2. PROFILES OF DRUGS AND EXCIPIENTS

To test the biological efficacy of the thiolated dendrimers for prolonged mucoadhesion and improved mucosal penetration, three model drugs, acyclovir (ACY), amphoterecin B (AmB) and paclitaxel (PAC), were selected.

Brief account of the physico-chemical properties, pharmacokinetics, indications, dosage and adverse reactions and analytical method development and method of validation of these three drugs are discussed in this chapter.

Also the physiochemical properties, applications of dendrimers and the thiol introducing ligands (Cysteamine & 2-iminothiolane) are discussed in this chapter.

2.1. Acyclovir drug profile

Acyclovir was the first specific antiviral drug to become widely used against herpes viruses, particularly Herpes Simplex Viruses (HSV) types I and II and Varicella Zoster. It is widely used in the treatment of various ocular viral diseases. The topical application of acyclovir is limited due to low corneal penetration of the drug and by its poor water solubility. Many strategies have been developed to improve therapeutic efficacy of the acyclovir such as chemical modification, liposomes, nanoparticles. However they have not met the therapeutic requirement. Hence there should be a strong delivery system overcoming the limitations of earlier strategies. Acyclovir is a
white powder consisting of elongated rectangular crystals, it is prepared by chemical synthesis.

2.1.1. Structure

![Chemical Structure of Acyclovir](image)

**Chemical name**

9-[(2-Hydroxyethoxy)methyl] guanine 2-Amino-1,9-dihydro-9-(2-hydroxyethoxymethyl)-6H-purin-6-one.

**Molecular formula** \( \text{C}_8\text{H}_{11}\text{N}_5\text{O}_3 \)

**Molecular weight** 225

**Category** Anti Viral

2.1.2. Solubility

Acyclovir is slightly soluble in water; insoluble in ethanol; practically insoluble in most organic solvents; soluble in dilute aqueous solutions of alkali hydroxides and mineral acids.

Solubility of acyclovir is defined as

- in ethanol \(< 1 \text{ in } 5000\)
- in water \(< 1 \text{ in } 400\)

2.1.3. Analytical profile

Various methods for the identification and quantitative estimation of acyclovir in pharmaceutical formulations and biological
fluids have been reported in the literature, which include spectrophotometric procedures\textsuperscript{5-7}, HPLC procedures\textsuperscript{8-11}, and more recently enzyme immunosorbent assay procedures\textsuperscript{12}.

2.1.4. Biological profile

2.1.4.1. Mechanism of action

Acyclovir is a synthetic purine nucleoside analogue with in vitro and in vivo inhibitory activity against HSV 1 & HSV 2 and Varicella Zoster Virus (VZV). The inhibitory activity of acyclovir is highly selective due to its affinity for the enzyme thymidine kinase (TK) encoded by HSV and VZV. This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleoside analogue. The monophosphate is further converted into triphosphate by a number of cellular enzymes. IN vitro, acyclovir triphosphate stops replication of herpes viral DNA. This is accomplished in 3 ways: 1) competitive inhibition of viral DNA polymerases 2) incorporation into and termination of the viral DNA chain and 3) inactivation of the viral DNA polymerases. The greater antiviral activity of acyclovir against HSV compared to VZV is due to its more efficient phosphorylation by the viral TK\textsuperscript{13}.

2.1.4.2. Drug resistance

Resistance of HSV and VZV to acyclovir can result from qualitative and quantitative changes in the viral TK and/or DNA polymerases. Clinical isolates of HSV and VZV with reduced susceptibility to acyclovir have been recovered from immune compromised patients, especially with advanced HIV infection. TK
negative mutants may cause severe diseases in infants and immune compromised adults.

2.1.4.3. Pharmacokinetics

The intravenous infusion of acyclovir as the sodium salt produces plasma acyclovir concentrations that demonstrate a biphasic pattern. The infusion over one hour of a dose equivalent to 5 mg of acyclovir per kg body weight in adults has produced steady state plasma concentrations with a peak of 9.8 µg/ml and trough of 0.7µg/ml. Acyclovir is excreted through the kidney by both glomerular filtration and tubular secretion. The terminal or beta phase half life reported to be about 2-3 hrs for adults without renal impairment. Most of a dose by intravenous infusion is excreted with only up to 14% appearing in the urine as the inactive metabolite 9-carboxymethoxymethylguanine. There is wide distribution including into the CSF where concentrations achieved are about 50% of those achieved in plasma. Protein binding is reported to in the range from 9 to 33%. About 15-30% of a dose of acyclovir given oral is considered to be absorbed from the gastrointestinal tract. A dose of 200 mg acyclovir every 4 hours by mouth is reported to produce maximum and minimum steady state plasma concentrations of 0.7 and 0.4 µg/ml respectively. The orally active prodrugs, descyclovir and valacyclovir have been developed to overcome this poor absorption. Acyclovir crosses the placenta and is distributed into breast milk in concentrations approximately 3 times higher than those in maternal
serum. Absorption of acyclovir is usually slight following topical application to intact skin, although it may be increased by changes in formulation. Acyclovir is absorbed following application of a 3% ointment to the eye giving a relatively high concentration of 1.7 µg/ml in the aqueous humour but negligible amounts in the blood.

2.1.4.4. Adverse Effects

Acyclovir is generally well tolerated. When administered intravenously as acyclovir sodium it may cause local reactions at the injection site with inflammation and phlebitis; these reactions may be associated with extravasation that leads rarely to ulceration. Renal impairment may occur in a few patients, it is usually reversible and is reported to respond to hydration and/or dosage reduction or withdrawal, but may progress to acute renal failure. The risk of renal toxicity is increased by conditions favouring deposition of acyclovir crystals in the tubules such as when the patient is poorly hydrated, has existing renal impairment or when the drug is given at high dosage of by rapid bolus injection. Some patients receiving systemic acyclovir may experience transient increase in blood concentrations of urea and creatinine though this is more acute with intravenous administration. Occasional adverse effects following systemic administration include increased serum bilirubin and liver enzymes, haematological changes, skin rashes, fever, headache, dizziness and gastrointestinal effects such as nausea, vomiting and diarrhoea. Neurological effects including lethargy, somnolence, confusion,
hallucinations, agitation, tremors, psychosis, convulsions and coma have been reported in a small number of patients, particularly in those receiving intravenous acyclovir and with predisposing factors such as renal dysfunction. Accelerated diffuse hair loss has also been reported. Topical application of acyclovir, especially to genital lesions may sometimes produce transient stinging, burning, itching or erythema. Eye ointments may occasionally produce transient stinging, superficial punctuate keratopathy, blepharitis or conjunctivitis.

There has been no evidence of bone marrow toxicity in patients given acyclovir following bone marrow transplantation. There has also been a report of inhibition of human peripheral blood lymphocytes in samples taken from healthy subjects given acyclovir.

Transient renal impairment occurred in 2 adequately hydrated patients following acyclovir 10 mg/kg body weight infused over 1 hour every 8 hrs. Acute real failure and coma observed in some patients receiving high dose of oral therapy.

Some patients have developed reversible neurological symptoms including tremor, agitation, nausea, lethargy, mild dis-orientation speech up on administration of acyclovir in doses ranging from 0.75-3.6 Kg/m² body surface daily by intravenous infusion.

A report of vesicular lesions associated with intravenous administration of acyclovir in a patient thought to have herpes simplex encephalitis. Acyclovir has been associated with vasculitis.
2.1.4.5. Precautions

Acyclovir should be administered with caution to patients with renal impairment and doses should be adjusted according to creatinine clearance. Parenteral administration should be by slow intravenous infusion over one hour to avoid precipitation of acyclovir in the kidney; rapid or bolus injection should be avoided and adequate hydration maintained. The risk of renal impairment is increased by the concomitant use of other nephrotoxic drugs. Intravenous acyclovir should also be used with caution in patients with underlying neurological abnormalities with significant hypoxia or with serious hepatic or electrolyte abnormalities.

2.1.4.6. Interactions

Probenecid is reported to block the renal clearance of acyclovir. The risk of renal impairment is increased by the concomitant use of other nephrotoxic drugs.

2.1.4.7. Pharmaceutics

Acyclovir is available from several manufacturers. Preparations containing acyclovir are available for oral, topical and ophthalmic administration and acyclovir sodium for injectable formulation.

2.1.4.8. Therapeutic uses

Indications:

- Treatment of herpes simplex keratitis
• Treatment and prophylaxis of herpes simplex infections of skin and mucous membranes in immune competent individuals

• Treatment of severe and/or generalized herpes simplex infections in immunocompromised and immunocompetent individuals.

• Treatment of varicella-zoster infections in immunocompromised and immunocompetent individuals.

• Prophylaxis of herpes simplex, varicella-zoster, and cytomegalovirus infections in the immune compromised patients.

Other uses:

• Improvement of survival in patients with AIDS.

2.1.5. Analytical method used in the current investigation and its validation

The amount of acyclovir was estimated by using reported HPLC method with minor modifications\textsuperscript{14}. Two separate calibration curves were constructed between peak area vs. concentration of acyclovir, one for estimation in dendrimeric vesicles and in \textit{in vitro} release studies and the other in aqueous humour for estimation of the drug in \textit{in vivo} studies.

2.1.5.1. Instrumentation

The HPLC system (Agilent 1100) was operated in a binary mode with a photodiode array detector, auto injector and column oven. The
analysis was performed at 254 nm on a reversed phase C18 column (Inertsil, 250 mm×4.6 mm, 5 μm) maintained at 25° C.

2.1.5.2. Chromatographic conditions

The mobile phase comprised of 0.02M potassium dihydrogen phosphate - acetonitrile in the ratio of 99:1, filtered before use through a 0.45 μm membrane filter and pumped from the respective solvent reservoirs at a flow rate of 1.0 mL/min. Eluents were monitored using UV detection at a wavelength of 254 nm. The volume of injection port was 20 μL.

2.1.5.3. Preparation of stock solution and standard solutions

Stock solution of ACY was prepared by dissolving 25 mg of acyclovir in 25 mL of methanol in 25 mL volumetric flask. The stock solution of acyclovir was subsequently diluted with mobile phase to obtain a series of standard solutions containing 10, 50, 100, 250, 500 and 1000 ng of acyclovir in 1.0 mL. All standard solutions were filtered through 0.45 μm membrane filter and 20 μL of the sample was injected in to HPLC column.

The concentrations of acyclovir and the corresponding peak area are given in Table 2.1. The calibration curve was plotted between concentration of acyclovir and corresponding peak area (shown in Fig 2.2).
2.1.5.4. Estimation of acyclovir in aqueous humour

**Aqueous humour collection**

Aqueous humour was aspirated from the limbus region of rabbits with a heparin-rinsed glass syringe connected to a 27-gauge needle. After the collection aqueous humour was centrifuged (5000 rpm) and stored at –80°C until analysis.

**Sample preparation**

Acyclovir working standard solutions in the range of 50-1000 ng/mL were prepared by serial dilution of a methanolic stock solution (1 mg/ml) using the mobile phase. 100 µl of working standards of acyclovir were added to the aqueous humour samples (100 µl) and vortexed for few seconds. 1ml of 2% (w/v) ZnSO₄·7H₂O was added to these samples and for deproteinization, vortexed and centrifuged at 3000 rpm for 15 minutes. The supernatant was collected, filtered using 0.2 µm membrane (Millipore) and estimated for the acyclovir content using the above described conditions under Sec.2.1.5.2.

**2.1.5.5. Quantification and assay validation**

Aqueous humour samples were quantified for acyclovir by plotting peak areas against acyclovir concentrations and respective acyclovir concentrations in the samples were calculated by using least squares linear regression equation.
• **Linearity**

To evaluate linearity, aqueous humour calibration curves (50 ng/ml to 1000 ng/ml) were prepared and assayed in triplicate.

• **Accuracy and precision**

Accuracy and precision were also determined by analyzing three concentrations (100 ng/ml, 500 ng/ml, and 1 µg/ml), three samples each over three days.

• **Specificity**

Specificity was verified by analyzing three independent blank aqueous humour samples and comparing these chromatograms with those obtained after spiking the aqueous humour samples from the same source with acyclovir.

• **Stability**

Stability of the acyclovir in the aqueous humour samples was estimated by spiking 500 ng/ml of acyclovir to aqueous humour and analyzed after storage at -20°C for 24 hrs and 48hrs.

• **Recovery**

Recovery of the precipitation procedure was conducted by adding 500 ng of acyclovir to the pre-analyzed aqueous humour drug samples containing 500 ng/mL of acyclovir and subjected them to the HPLC method (shown in Table 2.3). Three replicate
(n=3) spiked aqueous humour samples at these concentration levels were subjected for analysis to calculate mean recovery.

### 2.1.6. Results and discussion

In the reported method the mobile phase was ammonium acetate (10mm; pH 6.8)- acetonitrile at 99:1 ratio. However in the present method the mobile comprised of 0.02M potassium dihydrogen phosphate - acetonitrile in the ratio of 99:1 and resulted in a good resolution of acyclovir. The retention time of all the chromatograms was found to be 8.3 min. Each of the samples was injected three times and the same retention times were observed in all the cases with a variation of ± 0.05 min. A good correlation (r = 0.9998) between the concentration of acyclovir and peak area values was observed indicating the linearity of the method. The amount of acyclovir was calculated using the corresponding regression equations, shown in Fig.2.3. There is no precipitation of the drug during the dilution with the mobile phase used for the analysis of the acyclovir. The chromatograms of the blank aqueous humor and test sample were given in Fig.2.4.

#### 2.1.6.1. Linearity and lower limit of quantification

Calibration graph was constructed by plotting peak area of acyclovir on ordinate and different concentrations of acyclovir on abscissa (Fig. 2.3). The graph was found to be linear in the concentration range of 50 ng/ml to 1000 ng/ml in aqueous humour. The correlation coefficients were greater than 0.99 for all the matrices.
confirming the linearity (Table 2.3). The lower limit of quantification (LOQ) was defined as the lowest concentration analyzed with acceptable accuracy and precision was 50 ng/ml for aqueous humour and limit of detection (LOD) was found to be 10 ng/ml.

2.1.6.2. Accuracy and precision

The overall accuracy of the method, expressed in terms of relative standard deviation (RSD) from the true values lay between 90-110% for the concentrations investigated. Inter- and intra-day statistics for accuracy and precision are presented in Table 2.4. The precision of the method, given by RSD was less than 5%. At this retention time no other peaks from endogenous aqueous humour components were observed. The results of the study indicated that the method was sensitive, precise and accurate.

2.1.6.3. Specificity

Representative chromatogram of blank aqueous humour sample was compared with blank aqueous humour spiked with acyclovir at the lower limit of quantification. No interference’s of aqueous humour were detected at the retention time (RT) of acyclovir (Fig 2.3). It was further confirmed by comparing the UV absorption spectra of the test sample with that obtained after injecting methanolic solution of the pure drug.
2.1.6.4. Stability

The satiability data indicated (Table 2.4) the acyclovir stability at least for 48hrs in the biological matrix.

2.1.6.5. Recovery

The percentage recovery values (Table 2.4) revealed that this procedure is suitable for the complete recovery of acyclovir from the aqueous humour samples.

Table 2.1: Linearity data of acyclovir in acetonitrile / aqueous humor.  \( x=\text{Concentration (ng/ml)}; y=\text{Area} \)

<table>
<thead>
<tr>
<th>Matrix/Solution</th>
<th>Linearity range (ng/ml)</th>
<th>Regression Equation</th>
<th>Correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>10 to 1000</td>
<td>( Y=102.8x+369.7 )</td>
<td>0.999</td>
</tr>
<tr>
<td>Aqueous Humour</td>
<td>50-1000</td>
<td>( Y=368.7x+3292.12 )</td>
<td>0.999</td>
</tr>
</tbody>
</table>
Table 2.2: Concentration versus peak area values for the estimation of acyclovir in mobile phase

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Peak area (mean, n=3)</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>912</td>
<td>11</td>
</tr>
<tr>
<td>50</td>
<td>4878</td>
<td>18</td>
</tr>
<tr>
<td>100</td>
<td>9768</td>
<td>17</td>
</tr>
<tr>
<td>250</td>
<td>24845</td>
<td>24</td>
</tr>
<tr>
<td>500</td>
<td>55064</td>
<td>21</td>
</tr>
<tr>
<td>1000</td>
<td>99315</td>
<td>24</td>
</tr>
</tbody>
</table>

Fig. 2.2: Calibration curve for the estimation of acyclovir in mobile phase

Table 2.3: Concentration versus peak area values for the estimation of acyclovir in aqueous humour

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Peak area (mean, n=3)</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>21489</td>
<td>9</td>
</tr>
<tr>
<td>100</td>
<td>40913</td>
<td>11</td>
</tr>
<tr>
<td>250</td>
<td>92082</td>
<td>14</td>
</tr>
<tr>
<td>500</td>
<td>191812</td>
<td>14</td>
</tr>
<tr>
<td>1000</td>
<td>370702</td>
<td>21</td>
</tr>
</tbody>
</table>

Fig. 2.3: Calibration curve for the estimation of acyclovir in aqueous humour
Fig. 2.4: HPLC Chromatograms of a) blank aqueous humour b) test sample
2.1.7. Validation summary

The results of the study indicated that the method was sensitive, precise and accurate and is suitable for the estimation of acyclovir in aqueous humour samples during the in vivo studies.

Table 2.4. A summary of the different validation parameters

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Validation parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Linearity</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>50 to 1000 ng/ml</td>
</tr>
<tr>
<td></td>
<td>Goodness of fit</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>2</td>
<td><strong>Sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LLOQ</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td></td>
<td>LOD</td>
<td>5 ng/ml</td>
</tr>
<tr>
<td>3</td>
<td><strong>Accuracy</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
<td>90% to 110%</td>
</tr>
<tr>
<td></td>
<td>Inter-day</td>
<td>90% to 110%</td>
</tr>
<tr>
<td>4</td>
<td><strong>Precision</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
<td>&lt; 15%</td>
</tr>
<tr>
<td></td>
<td>Inter-day</td>
<td>&lt; 15%</td>
</tr>
<tr>
<td>5</td>
<td><strong>Stability</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Short-term stability</td>
<td>102.5%</td>
</tr>
<tr>
<td></td>
<td>Long-term stability</td>
<td>98.5%</td>
</tr>
<tr>
<td>6</td>
<td><strong>System suitability</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retention time (% CV)</td>
<td>2.1%</td>
</tr>
<tr>
<td></td>
<td>Peak area (% CV)</td>
<td>0.1%</td>
</tr>
<tr>
<td>7</td>
<td><strong>Recovery</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration (500 ng/ml)</td>
<td>98.92%</td>
</tr>
</tbody>
</table>


2.2. Amphoterecin B drug profile

Amphotericin B is an amphoteric polyene heptane macrolide antibiotic possessing 7-conjugated double bonds in trans position and 3-amino, 3, 6-dideoxymannose (Mycosamine) connected to polyene ring by glycosidic bond. The name itself is termed from amphoteric behavior of drug, having presence of carboxylic group on main ring and primary amine on sugar ring. Amphotericin B has been shown to be rigid and rod shaped with hydrophilic group of macrolide ring forming an opposite phase to lipophilic polyene portion\(^\text{15}\). The drug is sensitive to heat, light and is inactivated at low pH. Amphotericin B (AmB) is a polyene macrolide antifungal agent and the drug of choice for systemic fungal infection\(^\text{16}\). Unfortunately, it is poorly absorbed from the gastrointestinal tract due to its poor aqueous solubility. Many formulations have been designed and marketed by using novel excipients, but due to toxicity and high cost\(^\text{17}\) they met with little success. Hence there is a potential scope for developing a formulation with good oral bioavailability and minimum side effects.

2.2.1. Structure

![Structure of Amphoterecin B](image)

**Fig 2.5: Structure of Amphoterecin B**
**Chemical name**

[1R–(IR*,3S*,5R*,6R*,
35S,36S*,37S*)–3 3-[(3-Amin-3,6-dideoxy-β-D-annopyranosyl)oxy]–
1,3,5,6,9,11,17,37–octa hydroxy-15,16,18–trimethyl–13-oxo-14,39-
dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31–heptaene-36-
carboxylic acid.

**Molecular formula**  \( \text{C}_{47}\text{H}_{73}\text{NO}_{17} \)

**Molecular weight** 924.09

**Category** Anti-fungal agent

**2.2.2. Solubility**

Amphotericin B is practically insoluble in water, dehydrated alcohol, benzene, ether and toluene; slightly soluble in dimethyl formamide, Dimethyl sulfoxide and propylene glycol\(^\text{18}\). Storage at 2-8°C in airtight container protected from light\(^\text{19}\).

**2.2.3. Analytical profile of the drug**

Various methods for the identification and quantitative estimation of Amphotericin B (AmB) in pharmaceutical formulations and biological fluids have been reported in the literature, which include spectrophotometric procedures\(^\text{20-21}\), microbiological assay procedures\(^\text{22}\) and HPLC procedures\(^\text{23-28}\).

**2.2.4. Biological profile**

**2.2.4.1. Pharmacokinetics**
Amphotericin B reported to be a little or not absorbed drug from intestine. The drug after oral administration distributed to lungs, liver, spleen, kidneys, adrenal glands, muscle, and other tissues, reaches approximately two-thirds the concurrent plasma concentration in the fluids of peritoneum, synovium and aqueous humor; concentrations in cerebrospinal fluid (CSF) usually undetectable. The volume of distribution is approximately 4 L per Kg and the elimination half life is approximately 24 hrs, whereas terminal half life is 15 days. Amphotericin B largely bound to proteins (>95%) and is not removed by hemodialysis.29

2.2.4.2. Adverse Effects

**Acute reactions:** Adverse effect with IV infusion includes chills, fever, aches and pain all over, nausea vomiting and dyspnoea, thromboflebitis.

**Long-term toxicity:** Nephrotoxicity is most common. It occurs fairly uniformly and is dose related; manifestations are – azotemia, reduced glomerular filtration rate acidosis, hypokalemia and inability to concentrate urine. Anemia, due to bone marrow depression. CNS toxicity occur only on intrathecal injection.30

2.2.4.3. Drug Interactions

Patients who receive amphotericin B are generally quite ill for long time and most likely are receiving multiple-drug therapy for concurrent illnesses. Therefore, these patients may be at risk for drug interactions. A number of interactions have been identified.
Amphotericin B may increase the renal toxicity of cyclosporine and aminoglycosides. Antineoplastic agents, such as cisplatin and the nitrogen mustard compounds, may enhance the renal toxicity of amphotericin B, and these agents should be administered concomitantly only with great caution.

10-fold risk of acute pulmonary reactions (acute dyspnea, hypoxemia, and pulmonary infiltrates) in patients receiving leukocyte transfusions with amphotericin B concomitantly when compared with a population receiving leukocyte transfusions alone. However, slow administration of amphotericin B and avoidance of concomitant leukocyte transfusions have been proposed to minimize the potential interaction.

2.2.4.4. Therapeutic uses

- It is drug of choice in systemic mycosis.
- Also used in leishmaniasis.
- It is first drug of choice in number of fungal diseases such as candidiasis, cryptococcosis, sporotrichosis, aspergillosis, mucormycosis etc.

2.2.5. Analytical method used in the current investigation and its validation

To estimate the amount of amphotericin B trapped within the dendrimeric vesicles, in in vitro release studies and in biological samples, HPLC calibration curves of amphotericin B (peak area vs.
concentration) were constructed in the mobile phase and in plasma employing the previously reported method by Fukui et al.

### 2.2.5.1 Instrumentation

The HPLC system (Agilent 1100) was operated in a binary mode with a photodiode array detector, auto injector and column oven. The analysis was performed at 408 nm on a reversed phase C18 column (Inertsil, 250 mm × 4.6 mm, 5 μm) maintained at 25°C.

### 2.2.5.2 Chromatographic conditions

The mobile phase comprised of 10mM sodium acetate buffer (pH 4.0) - acetonitrile in the ratio of 17:11 %v/v filtered by using a 0.45 μm membrane filter and pumped from the respective solvent reservoirs at a flow rate of 1.0 mL/min. Eluents were monitored using UV detection at a wavelength of 408 nm. The volume of injection port was 100 μL.

### 2.2.5.3 Preparation of stock solution and standard solutions

Stock solution of AmB was prepared by dissolving 25 mg of AmB in 25 mL of methanol in 25 mL volumetric flask. The stock solution of AmB was subsequently diluted with mobile phase to obtain a series of standard solutions containing 5, 25, 50, 100, 250, 500 and 1000 ng of AmB in 1.0 mL. All standard solutions were filtered through 0.45 μm membrane filter and 100 μL of the sample was injected into HPLC column. The concentrations of AmB and the corresponding peak area are given in Table 2.5. The calibration curve was plotted between concentration of AmB and corresponding peak
area (shown in Fig 2.6). All the experiments were performed in triplicate and average values are reported.

2.2.5. 4. Estimation of Amphotericin B in rat plasma

Blood collection

Blood was collected from orbital sinus of Wistar rats into EDTA coated glass tubes. Plasma was separated immediately by centrifugation (5000 rpm) and stored at –80° C until further analysis.

Sample preparation

Working standard solutions were prepared by serial dilution of a methanolic stock solution (1 mg/ml). 100 µl of methanolic AmB working standards solutions (1, 50, 100, 250, 500 and 1000ng) were added to the plasma samples (100 µl) and vortexed for few seconds. 1 ml of extraction solvent acetonitrile was added to the samples and vortexed for 3 minutes. The samples were centrifuged for 15 minutes at 10,000 rpm and the organic layer was transferred into a clean dry glass test tube. The organic solvent was evaporated under nitrogen atmosphere and the residue was dissolved in 500 µl of mobile phase. This solution was filtered through a membrane filter (0.2 µm) and injected to the HPLC system.

2.2.5.5. Quantification and assay validation

Plasma homogenate samples were quantified by plotting peak areas against amphotericin B concentrations and respective amphotericin B concentrations were calculated by using least squares linear regression.
• **Linearity**

The linearity in plasma was evaluated by plotting the calibration curves of the AmB in the concentration range of 20 ng/ml to 1000 ng/ml. The assay was conducted in triplicate on three separate days.

• **Accuracy and precision**

For the evaluation of accuracy and precision of the method, three concentrations (20 ng/ml, 500 ng/ml, and 1 µg/ml), were determined by analyzing three samples each over three days.

• **Specificity**

Specificity of the method was verified by analyzing three independent blank plasma samples and comparing these chromatograms with those obtained after spiking the plasma samples with amphoterecin B.

• **Stability**

Stability of the amphoterecin B in the plasma samples was estimated by spiking 500 ng/ml of AmB to plasma and analyzed after storage at -20°C for 24hrs and 48hrs.

• **Recovery**

Recovery of the precipitation procedure was conducted by adding 20 ng, 1, 10 µg of amphoterecin B to the pre-analyzed plasma drug samples containing 20 ng/mL and 1, 10 µg/ml of amphoterecin B and subjected them to the HPLC method. Three
replicate \((n=3)\) spiked plasma samples at these different concentration levels were subjected for analysis to calculate mean recovery.

**2.2.6. Results and discussion**

The mobile phase resulted in a good resolution of AmB. The retention time of all the chromatograms was found to be 15.6 min (Fig. 2.8). Each of the samples was injected three times and the same retention times were observed in all the cases with a variation of ± 0.05 min. A good correlation \((r = 0.9998)\) between the concentration of AmB and peak area values was observed indicating the linearity of the method. The amount of AmB was calculated using the corresponding regression equations, shown in table 2.5. There is no precipitation of the drug during the dilution with the mobile phase used for the analysis of the AmB.

**2.2.6.1. Linearity and lower limit of quantification**

Calibration graph was constructed by plotting peak area of AmB on ordinate and different concentrations of AmB on abscissa. The graph was found to be linear in the concentration range of 20 ng/ml to 1000 ng/ml in plasma. The correlation coefficients were greater than 0.99 confirming the linearity (Table 2.5). The lower limit of quantification (LOQ) was 20 ng/ml for plasma and the lower limit of detection (LOD) was found to be 5.0 ng/ml.
2.2.6.2. Accuracy and precision

The overall accuracy of the method, expressed in terms of relative standard deviation (RSD) from the true values lay between 90-110% for the concentrations investigated. The precision of the method, given by RSD was less than 5%. At this retention time no other peaks from endogenous plasma components were observed. Inter- and intra-day statistics for accuracy and precision are presented in table 2.8.

2.2.6.3. Specificity

Representative chromatogram of blank plasma sample was compared with blank plasma spiked with amphoterecin B at the lower limit of quantification. No interference’s were detected at the retention time (RT) of amphoterecin B. It was further confirmed by comparing the UV absorption spectra of the test sample with that obtained after injecting methanolic solution of the pure drug.

2.2.6.4. Stability

The stability of AmB in the rat plasma was conducted for 48hrs and the data has proven the stability of AmB (Table 2.8.).

2.2.6.5. Recovery

The percentage recovery values (Table 2.8) revealed that this procedure is suitable for the complete recovery of AmB from the rat plasma samples.
Table 2.5: Linearity data of Amphoterecin B in acetonitrile and in plasma

<table>
<thead>
<tr>
<th>Matrix/Solution</th>
<th>Linearity range (ng/ml)</th>
<th>Equation</th>
<th>Correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>5 to 1000</td>
<td>$y = 262.8x + 649.5$</td>
<td>0.998</td>
</tr>
<tr>
<td>Plasma</td>
<td>20 to 1000</td>
<td>$y = 559.5x + 5254$</td>
<td>0.997</td>
</tr>
</tbody>
</table>
Table 2.6: Concentration versus peak area values for the estimation of amphotericin B in mobile phase

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Peak area (mean, n=3)</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1512</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>6324</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>12357</td>
<td>11</td>
</tr>
<tr>
<td>100</td>
<td>27003</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>60618</td>
<td>13</td>
</tr>
<tr>
<td>500</td>
<td>125238</td>
<td>17</td>
</tr>
<tr>
<td>1000</td>
<td>264522</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig. 2.6: Calibration curve for the estimation of amphotericin B in mobile phase

Table 2.7: Concentration versus peak area values for the estimation of amphotericin B in plasma

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Peak area (mean, n=3)</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>12670</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>20877</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>54685</td>
<td>8</td>
</tr>
<tr>
<td>250</td>
<td>134920</td>
<td>10</td>
</tr>
<tr>
<td>500</td>
<td>291852</td>
<td>15</td>
</tr>
<tr>
<td>1000</td>
<td>559121</td>
<td>18</td>
</tr>
</tbody>
</table>

Fig. 2.7: Calibration curve for the estimation of amphotericin B in plasma
Fig. 2.8: HPLC Chromatograms of Amphoterecin B
a) blank plasma b) test sample
2.2.7. Validation summary

The results of the study indicated that the method was sensitive, precise and accurate and is suitable for the estimation of AmB in rat plasma samples during the in vivo studies.

Table 2.8  A summary of the different validation parameters

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Validation parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Linearity</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>20,500 to 1000 ng/ml</td>
</tr>
<tr>
<td></td>
<td>Goodness of fit</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>2</td>
<td><strong>Sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LLOQ</td>
<td>5 ng/ml</td>
</tr>
<tr>
<td></td>
<td>LOD</td>
<td>2.5 ng/ml</td>
</tr>
<tr>
<td>3</td>
<td><strong>Accuracy</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
<td>90% to 110%</td>
</tr>
<tr>
<td></td>
<td>Inter-day</td>
<td>90% to 110%</td>
</tr>
<tr>
<td>4</td>
<td><strong>Precision</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
<td>&lt; 9%</td>
</tr>
<tr>
<td></td>
<td>Inter-day</td>
<td>&lt; 12%</td>
</tr>
<tr>
<td>5</td>
<td><strong>Stability</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Short-term stability</td>
<td>100.9%</td>
</tr>
<tr>
<td></td>
<td>Long-term stability</td>
<td>104.1%</td>
</tr>
<tr>
<td>6</td>
<td><strong>System suitability</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retention time (% CV)</td>
<td>1.1%</td>
</tr>
<tr>
<td></td>
<td>Peak area (% CV)</td>
<td>0.1%</td>
</tr>
<tr>
<td>7</td>
<td><strong>Recovery</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low concentration (20 ng/ml)</td>
<td>96.17%</td>
</tr>
<tr>
<td></td>
<td>Medium concentration (1µg/ml)</td>
<td>101.5%</td>
</tr>
<tr>
<td></td>
<td>High concentration (10 µg/ml)</td>
<td>98.9%</td>
</tr>
</tbody>
</table>
2.3. Paclitaxel drug profile

Paclitaxel is one of the most important lead compounds to obtain from a plant source. Paclitaxel (Taxol®), the first taxane in clinical trials, is active against a broad range of cancers that are generally considered to be refractory to conventional therapy. This has lead to the regulatory approval of paclitaxel in the U.S. and many other countries for its use in the palliative therapy of patients with ovarian and breast cancer resistant to chemotherapy. Paclitaxel has a low therapeutic index and is highly lipophilic and practically insoluble in water. Current formulation of paclitaxel in a non-aqueous vehicle containing Cremophor EL® causes allergic reactions and precipitation on aqueous dilution after intravenous administration. Moreover, the extensive clinical use of this drug is somewhat delayed due to the lack of appropriate delivery vehicles. Hence there is a need for the development of alternate formulation of paclitaxel having good aqueous solubility and at the same time free of any side effects. The challenge now is to develop suitable delivery systems for paclitaxel for successful therapy of cancer. Considering all these, carrier delivery systems including liposomes, micelles, microspheres, nanoparticles and particulate drug delivery systems seem to be the best possible approach towards the ideal dosage form which would bypass all the present limitations and provide a desirable means to the present therapy.
2.3.1. Structure

Fig. 2.9: Structure of paclitaxel

Chemical Name

5b, 20-epoxy-1,2a, 4, 7b, 10b, 13a-hexahydroxytax-11-en-9-one-4,10-diacetate-2-benzoate 13 ester with (2R, 3S)-N-benzoyl-3-phenylisoserine

Molecular formula $C_{47}H_{51}NO_{14}$

Molecular weight 853.92

2.3.2. Solubility

Paclitaxel is poorly soluble in an aqueous medium, but can be dissolved in organic solvents. Its solutions can be prepared in a millimolar concentration in a variety of alcohols, such as methanol, ethanol, tertiary-butanol as well as in DMSO. Non-aqueous solubility is found to be $\sim 46$ mM in ethanol, $\sim 20$ mM in methylene chloride or acetonitrile, $\sim 14$ mM in isopropanol$^{35}$. 
2.3.3. **Analytical profile**

Various analytical methods for the determination of paclitaxel using HPLC methods\(^{36-38}\) have been reported.

2.3.4. **Biological profile**

2.3.4.1. **Mechanism of action**

Paclitaxel has a unique mechanism of action and differs from that of other currently available anticancer agents. It aids polymerization of tubulin dimers to form microtubules, even in the absence of factors that are normally required for microtubule assembly (e.g. guanine triphosphate, GTP), and then stabilizes the microtubules by preventing depolymerization\(^{39}\). Paclitaxel mainly binds to microtubules, rather than to tubulin dimers\(^ {40}\). The binding site for paclitaxel is the N-terminal 31 amino acids of the β-subunit of tubulin in the microtubule, unlike the binding sites of colchicine, vinblastine and podophyllotoxin for GTP. The microtubules formed due to paclitaxel action are not only very stable but are also dysfunctional, leading to cell death. While the precise mechanism of action of the drug is not understood fully, paclitaxel disrupts the dynamic equilibrium within the microtubule system and blocks cells in the late G2 phase and M phase of the cell cycle, thereby inhibiting cell replication.
2.3.4.2. Pharmacokinetics

Following intravenous administration paclitaxel exhibits a biphasic decline in plasma concentrations\(^4^1\). The initial rapid decline represents distribution to the central compartment and elimination of the drug and the later phase is due in part, to the efflux of the drug from the peripheral compartment. The pharmacokinetics are non-linear and show wide variability. Terminal half-life was found to be in the range of 1.3–8.6 h (mean 5 h) and the steady-state volume of distribution was found to be \(~87.1\text{m}^2\), indicating extensive extravascular distribution, tissue binding, or both. More than 90% of the drug binds rapidly and extensively to plasma proteins. Paclitaxel is metabolised in the liver, with the major metabolic pathway apparently mediated by the cytochrome P450 isoform CYP2C8, although CYP3A4 may play a minor role. High paclitaxel concentrations have been reported in bile. Less than 10% drug in the unchanged form is excreted in the urine. Most of the drug is eliminated in feces\(^4^2\).

2.3.4.3. Incompatibility

The vehicle for paclitaxel injection, which contains alcohol and polyethoxylated castor oil, was found to leach the plasticiser diethylhexyl phthalate from some plastic administration sets.

2.3.4.4. Adverse effects, treatment and precautions

Paclitaxel produces severe dose-limiting bone marrow depression, the lowest of the white cell count usually occurring after
about 11 days, with recovery usually by day 15 to 21 after a dose. Myelosuppression may be less frequent and less severe when infusions are given over 3 rather than 24 hours.

Peripheral neuropathy may also be severe, and occasionally dose-limiting. Hypersensitivity reactions, with dyspnoea, hypotension, and angioedema have occurred, and premedication with corticosteroids and histamine antagonists were necessary to overcome these side-effects. Other adverse effects include alopecia, arthralgia and myalgia, gastro-intestinal disturbances, mucositis, ileus, seizures, encephalopathy, bradycardia and cardiac conduction abnormalities, chest pain, flushing, rashes, nail dystrophies, hepatic necrosis and elevation of liver enzyme values. Extravasation may result in tissue damage.

Paclitaxel is not recommended in patients with severely impaired hepatic function. The drug is formulated in polyethoxylated castor oil (Cremophor EL®) and should be avoided in patients with known hypersensitivity. Continuous cardiac monitoring should be performed in patients who have experienced previous significant conduction abnormalities while receiving paclitaxel.

Infusion of paclitaxel has been associated with myocardial infarction, sudden death and symptoms suggestive of heart failure. There is some evidence of cellular damage to the myocardium with paclitaxel-associated cardiac symptoms. Also it was observed that severe cardiovascular events occurred more frequently following the
use of paclitaxel in patients with non-small cell lung cancer than in those with breast or ovarian carcinoma.

Acute bilateral interstitial pneumonitis has been reported rarely in patients receiving paclitaxel, despite premedication with corticosteroids and histamine antagonists. Symptoms resolved on treatment with parenteral corticosteroids.

2.3.4.7. Interactions

Pre treatment with cisplatin may reduce the clearance of paclitaxel, resulting in increased toxicity, and when the two drugs are given in combination paclitaxel should be given first; presumably such a risk may also apply if paclitaxel is given to patients who have received other nephrotoxic drugs.

Pre treatment with fluorouracil has been reported to inhibit paclitaxel’s cytotoxic action, possibly by preventing tumor cells from entering the G2-M phases of the cell cycle. The effect has also occurred when the two drugs were given simultaneously, suggesting that combination therapy might not be appropriate.

2.3.5. Uses and administration

It is used for its antineoplastic action against malignant neoplasms of the breast and ovary, and in advanced non-small cell lung cancer, but has also been tried in other malignancies including tumors of the head and neck, prostate, and Kaposi’s sarcoma. It is administered as oral dosage and infusion therapy.
2.3.6. Analytical method used in the current investigation and its validation

In the present study the method described by Kim et al. was followed. The present study was carried out in isocratic mode.

2.3.6.1. Instrumentation

The HPLC system (Agilent 1100) was operated in a binary mode with a photodiode array detector, auto injector and column oven. The analysis was performed at 228 nm on a reversed phase C18 column (LiChrospher 100RP-18e, 250x4 mm, 5 μm) maintained at 25º C.

2.3.6.2. Chromatographic conditions

The mobile phase comprised of acetonitrile and water in the ratio of 6:4, filtered before use through a 0.45 μm membrane filter and pumped from the respective solvent reservoirs at a flow rate of 1.0 mL/min. Eluents were monitored using UV detection at a wavelength of 228 nm. The volume of injection port was 100 μL.

2.3.6.3. Preparation of stock solution and standard solutions

Stock solution of paclitaxel was prepared by dissolving 25 mg of paclitaxel in 25 mL of methanol in 25 mL volumetric flask. The stock solution of paclitaxel was subsequently diluted with mobile phase to obtain a series of standard solutions containing 10, 50, 100, 250 and 1000 ng of paclitaxel in 1.0 mL. All standard solutions were filtered.
through 0.45 µm membrane filter and 100 µL of the sample was injected in to HPLC column.

2.3.5.4. Estimation of paclitaxel in brain tissue

Brain tissue collection

Brain tissue was removed after sacrificing the animal, weighed and homogenized with PBS, pH 7.4. The homogenate was stored at -80°C until further analysis.

Sample preparation

Working standard solutions were prepared by serial dilution of a methanolic stock solution (1 mg/ml). Fixed volumes of working standards of paclitaxel were added to the brain tissue samples (100 µl) and vortexed for few seconds. About 1 ml of extraction solvent ethyl acetate was added to the samples and vortexed for 3 minutes. The samples were centrifuged for 15 minutes at 10,000 rpm and the organic layer was transferred into a clean dry glass test tube. The organic solvent was evaporated under nitrogen atmosphere and the residue was dissolved in 500 µl of mobile phase. This solution was filtered through a membrane filter (0.2 µm) and injected to the HPLC system. The concentrations of paclitaxel and the corresponding peak area are given in Table 2.10. The calibration curve was plotted between concentration of paclitaxel and corresponding peak area (shown in Fig 2.10).
2.3.5.5. Quantification and assay validation

Brain tissue homogenate samples were quantified by plotting peak areas against paclitaxel concentrations and respective paclitaxel concentrations were calculated by using least squares linear regression.

- **Linearity**

  To evaluate linearity, tissue calibration curves (10 ng/ml to 1000 ng/ml) were prepared and assayed in triplicate on three separate days.

- **Accuracy and precision**

  Accuracy and precision were also determined by analyzing three concentrations (10 ng/ml, 500 ng/ml, and 1 µg/ml), three samples each over three days.

- **Specificity**

  Specificity was verified by analyzing three independent blank plasma samples and comparing these chromatograms with those obtained after spiking the plasma samples from the same source with paclitaxel.

- **Recovery**

  Recovery of the precipitation procedure was conducted by adding 10 ng, 1 and 10 µg of paclitaxel to the pre-analyzed brain homogenate drug samples containing 10 ng, 1 and 10 µg of paclitaxel respectively, and subjected them to the HPLC method.
(shown in Table 2.12). Three replicate (n=3) spiked biological samples at these different concentration levels were subjected for analysis to calculate mean recovery.

2.3.6. Results and discussion

The mobile phase resulted in a good resolution of paclitaxel. Paclitaxel was eluted at 9.5 minutes with good resolution. The mobile phase composition was optimized at 60:40 (acetonitrile: water) at 1 ml/min with a full run time of 15 min. No interference was found between endogenous tissue components and paclitaxel in the present analytical conditions (Fig. 2.12).

2.3.6.1. Linearity and lower limit of quantification

Calibration graph was constructed by plotting peak area of paclitaxel on ordinate and different concentrations of paclitaxel on abscissa. The graph was found to be linear in the concentration range of 5ng/ml to 1000ng/ml in plasma and from 10ng/ml to 1000ng/ml in brain tissue homogenates. The correlation coefficients were greater than 0.99 for all the matrices confirming the linearity (Table 2.5). The lower limit of quantification (LOQ) was defined as the lowest concentration analyzed with acceptable accuracy and precision was 5ng/ml for plasma and 10ng/ml for tissues and the lower limit of detection (LOD) was found to be 2.5ng/ml.
2.3.6.2. Accuracy and precision

The overall accuracy of the method, expressed in terms of relative standard deviation (RSD) from the true values lay between 90-110% for the concentrations investigated. The precision of the method, given by RSD was less than 5%. At this retention time no other peaks from endogenous plasma components were observed. Inter- and intra-day statistics for accuracy and precision are presented in Table 2.6.

2.3.6.3. Specificity

Representative chromatogram of blank plasma sample was compared with blank plasma spiked with paclitaxel at the lower limit of quantification. No interference’s were detected at the retention time (RT) of paclitaxel. It was further confirmed by comparing the UV absorption spectra of the test sample with that obtained after injecting methanolic solution of the pure drug.

2.3.6.4. Recovery

The recovery of paclitaxel from plasma/tissue was carried out by a simple liquid extraction procedure. Different solvents were tried to enhance the recovery. Extraction with methanol (86.5%), dichloromethane (78.25%) and t-butyl methyl ether (87.2%) resulted in low yield. Ethyl acetate consistently gave good recovery (>95%) at low, medium and high concentrations. Hence for the present study ethyl acetate was used for extracting the drug from plasma/tissue homogenates.
The described HPLC method satisfies all the criteria of validation and hence suitable for analyzing paclitaxel from various biological samples with good accuracy, precision and recovery.

**Table 2.9: Linearity data of paclitaxel in acetonitrile and Brain homogenate**

<table>
<thead>
<tr>
<th>Matrix/Solution</th>
<th>Linearity range (ng/ml)</th>
<th>Equation</th>
<th>Correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>5 to 1000</td>
<td>$y = 338.14x + 607.49$</td>
<td>0.9989</td>
</tr>
<tr>
<td>Brain</td>
<td>10 to 1000</td>
<td>$y = 117.38x + 2796.4$</td>
<td>0.9981</td>
</tr>
</tbody>
</table>
Table 2.10: Concentration versus peak area values for the estimation of paclitaxel in mobile phase

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Peak area (mean, n=3)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1589</td>
<td>5</td>
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<tr>
<td>25</td>
<td>5746</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>13532</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>39980</td>
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<tr>
<td>250</td>
<td>90742</td>
<td>14</td>
</tr>
<tr>
<td>500</td>
<td>166721</td>
<td>14</td>
</tr>
</tbody>
</table>

Fig. 2.10: Calibration curve for the estimation of paclitaxel in mobile phase

Table 2.11: Concentration versus peak area values for the estimation of paclitaxel in brain tissue homogenate

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Peak area (mean, n=3)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1675</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>8742</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>15901</td>
<td>8</td>
</tr>
<tr>
<td>250</td>
<td>31458</td>
<td>12</td>
</tr>
<tr>
<td>500</td>
<td>64456</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig. 2.11: Calibration curve for the estimation of paclitaxel in brain tissue homogenate
Fig. 2.12: HPLC Chromatograms of Paclitaxel  a) blank b) test sample
2.3.7. Validation summary

The results of the study indicated that the method was sensitive, precise and accurate and is suitable for the estimation of paclitaxel in rat tissue samples during the in vivo studies. A summary of the different validation parameters is given in below table 2.12

**Table 2.12. A summary of the different validation parameters**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Validation parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Linearity</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>5, 10 to 1000 ng/ml</td>
</tr>
<tr>
<td></td>
<td>Goodness of fit</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>2</td>
<td><strong>Sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LLOQ</td>
<td>5 ng/ml</td>
</tr>
<tr>
<td></td>
<td>LOD</td>
<td>2.5 ng/ml</td>
</tr>
<tr>
<td>3</td>
<td><strong>Accuracy</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
<td>90% to 110%</td>
</tr>
<tr>
<td></td>
<td>Inter-day</td>
<td>90% to 110%</td>
</tr>
<tr>
<td>4</td>
<td><strong>Precision</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
<td>&lt; 12%</td>
</tr>
<tr>
<td></td>
<td>Inter-day</td>
<td>&lt; 12%</td>
</tr>
<tr>
<td>5</td>
<td><strong>Stability</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Short-term stability</td>
<td>105.5%</td>
</tr>
<tr>
<td></td>
<td>Long-term stability</td>
<td>101.70%</td>
</tr>
<tr>
<td>6</td>
<td><strong>System suitability</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retention time (% CV)</td>
<td>1.7%</td>
</tr>
<tr>
<td></td>
<td>Peak area (% CV)</td>
<td>0.1%</td>
</tr>
<tr>
<td>7</td>
<td><strong>Recovery</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low concentration (10 ng/ml)</td>
<td>98.92%</td>
</tr>
<tr>
<td></td>
<td>Medium concentration (1 µg/ml)</td>
<td>99.16%</td>
</tr>
<tr>
<td></td>
<td>High concentration (10 µg/ml)</td>
<td>96.85%</td>
</tr>
</tbody>
</table>
2.4. Dendrimers

Dendrimers represent a relatively new class of well defined monodisperse macromolecules with striking features such as controlled structure, globular shape, and a high density of “tunable” surface functional groups in their periphery\(^{44}\). A variety of dendrimers have been synthesized and tailored for diverse applications. Some of the commonly encountered types of dendrimers in biological application are based on poly amidoamines, polyamines, polyamides (polypeptides), poly (aryl ethers), and polyesters\(^{45}\). By far the most common dendrimer scaffold is that of polyamidoamine (PAMAM) dendrimers, which are commercially available with a wide variety of generations and peripheral functionalities\(^{46}\). The PAMAM dendrimers are biodegradable and biocompatible. The PAMAM dendrimers generally display concentration dependent toxicity and hemolysis. Here in the present project both anionic (Generation 3.5; -COOH terminated) and cationic terminated dendrimers (Generation 4.0; -NH\(_2\) terminated) are used.

2.4.1. Anionic PAMAM dendrimer

Generation: G 3.5

Molecular weight: 12,931

Terminal groups: 64 terminated carboxylic groups
2.4.2. Cationic PAMAM dendrimer

Generation: G 4.0
Molecular weight: 14,215
Molecular Size: 4.0 nm / 45Å
Terminal groups: 64 terminated amine groups

Fig: 2.13. Structure of PAMAM dendrimer

Fig: 2.14. Structure of PAMAM dendrimer
2.5. Thiol introducing ligands

Thiol introducing ligands bear thiol moieties on the surface and during conjugation reaction with polymer the thiol groups are transferred to polymer to form thiomers. Here in the present work two ligands, cysteamine and 2-iminothiolane, are used in the thiolated dendrimer preparation. 2-iminothiolane is widely used reagent for the disulfide linked cleavable dimmers in the proteins thiolation of polysaccharides and an effective RNA cross linking agent\textsuperscript{47}.

2.5.1. Cysteamine HCl

Structure:

\[
\begin{align*}
\text{HS} & \quad \text{2 HCl} \\
\text{NH}_2 & \quad \text{2 HCl}
\end{align*}
\]

Fig: 2.15. Structure of Cysteamine HCl

Chemical name: C\textsubscript{2}H\textsubscript{7}NS 2HCl

Molecular weight: 77.14

CAS number: 60-23-1

IUPAC name: 2-aminoethanethiol

2.5.2. 2-Iminothiolane HCl (Taut’s reagent)

Structure:

\[
\begin{align*}
\text{S} & \quad \text{NH}_2^+ \text{Cl}^- \\
\end{align*}
\]

Fig: 2.16. Structure of 2-Iminothiolane HCl

Chemical name: C\textsubscript{4}H\textsubscript{7}NS.HCl

Molecular weight: 137.6

CAS number: 4781-83-3
REFERENCES


