOQ were computed. To ascertain the amount of acetonitrile/dichloromethane in microspheres, known weight of microspheres were taken in head space vial and chromatograms were obtained through gas chromatography (GC-2014, Shimadzu). The detector in combination with column used for the analysis was; flame ionization detector (FID) and DB-624, 30 m x 0.25 mm (fused silica, 1.0 μm film) respectively [147].

4.5.8 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscope (CLSM) is a technique mostly used for achieving high resolution optical images. The important feature of the CLSM is its ability to produce in focus images of thick specimens. Confocal microscopy provides a substantial improvement in lateral resolution and also used to procure an exclusive approach to investigate the internal structure of the microspheres and drug distribution pattern as a nondestructive visualization technique [147].

The method based on fluorescent imaging with a Carl Zeiss LSM 510 confocal laser scanning microscope (CLSM, Carl Zeiss Micro imaging, Inc., Thornwood, NY) was used. The microspheres were prepared using rhodamine as well as coumarine having fluorescence spectrum of 525 nm (excitation)-555 nm (emission) and 458 nm (excitation)-540 nm (emission) respectively [272]. The stavudine with the particular dyes was used in the drug/polymer ratio of 1:10, which was low enough to prevent quenching of the fluorescent labeling.

Analytical instrument equipped with an Enterprise UV laser and a Carl Zeiss inverted Axiovert 100 M microscope was employed for visualization of permanent slide of these labeled and dried microspheres [273]. The fluorescent dye was excited by UV laser at 525 nm along with filters (450 nm, 520 nm) were used in combination with a Plan-Neofluar 100X oil immersion objective lens with numerical aperture of 1.2 to build images. The laser power and detection gain were set at 150 μW and 710 units respectively. The pinhole was 170 μm, which resulted in an optical slice of less than 2.5 μm. The images were scanned by 16 bit plane mode at a scan speed of 6.40 μs/pixel and the image size was 512×512 pixels [147, 274].
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4.6 Evaluation of Microspheres

Not only the parametric quality but also the characteristic behavior of the prepared biodegradable polymeric microspheres was justified through the characterization studies. The microparticles were further evaluated on certain specific parameters.

4.6.1 In-vitro Drug Release Studies

The release from the microspheres is dependent both on diffusion as well as polymeric degradation of the matrix. The possible mechanisms of drug release include; initial burst release contributed through the drug adhered over the microsphere surface, release through the pits and pores which is dependent on microspheres structure [14], diffusion through the intact polymer barrier, which depends on intrinsic polymeric properties as well as its core solubility, diffusion through a water swollen barrier which is governed through polymer hydrophilicity, which in turn depends on molecular weight of the polymer, erosion and bulk degradation of the polymer [41,44]. The release of active from the polymeric matrix is mostly affected by the rate of erosion and hydrolysis of polymer chains, leading to formation of channels in polymeric matrix mesh.

Known weight of microspheres were suspended in 1.5 ml of PBS (phosphate buffered saline; pH 7.4) in eppendorf tubes. The tubes were placed in incubator shaker (37°C) at 50 rpm or strokes [147]. At defined time intervals, samples were centrifuged at 7000 rpm at room temperature. Supernatants were withdrawn and replaced with fresh PBS for sustaining the sink conditions till completion of the dissolution study. Stavudine was estimated in supernatants spectrophotometrically at 267 nm [147].

4.6.2 Drug Release Kinetics

To investigate the release kinetics of the final formulation, data obtained from in vitro drug release studies were fitted in various kinetic models: Zero order (Equation 3) as cumulative amount of drug released vs time, first order (Equation 4) as log cumulative percentage of drug remaining vs time and Higuchi’s model (Equation 5) as cumulative percentage of drug released vs square root of time.

A. Zero order rate:

\[
C = K_0 t
\]  

[3]
Where \( K_0 \) is the zero order rate constant expressed in units of concentration/time
and \( t \) is the time in minutes.

**B. First order equation:**

\[
\log C = \log C_0 - \frac{Kt}{2.303}
\]  

[4]

Where \( C_0 \) is the initial concentration of drug, \( K \) is the first order constant and
\( t \) is the time.

**C. Higuchi model:**

\[
Q_t = Kt^{1/2}
\]  

[5]

Where \( Q_t \) is the amount of drug release in time \( t \), \( K \) is the kinetic constant and
\( t \) is the time in minutes.

**D. Hixson-Crowell cube root law:**

\[
Q_{0}^{1/3} - Q_{t}^{1/3} = K_{HC}t
\]  

[6]

Where \( Q_0 \) is the initial amount of the drug in the formulation, \( Q_t \) is the amount
of the drug released at time \( t \) and \( K_{HC} \) is the Hixson-Crowell rate constant (Equation 6).

**E. Korsmeyer-Peppas equation:**

To assess the mechanism of drug release from the stavudine loaded microspheres,
drug release profile was plotted with Korsmeyer-Peppas equation as log cumulative
percentage of drug released vs log time (Equation 7). The release exponent \( n \) and \( K \) value
were calculated from the slope of the straight line.

\[
\frac{M_t}{M_x} = Kt^n
\]  

[7]

Where \( M_t \) represents amount of the released drug at time \( t \), \( M_x \) is the total
amount of drug released after an infinite time, \( K \) is the diffusional characteristic of
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drug/polymer system constant and ‘*n*’ is an exponent that characterizes the mechanism of drug release. If the exponent ‘*n*’ = 0.5 then the drug release mechanism is Fickian diffusion, if ‘*n*’ < 0.5 the mechanism is quasi Fickian diffusion, if ‘*n*’ = 0.5-1.0 then it is non Fickian or anomalous diffusion, if ‘*n*’ =1.0 mechanism is non-Fickian case II diffusion and if ‘*n*’ > 1.0 mechanism is non-Fickian super case II.

### 4.6.3 Stability Studies

Stability testing of biodegradable polymeric microspheres on storage is of great concern as it is major resistance in the development and scale up of marketed preparations [147, 148]. It is an important issue to be taken care in order to evaluate and demonstrate the patient safety, clinical as well as therapeutic efficacy, and quality of the drug which is to be maintained during its maximal time of storage and intended use. The theme of stability testing is to provide detailed evidential data on variation of the quality of a drug substance or drug product with due course of time under the influence of storage conditions. The generated stability data is always an asset as it accomplishes to establish the recommended storage conditions and shelf-life [15, 37].

For evaluation of storage stability, biodegradable microspheres of different polymers including Poly-Lactide-co-glycolide (PLGA), Polylactic acid (PLA) and Poly-L-lactic acid (PLLA) with a fixed drug to polymer ratio (1:50) were sealed in vials (10mL capacity) and stored at 2-8 °C and 25±2 °C for predetermined period of time (30, 60 and 90 days). [209] The stability of polymeric microspheres were assessed by monitoring the shape and surface topography with texture and morphology of fractured or sectioned surfaces by scanning electron microscopy (SEM). Size of the stored microspheres as well as tendency of agglomeration on storage was investigated through particle size distribution using Malvern Mastersizer 2000. Differential scanning calorimetry (DSC) was performed to find out any incompatibility between the drug molecule and the polymer network on storage whereas percentage residual drug content was also calculated by investigating the percentage entrapment efficiency over specified duration of time [275-281].

### 4.6.4 Ex-vivo Evaluation

Other than in vitro evaluation the microspheres were evaluated ex-vivo. The evaluation criteria includes cellular uptake/engulfment studies which are certainly a
significant parameter for microsphere system which need to be targeted to destroy the hidden HIV form their reservoir sites. The engulfment studies itself generate a demand for the cytotoxicity studies or the cell viability studies in concurrent combination of hemolysis studies as these are the criteria which need to be fulfilled before the microparticles are directed for the targeting the host reservoirs [60].

Histopathological studies were also performed to rule out any possibility of tissue level toxicity as the biodegradable polymeric microspheres were used as parenteral depot system for sustaining the d4T for extended period of time to avoid the plasma level fluctuation of stavudine for minimized adverse effects and enhanced therapeutic benefits in maintaining the viral load to undetectable levels leading to improvement in quality of life of the patient with HIV.

(A) Cell Viability/Cytotoxicity Studies

The biodegradable polymeric microparticulate systems were developed for targeted delivery of stavudine towards macrophages for complete eradication of HIV from their host reservoirs which suggest the need for cellular toxicity studies [169]. Cell viability or cytotoxicity studies with determination of subtoxic concentration were performed using J774 A1 macrophages cell line which was maintained as an adherent culture in humidified atmosphere supplemented with Fetal Bovine Serum (FBS) and antibiotics in a humidified 5% CO₂/air atmosphere at 37±2°C [170, 171].

For experiments, cells were detached mechanically and adjusted to the required concentration of viable cells by enumeration in a hemocytometer. Flat-bottom tissue culture plates (Corning Incorporated, Corning NY, USA) with 96 wells were used for the experiment. One-hundred microlitres of 10% Roswell Park Memorial Institute (RPMI) medium was added in all the 96 wells of the flat-bottom tissue culture plates.

Drug loaded biodegradable polymeric microspheres as well as free stavudine (d4T) were exposed to ultraviolet radiations for 30 minutes for sterilization to avoid any possible contamination. To the second row of columns II, III, IV, V and VI, 200 µL of free d4T solution and equivalent amount of d4T loaded biodegradable polymeric microspheres suspension were added respectively [269]. From the second row of each column, 100 µL of sample was transferred to the third row, then 100 µL from the third row to the fourth row, and likewise serial dilutions were made until the last row (each well contained 5000, 2500, 1250, 625, 312.5, 156.2, 78.1, 39.0, 19.5 and 9.75 µM).
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J774 A1 cell suspension (100 µL; 0.1×10^6 cells/100 µL) was added in each well and mixed thoroughly. The plate was incubated at 37±2°C for 2 days in a CO₂ incubator (Forma Scientific Inc., USA). On the second day post infection, cell toxicity was determined by the quantitative scores obtained through [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay [270]. MTT assay is an established calorimetric method based on determination of cell viability, utilizing the reaction of a water soluble tetrazolium salt yielding a yellowish solution, to each culture being assayed. MTT is converted into an insoluble purple formazan dye by mitochondrial dehydrogenase enzymes. Only active mitochondrial dehydrogenases of living cells will convert MTT into the insoluble purple formazan dye (Fig. 12).

After completion of incubation period and introduction of absolute isopropanol, cells are lysed and the precipitated formazan is dissolved. Formazan concentrations are quantitatively determined by measuring the optical density (OD) at 570 nm with background correction of the OD at 690 nm. All the experiments were performed in triplicate using separate 96 well U bottom tissue culture plates for reproducibility [271].

Figure 12: Well plate used for MTT assay for cytotoxicity study of stavudine loaded microspheres of RESOMER® 505H.

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(B) Cellular Internalization/Uptake Studies

The extended release biodegradable microspheres could also be used as targeted drug delivery systems for achieving better therapeutic benefits as these MS systems will be treated as foreign particles by the immune system of the body which will thereby stimulate the phagocytic cells undergoing phagocytosis and inducing host defense reactions [60, 169].

Upon particle phagocytosis, the mononuclear phagocytic system (MPS) increases the production of cytokines and reactive oxygen intermediates (ROI), which are involved in host defense mechanism. Thus, in addition to selective drug delivery for the HIV virus infecting the macrophages the ingestion of microparticles may result in the activation of the same for longer duration and, subsequently, enhance the host defense functions of the immune system; which is the ultimate goal of the therapy [59].

In current trends flowcytometry based assays have been proposed to access cell-microparticle associations by qualitatively measuring the increase in the sideward scattering of cells incubated with non fluorescent particles. Novel qualitative measurements are based on the fact that after internalization of the microparticles, the granularity of the cells increases, which concomitantly increases the sideward scattering intensity. Differentiation of side scattering patterns for the microparticles that bind on the cells to those that are internalized by the cells could be done by combining the light microscopy with flowcytometry [60, 270].

J774 A1 macrophages cell line was employed for phagocytic uptake studies of PLGA microspheres. The cells were maintained in complete medium consisting of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 10% heat inactivated Fetal Calf Serum (FCS) and 0.1% antibiotic-antimyotic solution and incubated at 37±2°C and humidified atmosphere of 5% CO₂/air [60]. For the experiments, macrophages were washed and detached mechanically. They are collected by centrifugation and counted by Trypan blue exclusion; then 2×10⁵ cells in 400 μL of complete medium were added per well into required number of plates and incubated overnight to adhere; then washed with phosphate-buffered saline pH 7.4 to remove non-adherent cells and FCS [169-171]. Specified quantity of drug loaded microspheres was utilized in accordance with the predetermined subtoxic concentration of the drug. Both were exposed to ultraviolet radiations for 30 minutes for sterilization to avoid any
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possible contamination before preparation of free d4T solution and suspension of the drug loaded biodegradable polymeric microspheres.

Firmly adhered J774 A1 macrophages cells were co-incubated with sterilized polymeric microspheres of Resomer 505H at the concentration of 100 μM, 75 μM, 50 μM, 25 μM, 10 μM and 1 μM equivalent to pure d4T for predetermined time period to allow phagocytosis of the particles by macrophages cell line and analyzed after the complete tenure of the experiment [59]. At the end of incubation duration the cells were harvested, excess of formulation along with cell surface bound and non-ingested microspheres were removed by aspirating the media and washing five times with ice-cold Phosphate Buffered Saline (PBS) containing 20 mM Ethylene Diamine Tetra Acetate (EDTA) and resuspended in Flow Cytometry Staining (FACS) buffer (BD Biosciences, USA). Simultaneously the cells were fixed with methanol for 5 min and stained with giemsa for 45 min at the same time intervals for light microscopy (Nikon). Excess dye was washed off with PBS [60, 170].

Cell internalization was then measured with a flow cytometer (BD FACS Calibur, USA) in accordance with the increment in cell granularity as well as cellular density. For each sample 10,000 events were collected. The obtained shifted scatter patterns due to uptake of MS were revealed through dot plots as well as supported by the oil immersion microscopy at 1000X magnification; which have discriminated the internalized as well as cell surface bound microparticles [60, 270].

Microsphere uptake by macrophages was calculated by counting phagocytic cells by light microscopy at 200X magnification. MS were assayed in triplicate and in each well 100 macrophages were evaluated. Results were expressed as percentage of macrophages that had ingested at least one MS as well as phagocytic index (PI) i.e. the mean number of particles ingested per macrophage.

(C) Hemolysis Studies

The use of biodegradable polymeric microparticulate systems for parenteral delivery is subjected to hemolytic toxicity studies. Rat blood was collected in centrifuge tubes which have heparin as an anticoagulant and undergo centrifugation at 2500 rpm for 10 min for pellet formation. Cells were washed, thrice with 30 ml of phosphate-buffered saline (pH 7.4) [59, 60]. The third supernatant was clear and colorless. One milli liter of hematocrit was suspended in 100 ml of PBS and stored at 48°C. For hemolysis test, 1 ml
of the hematocrit suspension in PBS was mixed with 1 ml of the test compound (d4T) at concentrations of 5000, 2500, 1250, 625, 312.5, 156.25, 78.125 µM as well as the formulation containing the above equivalent drug concentration.

Similarly the equivalent drug solution was also mixed with the hematocrit suspension. These mixtures were incubated in shaker bath shaking at 100 strokes per minute for 1 h at 37°C followed by centrifuged at 2500 rpm for 10 min. The resultant sample was subjected to measurement of optical density (OD) at 540 nm [269-271]. Negative and positive controls were prepared by incubating 1ml PBS and hematocrit suspension with 0.1% w/v Triton X-100 in Elga respectively. The assays were carried out in triplicate. Percentage hemolysis was calculated by the following formula.

\[
\text{Hemolysis} (\%) = \frac{\text{AS} - \text{AN}}{\text{AP} - \text{AN}}
\]

Where, AS, AP, and AN are the OD\textsubscript{540} of sample, positive control, and negative control, respectively.

**D) Histopathological Studies/In-vivo Biocompatibility**

The evaluation of the histological changes as well as biocompatibility of injectable biodegradable polymeric microspheres system requires an understanding of the inflammatory and healing responses in accordance to the delivery systems. The tissue or cellular host responses to injury are generally indicated by inflammation, foreign body responses and wound healing [35]. The response to injury is initiated by injection of the formulation within a solvent vehicle in the case of microspheres. Inflammation and healing are produced by the activation of humoral and cellular mechanisms. The rate of biodegradation may be governed by porosity of the microspheres. This may be crucial in catalyzing the rate of the process, especially when the pore dimensions are sufficiently large enough to permit cellular migration into the pores of the microsphere. Porosity may also improve the diffusion of oligomers and low molecular weight degradation products which are having carboxylic chain ends may facilitate the autocatalytic degradation of the polymer [36].

Microscopic observations of the phagocytic studies of the microspheres have shown that the phagocyted microspheres were gradually degraded within the macrophage interior with incubation time. The rate of microsphere degradation in the cells was controlled by changing the monomer composition of the copolymers and
molecular weight. Homogeneous hydrolysis resulted in degradation and resorption which reduces the size of the larger microparticulate systems to a point where they become vulnerable to phagocytosis by macrophages and foreign body giant cells which led to the foreign body reaction surrounding the larger microspheres [35-37]. The degradation of microspheres with a size range favorable to phagocytosis will be rapid and most probably much greater than that observed for the smaller microparticulate systems.

The acid and other agents produced by macrophages and foreign body giant cells present at the interface of the biodegradable microsphere can facilitate the biodegradation process. The interfacial pH between the macrophages, foreign body giant cells and the surface of biodegradable polymeric microsphere may be much lower than buffered physiological systems (approximately 7). Phagolysosomal vacuoles within macrophages have been identified as having a pH as low as 3. Thus, cells of the foreign body reaction may produce acidic concentrations which are comparatively more acidic than extracellular fluid [38].

For the experiment revealing the in vivo biocompatibility and histological changes with due course of time, biodegradable polymeric microspheres were administered to female SD rats. Animals were divided into three groups (n=3), the animals were anaesthetized with diethyl ether and skin was shaved off. The blank polymeric microspheres of Resomer 505H with a dose of 10 mg/kg were injected subcutaneously at the dorsal side of the animals after constitution with a viscous aqueous dispersion (1% carboxy methyl cellulose, w/v and 0.5% Tween-80, v/v).

Animals were sacrificed at the predetermined time intervals of 15, 30 and 60 days. An incision was made on the back of the rat to remove the tissue around the place of the injection. The removed tissue was divided into pieces and a piece of the removed tissue, fixed with formalin solution (10% v/v), was immersed in paraffin [37, 38]. Sections (10 mm) were cut with a paraffin microtome (Minot type). Samples were dyed using the alcin blue hemalum picro-indigo and the hematoxyline-eosin methods. The fixed slides were observed for the histological changes if any [162].

4.6.5 In-vivo Pharmacokinetic Studies

In-vivo pharmacokinetic studies were performed to determine plasma concentration time profiles of pure d4T as well as drug loaded biodegradable polymeric microspheres. Pharmacokinetic studies were conducted in female SD rats, weighing ~
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250 g as per the protocol approved by the Institutional Animal Ethical Committee, Panjab University, Chandigarh to evaluate the in-vivo performance of stavudine loaded microspheres [147]. They were housed in cages and maintained on a 12 h light/dark cycle at room temperature and relative humidity (RH) of 45-55%. The animals were fed with standard rat food and water ad libitum. Before the drug release experiments were carried out, animals were acclimatized to laboratory conditions for one week prior to dosing.

Animals were divided into four groups; one of the groups was used as control (n=4) and the other three groups were used as test animals (n=5). The animals were anaesthetized with diethyl ether. The drug loaded microspheres of Resomer 505H, PLA and PLLA, were injected subcutaneously at the back of test animals of each group with a dose of 30 mg/kg [267]. The microspheres were injected after constitution with a viscous aqueous dispersion (1% carboxy methyl cellulose, w/v and 0.5% Tween-80, v/v); whereas control group was given subcutaneous injection of stavudine, equivalent to the encapsulated drug into the microspheres.

Serial blood samples (0.5 ml) were collected from the retro-orbital plexus using heparinized capillary tube at predetermined time intervals of 0.5, 1.0, 1.5, 2.0, 4.0, 8.0, 12.0 and 24.0 h for control group and 1.0, 2.0, 4.0, 8.0, 12.0 and 24.0 h, 2.0 day, 4.0 day, 6.0 day, 8.0 day, 10 day, 15.0 day and 30.0 day for test animals respectively. The blood samples were centrifuged immediately at 5000 X g for 10.0 minutes and separated plasma was stored at -80°C until analysis. Plasma samples kept at -80°C were thawed at room temperature, 200 µl of the blank plasma were taken into a microcentrifuge eppendorf tube and added with 200 µl of 10% perchloric acid solution to precipitate plasma proteins [267]. The mixture was shaken for 30 seconds. The samples were centrifuged at 3000 X g for 10.0 minutes, the supernatant was transferred to microcentrifuge eppendorf tube and 20 µl was injected directly into the chromatographic system.

Plasma concentration time profiles were evaluated by data fitting to suitable compartmental approach. Various pharmacokinetic parameters including elimination half life (T1/2), Cmax (maximum concentration in plasma), Tmax (time to attain maximum plasma concentration), total area under the curve (AUCtotal), Vd (volume of distribution), Cl (clearance), Ke (elimination rate constant) and MRT (mean residence time) were evaluated. All the above mentioned pharmacokinetic parameters were calculated for individual animals using Kinetica 5.0 software.
4.7 Preparation of Unit/Multiparticulate Systems of Stavudine

4.7.1 Preparation of Matrix Tablets of Stavudine

Matrix tablets of stavudine were prepared by direct compression employing various polymers like carbopol 971 P, carbopol 71G NF and poly-ethylene oxide (PEO). Micro crystalline cellulose (MCC) was used as the directly compressible filler, magnesium stearate as the lubricant and talc as the glidant. The drug, polymer and the filler were sieved through mesh # 30, accurately weighed and then mixed thoroughly to obtain a uniform blend [28]. The mixture was thoroughly blended with the lubricant and the glidant previously sieved through mesh # 60 and compressed on rotary tablet press using 12 mm standard concave punches. 500 mg of powder mixtures were weighed individually and compressed. Table 6 shows the composition of matrix tablets prepared using varying proportions of different polymers.

Table: 6. Composition of matrix tablets prepared using different polymers.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Stavudine</th>
<th>MCC</th>
<th>DC</th>
<th>Carbopol 971P</th>
<th>Carbopol 71G NF</th>
<th>PEO</th>
<th>Mag. stearate</th>
<th>Talc</th>
</tr>
</thead>
<tbody>
<tr>
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<td>385</td>
<td></td>
<td></td>
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<td>5</td>
<td>10</td>
</tr>
<tr>
<td>ST 2</td>
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<td>385</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>CP 1</td>
<td>100</td>
<td>285</td>
<td>100</td>
<td></td>
<td>100</td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>CP 2</td>
<td>100</td>
<td>185</td>
<td>200</td>
<td></td>
<td>200</td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>CP 3</td>
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<td>85</td>
<td>300</td>
<td></td>
<td>100</td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>CG 1</td>
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<td>285</td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>CG 2</td>
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<td>185</td>
<td>200</td>
<td></td>
<td>200</td>
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<td>10</td>
</tr>
<tr>
<td>PEO 20</td>
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<td></td>
<td></td>
<td>100</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>
4.7.2 Preparation of Stavudine Pellets

Extrusion-spheronization is a process by which the pellets are produced from mixture of solids and liquid by involvement of forming and shaping forces. In every trial of pellet preparation the extruder speed, spheronization speed and spheronization time were optimized as these are the factors which can affect the various pellets properties. The extruder speed was set at 30 rpm, spheronization speed was 2000 rpm and spheronization time was 10 minutes. All the pellet batches were dried in an oven at 50 °C for a period of 12 h [29].

The drug (50 gms) and MCC (10 gms) were sifted through mesh # 30 and blended for 10 minutes, after blending the mixture was again sifted through the same sieve to insure thorough mixing. The resulting blend was granulated using water as granulating fluid and then subjected to extrusion followed by spheronization.

4.7.3 Coating of Stavudine Pellets

The prepared pellets were coated using GLATT particle coater with air suspension technology. The rationale for coating of pellets was to optimize and control the release of d4T for prolonged duration of time [33]. The pellets were coated to different coating levels which were based on the principle of fluidized bed coating.

A. Preparation of Coating Solution

The coating dispersion (5.0% w/v) consisted ethyl cellulose (20 cps), talc (5% w/w of polymer), dibutyl phthalate (12.5% w/w of polymer) in the mixture of isopropyl alcohol and water (9:1). The mixture was vortexed to obtain a uniform dispersion, which was continuously stirred during the coating process. The different optimized process parameters include inlet air temperature (35-40 °C), product temperature (25-30 °C), fluidized air pressure (0.9-1.00 Mpa), atomization air pressure (0.5-0.6 Mpa), rate of spray (2-3 ml/min).

4.8 Evaluation of Stavudine Tablets

The formulated matrix tablets of stavudine were evaluated for various parameters which are as follows:
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4.8.1 Dimensional Analysis
The diameter of the tablets depends invariably on the dimension of the dies and punches used. The thickness may vary with no change in weight because of difference in density of the compressed blend, the pressure applied for compression of the tablets as well as the speed of tablet compression. Thickness of the formulated tablets was noted for all the batches with the aid of a Vernier caliper.

4.8.2 Hardness
The resistance of the tablet to chipping, abrasion or breakage under conditions of storage, transportation, and handling before usage depends on its hardness. If the tablet is too hard, it may not disintegrate in the required period of time or meet the dissolution specification. If it is too soft, it will not withstand the handling during subsequent processing such as coating or packaging and shipping operations. The hardness of the formulated matrix tablets of stavudine was determined using Monsanto Hardness tester.

4.8.3 Friability
The test for friability of tablets is designed to evaluate the ability of the tablet to withstand abrasion in packaging, handling and shipping. Friability of the tablets was determined using Roche friability tester. Percent friability was calculated using the following formula:

\[
\text{% Friability} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100
\]

4.8.4 Drug Content
Ten tablets from each batch were weighed and powdered in a mortar and pestle. Powder equivalent to 100 mg of the drug was weighed and dispersed in 100 ml of purified water. The solution was sonicated for 5 minutes, filtered through 0.22 μm membrane filter and suitably diluted with purified water. The drug was then analyzed using UV-Visible spectrophotometer (λ_{max}=267 nm) and the drug content was computed. The determination was done in triplicate.
4.8.5 *In-vitro* Release Studies

The *in-vitro* release profile of the tablets was determined in triplicate using USP type II apparatus. The paddle speed was 50 rpm and dissolution media was pH 6.8 phosphate buffer maintained at 37 ± 0.5 °C. Samples were withdrawn at predetermined time intervals over a period of 24 hours and replaced with the fresh dissolution medium. The samples were analyzed by ultra-violet spectroscopy at 267 nm after suitable dilutions.

4.8.6 Drug Release Kinetics

The release pattern of the various formulated batches of tablets was evaluated to check the goodness of fit for different models of release kinetics.

4.8.7 Determination of Swelling Index

The determination of the swelling index for various optimized batches of tablets with different polymers was conducted. The test was carried out in 80 ml of pH 6.8 phosphate buffer. The tablets were kept in individual beakers containing the medium and placed in a constant temperature water bath for a period of 24 h at 37 °C. The diameters of the tablets were recorded at various time intervals by placing the beaker on a graph paper. The swelling index was determined by using following formula.

$$\text{Swelling index} = \left( \frac{D_t - D_0}{D_0} \right) \times 100$$

Where, ‘$D_0$’ and ‘$D_t$’ are the initial diameter and the diameter at time ‘$t$’ respectively.

4.8.8 Stability Studies

The stability of the stavudine in matrix tablets was determined by exposing them to the accelerated conditions. The tablets of the selected batches were stored at 40 ± 2 °C and 75 ± 5 % RH for one month. The formulations were observed and analyzed for changes in physical appearance, color, incompatibilities and drug content at the end of the study period of 15 and 30 days [282].
4.9 Characterization and Evaluation of Stavudine Pellets

The prepared matrix pellets of stavudine were characterized and evaluated for various enlisted parameters [29, 33].

4.9.1 Size Analysis

The size analysis of all the batches of the pellets was done using Malvern Mastersizer 2000 using dry dispersion technique at an air pressure of 1 bar and a feed rate of 25%. Various parameters like mean diameter \( [D (0.5)] \), and span values were calculated and size distribution graphs were plotted.

4.9.2 Shape Analysis

The shape analysis was performed by image analysis. The pellets were mounted on a Nikon microscope fitted with camera and the images of the pellets were analyzed using Nikon digital sight DS-L. The area of the pellets, maximum and minimum radii were calculated from which various shape factors were calculated as per the following formulae: [283, 284]

\[
Elongation = \frac{Maximum \ radius}{Minimum \ radius}
\]

\[
Rectang = \frac{Area}{4 \times Maximum \ radius \times Minimum \ radius}
\]

\[
Roundness = \frac{Area}{\pi \times Maximum \ radius^2}
\]

4.9.3 Bulk and Tapped Densities

Bulk density of the pellets was determined by gently pouring accurately weighed pellets into a graduated 100 ml measuring cylinder. The volume thus occupied was utilized to calculate the bulk density. The cylinder was then tapped 500 times from a
height of 14 mm and the volume was noted. It was tapped further 750 times, until no further decrease in volume was observed and the final volume was used to calculate the tapped density. Hausner ratio \( (H_r) \) and Carr index \( (I_c) \) were thus calculated using the following formulae:

\[
Hausner\ ratio = \frac{\rho_{bulk}}{\rho_{tapped}}
\]

\[
Carr\ index = \left[1 - \frac{\rho_{bulk}}{\rho_{tapped}}\right] \times 100
\]

Where, ‘\( \rho_{bulk} \)’ and ‘\( \rho_{tapped} \)’ are bulk and tapped densities respectively.

4.9.4 Flow Properties

The flow properties were characterized in terms of angle of repose and flow rate. For the determination of the angle of repose, in triplicate, known weight of pellets were allowed to flow freely through a funnel on to a smooth, vibration-free, horizontal surface from a height of 3 cm. Diameter and height of the heap thus formed were measured and the angle of repose calculated thereof using the following formula:

\[
\theta = \tan^{-1}\frac{h}{r}
\]

Where, ‘\( \theta \)’ is the angle of repose and ‘\( h \)’ and ‘\( r \)’ are the height and the radius of the heap respectively.

The flow rate was calculated as the time taken for 10 g of pellets to flow through a funnel. The determinations for the angle of repose and flow rate were done in triplicate and the average value with standard deviation was calculated.

4.9.5 Drug Content

The drug content of the pellets was determined by triturating 5 g of pellets using mortar and pastle. The amount equivalent to 100 mg of stavudine was weighed \( (n=3) \) and dissolved in known volume of purified water. The solution was sonicated for 5 minutes, filtered through 0.22 \( \mu \) membrane filter and suitably diluted with purified water. The drug
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was then analyzed using UV-Visible spectrophotometer ($\lambda_{\text{max}} = 267 \text{ nm}$) and the drug content was calculated.

4.9.6 *In-vitro* Release Studies

Suitable dissolution characteristics are an important property of oral solid dosage forms since, drug absorption and physiological activity depend on having the drug substance in dissolved state. The dissolution testing is intended to provide a step toward the evaluation of the physiological availability of the drug substance. The *in-vitro* release profile of the pellets was determined in triplicate using USP type II (paddle type) apparatus using 1000 ml of 6.8 phosphate buffer maintained at $37 \pm 0.5 \degree\text{C}$. The paddle speed was 50 rpm [146, 147]. Samples were withdrawn at predetermined time intervals over a period of 24 hours and replaced with the fresh dissolution medium. The samples were analyzed by ultra-violet spectroscopy at 267 nm after suitable dilutions.

4.9.7 Drug Release Kinetics

The release pattern of the various batches of pellets coated with ethyl cellulose at different coating levels was evaluated to check the goodness of fit for different models of release kinetics.

4.9.8 Stability Studies

To determine the stability of the stavudine matrix pellets, pellets of batch SP 4 was stored at $40 \pm 2 \degree\text{C}$ and $75 \pm 5\% \text{ RH}$ for one month. The formulation was observed for changes in physical appearance, incompatibilities, color and analyzed for drug content at the end of the study period of 15 and 30 days.