8.1. Introduction

Skin acts as the first barrier of the organism towards the external environment. Even though skin is said to efficiently prevent penetration of molecules heavier than 0.5 to 1 kDa, several studies tend to show that nanotechnology well above this limit are able to cross the epidermis and reach the dermis\(^1\). The diffusion process was found to depend on particle size (10–200 nm) and surface charge, as well as on collagen concentration (1.5–5 mgmL\(^{-1}\)). The presence of human dermal fibroblasts within the hydrogels also significantly impacted on the behaviour of the particles\(^1\).

Liposomes are vesicles of varying size consisting of a spherical lipid bilayer and an aqueous inner compartment that are generated in vitro. Liposomes were first reported as carriers for transdermal drug delivery in 1980\(^2\). However, liposomes were shown to be unsuitable vesicular carriers for the penetration of the skin barrier due to its rigidity which hinders their passage though the skin barrier. Recently, novel vesicular carriers were designed, including ultradeformable liposomes, ethosomes, binary ethosomes, solid lipid nanoparticles and niosomes\(^3\).

Ultradeformable liposomes (transfersomes) consist of phospholipids, an edge activator that increases deformability of the phospholipid bilayers. Applied on the skin surface, these elastic vesicles are able to squeeze through intercellular regions of the stratum corneum under the influence of the water-activity gradient. After passage, transfersomes were distributed between the cells via intercellular route, and accumulated in the subcutaneous tissue. Moreover, transfersomes can effectively protect the drug against undesired rapid clearance from cutaneous blood vessels increasing the drug circulation time and bioavailability\(^4\).

Azathioprine (Imuran) is a drug used to treat swelling and pain in arthritis. It belongs to a class of medications called disease-modifying antirheumatic drugs (DMARDs) or immunosuppressants. This class of medicines can decrease joint damage and disability. The most common diseases treated with Imuran are dermatomyositis, polymyositis, systemic lupus erythematosus, vasculitis, multiple sclerosis, myasthenia gravis, autoimmune hepatitis and inflammatory bowel disease. It also is used to prevent rejection of transplanted organs\(^5\).
The most common side effects associated with oral ingestion of AZT are nausea, vomiting, decreased appetite, liver function abnormalities, low white blood cell counts, and infection\(^6\). Azathioprine can cause liver toxicity. Less often, azathioprine may cause hepatitis (liver swelling or damage), pancreatitis (swelling or damage to the pancreas gland behind the stomach, which can cause abdominal pain) or an allergic reaction that may include a flu-like illness or a rash\(^7\).

Moses et.al.\(^8\) reported a case of cholestatic hepatitis developed one week after exposure to AZT. The subsequent prolonged cholestatic phase was followed by full clinical remission. This problem can be solved by changing route of administration.

Transdermal drug delivery is defined as the non-invasive delivery of medications through the skin surface. They ensure controlled absorption and more uniform plasma drug concentrations. Bioavailability is improved by avoiding first-pass hepatic metabolism and enzymatic or pH-associated deactivation. Delivery of the drug is via a simple and painless application. There is increased flexibility in terminating drug administration by removal from skin surface. Patient compliance is improved as formulations are simple, non-invasive, and convenient\(^9\).

It was previously proved that topical application of azathioprine shows safety and efficacy in treated diseases. Systemic effect of drug was reduced and formulation works as well as oral delivery\(^10\). Azathioprine liposomes and azathioprine silica nanoparticles were evaluated and optimized to get better efficacy and improvement of bioavailability. Authors proved that the formulations were stable and fulfil their rationals. They can target the diseased site and release the drug in a controlled manner and produces synergetic effect to the inflammatory sites\(^11\).

The present study was carried out to develop and optimize azathioprine Transfersomes by thin film hydration technique. A trial and error method was used to evaluate edge activator and its proportion with phospholipid for better deformability, entrapment efficiency, and drug penetration. Pre-formulation and drug-excipient compatibility was also required to find out drug purity and experiment feasibility.

**8.2. Pre-formulation Study**

Pre-formulation studies have a significant part to play in anticipating formulation problems and identifying logical path in both liquid and solid dosage form
technology. This information decides many of the subsequent events and approaches in formulation development. This first learning phase is known as Pre-formulation. From this stage, physicochemical properties of Active Pharmaceutical Ingredients (APIs) are characterized. It involves the application of biopharmaceutical principles to the physicochemical parameters of drug substance\textsuperscript{12}.

**Solubility determination**

The solubility of a substance is the amount of that substance that will dissolve in a given amount of solvent. Solubility is a quantitative term\textsuperscript{13}. Drugs must be water soluble so our bodies can absorb them. Yet, there is more than what meets the eye. This problem is a complex one with far-reaching components that make it a critical problem for address in drug development. Drugs that are not-polar are not soluble in water and thus cannot be absorbed by tissue very well. So this is very important determination parameter\textsuperscript{14}.

Azathioprine solubility was determined using various solvents like chloroform, ether, ethanol, methanol, acetone and water. Different dilute alkali solutions were also tried for solubility determination.

**Melting point**

The melting point (or, rarely, liquefaction point) of a solid is the temperature at which it changes state from solid to liquid at atmospheric pressure. At the melting point the solid and liquid phase exists in equilibrium\textsuperscript{15}.

The melting point of a drug can be measured using three techniques\textsuperscript{15}:-

1. Capillary Melting
2. Hot Stage Microscopy
3. Differential scanning calorimetry (DSC) or thermal Analysis.

Differential scanning calorimetry (DSC) monitors heat effects associated with phase transitions and chemical reactions as a function of temperature and is a very informative method in physical characterization of a compound. Changes in melting temperature and energy gives information about, for instance, content of amorphous material. Thus, the melting endotherm can be used for determination of purity of the sample\textsuperscript{16}.
Therefore, Melting point of Azathioprine was measured by DSC thermogram. In DSC study, the instrument measures the amount of energy required to keep the sample at the same temperature as the reference i.e. it measures the enthalpy of transition\textsuperscript{16}.

**Calibration curve of Azathioprine**

An analytical technique is a method that is used to determine the concentration of a chemical compound or chemical element. Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. There are a wide variety of techniques used for analysis, from simple weighing (gravimetric analysis) to titrations (titrimetric) to very advanced techniques using highly specialized instrumentation.

The most common technique used in analytical chemistry is Spectroscopy, based on the differential interaction of the analyte along with electromagnetic radiation\textsuperscript{17}. Most of drugs have aromatic rings and/or double bonds as part of their structure and absorb light in UV range, UV spectroscopy being a fairly accurate and simple method is a performed estimation technique at early preformulation stages\textsuperscript{12}.

Calibration curve of azathioprine was taken in methanol. Concentration of stock solution was 100 mcg/ml. Serial dilutions were made to get concentrations 0.4, 0.8, 1.2, 1.6 and 2 mcg/ml respectively. Scanning was done from 200 to 400 nm to determine absorption spectrum maxima and linearity.

**Drug-Excipients compatibility study (DECS)**

Drug is an active part of dosages form and it is mainly responsible for therapeutic value. Excipients are substances which are included along with drugs being formulated in a dosage form so as to impart specific qualities to them\textsuperscript{18}. Any physical or chemical interaction between drug and excipient can affect bioavailability and stability of drug. By performing DECS we can know the possible reaction before formulating final dosage form. Using DECS data we can select the suitable type of the excipient with the chemical entities emerging in drug discovery programs. Now, USFDA has made it compulsory to submit DECS data for any new coming formulation before its approval\textsuperscript{19}.
All the samples of drug-excipient blends are kept for 1-3 weeks at specified storage conditions. Then sample is physically observed for (1) caking, (2) liquefaction, (3) discoloration, (4) odor, (5) gel formation. It is then assayed by DSC. It requires only 5 mg of drug in a 50% mixture with the excipients to maximize the likelihood of obscuring an interaction. Mixture should be examined under N2 to eliminate oxidative and pyrolytic effects at a standard heating rate on DSC, over a temperature range, which will encompass any thermal changes due to both the drug and appearance or disappearance one or more peaks in thermograms of drug excipient mixtures are considered of indication of interaction\(^9\).

The physicochemical compatibility between drug and lipid in the transfersomes was studied by Differential Scanning Calorimeter at Nootan Pharmacy college, Visnagar. The samples of drug, lipid and its physical mixture were placed in aluminium pans and heated from ambient temperature to 250\(^0\)C in an atmosphere of nitrogen at a pre-programmed heating rate of 10\(^0\)C min\(^{-1}\). The thermo-grams obtained for AZT, lipid and physical mixtures of AZT with lipid were compared.

### 8.3. Materials and Methods

Azathioprine was procured as a gift sample from RPG life science, Ankleshwar. Span 20, Tween 20, Span 80 and Tween 80 was purchased from S.D Fine Chemical Ltd.
Mumbai, India. Phospholipon 90 G was gifted from Lipoid AG, Germany. All other ingredients used were of analytical grade.

**Preparation method of Azathioprine Transfersomes**

Transfersomes containing Azathioprine were prepared with slight modification. First, phospholipid (PC), edge activator (EA) and drug Azathioprine were taken in a clean, dry, round bottom flask, and then this lipid mixture was dissolved in organic solvents i.e. chloroform: methanol (2:1). The organic solvents were evaporated until complete dryness under reduced pressure using a rotary evaporator (Remi Instruments, Mumbai, India). The deposited lipid film was hydrated with PBS (pH 7.4) by 50 rpm for 1 h at 50°C and the resulting vesicles were swollen for 2 h at room temperature to get large multilamellar vesicles (LMLV). The thick suspension thus obtained was broken by probe sonication (53 KHz) for 30 min at 4°C for achieved desired vesicle size\(^2\). Composition of AZT-TFS is described in Table 8.1.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azathioprine</td>
<td>50 mg/ml (5% w/v)</td>
</tr>
<tr>
<td>PC</td>
<td>Phopholipon 90 G</td>
</tr>
<tr>
<td>EA</td>
<td>SPAN 80, TWEEN 80</td>
</tr>
<tr>
<td>PC+EA</td>
<td>100 mg/ml (10% w/v)</td>
</tr>
<tr>
<td>Chloroform: Methanol</td>
<td>25 % v/v (2:1)</td>
</tr>
<tr>
<td>PBS 7.4</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Table 8.1: Composition of AZT TFS**

8.4. **Optimization of Azathioprine Transfersomes**

In optimization of a design, the design objective could be simply to minimize the cost of production or to maximize the efficiency of production.

Pharmaceutical optimization has been defined as the implementation of systematic approaches to establish the best possible combination of materials and/or process variables under a given set of conditions that will result in the production of a quality pharmaceutical product with predetermined and specified characteristics each time it is manufactured\(^1\).
Trial and error is a fundamental method of solving problems. It is characterized by repeated, varied attempts which are continued until success, or until the agent stops trying. It is an unsystematic method which does not employ insight, theory or organised methodology. According to W.H. Thorpe, the term was devised by C. Lloyd Morgan after trying out similar phrases "trial and failure" and "trial and practice". Edward Thorndike showed how to manage a trial and error experiment in the laboratory. Trial and error is also a heuristic method of problem solving, repair, tuning, or obtaining knowledge. This approach is far more successful with simple problems and in games, and is often resorted to when no apparent rule applies. This does not mean that the approach need be careless, for an individual can be methodical in manipulating the variables in an attempt to sort through possibilities that may result in success. Nevertheless, this method is often used by people who have little knowledge in the problem area. It is possible to use trial and error to find all solutions or the best solution, when a testably finite number of possible solutions exist. The scientific method can be regarded as containing an element of trial and error in its formulation and testing of hypotheses.

In this investigation, trial-and-error method was used. Edge activators are bilayer softening component, such as biocompatible surfactant in to which an amphiphilic drug is added to increase lipid bilayer flexibility and permeability. An edge activator is often a single chain surfactant that destabilizes the lipid bilayer of the vesicles and increases the deformability of the bilayer by lowering its interfacial tension.

Span 80 and Tween 80 were used to optimize their action on deformability. Proportion of edge activator concentration can be varied from 5-25% of phosphatidyl choline shows a variable effect in entrapment efficiency. Present study varies edge activator concentration from 1-9% of lipid to optimize ratio of PC: EA for best entrapment efficiency and deformability of AZT-TFS (Table 8.2). Total 18 batches were taken to optimize formulation. Batches composition were selected in such a way that concentration of edge activator were in an increasing quantity. So effect of edge activator and its amount can give significant results.
8.5. Characterization of Azathioprine Transfersomes

Azathioprine transfersomes were characterized using following parameters.

- Vesicle encapsulation efficiency
- Deformability Index (Vesicle Elasticity Measurement)
- Particle Size and Surface Charge.
- Transmission Electron Microscopy
- Fourier Transform infrared Spectroscopy (FTIR)
- Physical stability

Vesicle encapsulation efficiency

Prepared vesicular formulations were separated from unentrapped drug by the centrifugation method. The vesicular fraction was added with minimum amount of triton X-100 (0.5%, w/v) to disrupt the vesicles and liberated drug was estimated spectrophotometrically\(^2\).

Deformability Index (Vesicle Elasticity Measurement)

Comparative measurement of elasticity of the bilayer of transfersomes was carried out by extrusion measurement. Briefly, the vesicles were extruded through polycarbonate filter with a pore size of 100 nm at 2.5 bar pressure. The elasticity of vesicle was expressed in terms of deformability index which is proportional to \( j(rv/rp)^2 \) where, \( j \) is

Table 8.2: Optimization batches of AZT-TFS

<table>
<thead>
<tr>
<th>PC:EA</th>
<th>%W/V PC</th>
<th>BATCH NO.</th>
<th>%W/V SPAN 80</th>
<th>BATCH NO.</th>
<th>%W/V TWEEN 80</th>
</tr>
</thead>
<tbody>
<tr>
<td>9:1</td>
<td>9</td>
<td>A1</td>
<td>1</td>
<td>A10</td>
<td>1</td>
</tr>
<tr>
<td>8:2</td>
<td>8</td>
<td>A2</td>
<td>2</td>
<td>A11</td>
<td>2</td>
</tr>
<tr>
<td>7:3</td>
<td>7</td>
<td>A3</td>
<td>3</td>
<td>A12</td>
<td>3</td>
</tr>
<tr>
<td>6:4</td>
<td>6</td>
<td>A4</td>
<td>4</td>
<td>A13</td>
<td>4</td>
</tr>
<tr>
<td>5:5</td>
<td>5</td>
<td>A5</td>
<td>5</td>
<td>A14</td>
<td>5</td>
</tr>
<tr>
<td>4:6</td>
<td>4</td>
<td>A6</td>
<td>6</td>
<td>A15</td>
<td>6</td>
</tr>
<tr>
<td>3:7</td>
<td>3</td>
<td>A7</td>
<td>7</td>
<td>A16</td>
<td>7</td>
</tr>
<tr>
<td>2:8</td>
<td>2</td>
<td>A8</td>
<td>8</td>
<td>A17</td>
<td>8</td>
</tr>
<tr>
<td>1:9</td>
<td>1</td>
<td>A9</td>
<td>9</td>
<td>A18</td>
<td>9</td>
</tr>
</tbody>
</table>
the amount of suspension, which was extruded in 5 min through a polycarbonate filter of 100 nm pore size, \( r_v \) the size of vesicle and \( r_p \) the pore size of membrane\(^{24}\).

**Particle size and surface charge**

The droplet size and zeta potential of the transfersomes was determined by a Malvern Particle sizer and Zeta Potential Analyzer (Malvern Instruments Ltd., UK) at M. S. University of Baroda, Vadodara. 1 ml of the transfersome suspension was diluted with deionized water.

**Transmission Electron Microscopy**

Transmission Electron Microscopy (TEM) was used to visualize the transfersomal vesicles using TEM microscope (Model: Philips Tecnai 20 G2, Holland) at SICART, Vallabh Vidhynagar. The vesicles were dried on a copper grid and adsorbed with filter paper\(^{24}\). After drying, the sample was viewed under the microscope at 10–100 k magnification at an accelerating voltage of 100 kV.

**Fourier Transform Infrared Spectroscopy**

Fourier transform infrared spectroscopy (FTIR) study was carried out to confirm structure of formulation. FTIR spectra of pure drug, lipid and formulated Transfersomes containing drug were recorded on FTIR Spectrophotometer (SHIMADZU, Japan) at Ratnamani Health care, Indrad. The scanning range was from 4000 to 600 cm\(^{-1}\) and the resolution was 1 cm\(^{-1}\). The scans were evaluated for presence of principal peaks of drug, shifting and masking of drug peaks, and appearance of new peaks due to excipient interaction\(^{24}\).

**Physical stability**

This study was carried out in stability chambers at Ratnamni health care. After measuring the initial percentage entrapment of the drug in the various formulations, the three batches of the same formulation were stored in sealed glass ampoules at different temperature\(^ {25}\). Physical stability was carried out at according to ICH, at refrigeration temperature (4±2 °C) and room temperature (25±2 °C/65% RH ± 5% RH) for a period of 3 months. After every month, percentage entrapment of the drug was determined in the formulations to study the stability of transfersomes at different
conditions and amount of drug leaked out at body temperature. The percentage drug lost was calculated taking the initial entrapment of drug as 100%.

### 8.6. Results & Discussion

The present investigation was undertaken to formulate and evaluate Transfersomes of Azathioprine by thin film hydration method using Phospholipon 90 G as a lipid and Span 80 and Tween 80 as an edge activator to enhance penetrability through skin. Optimization of formulation was undertaken using trial-and-error method and stability of formulation was determined at different storage temperature.

**Preformulation study**

By comparing the physicochemical properties of each drug candidate with in a therapeutic group, the preformulation scientist can assist the synthetic chemist to identify the optimum molecule, provide the biologist with suitable vehicles to elicit pharmacological response and advise the bulk chemist about the selection and production of the best excipients with appropriate particle size and morphology for subsequent processing.

**Solubility determination**

Azathioprine was insoluble in water, ethanol, chloroform, and ether. It was soluble in dichloromethane. It was freely soluble in alkali hydroxides and carbonates such as sodium chloride, sodium or potassium carbonate, phosphate buffers (pH 5.8 to 8.0) etc. Azathioprine is stable in solution at neutral or acid pH.

**Melting point determination**

DSC thermogram of azathioprine explains that melting point of drug was 261.83 °C (Figure 8.2). Reported range of melting point of AZT is 245-260 °C. So AZT has confirmed its physicochemical property.
Figure 8.2: DSC spectra of Azathioprine

**Calibration curve of Azathioprine for %EE**

A *calibration curve* is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. UV spectroscopy being a fairly accurate and simple method is a performed estimation technique at early preformulation stages.

The solutions of azathioprine were scanned on spectrophotometer in the UV range 200 - 400 nm. In spectrum Azathioprine showed absorbance maximum at 281 nm (Figure 8.3). The spectrum was recorded at 281 nm. The calibration plot was constructed as Absorbance vs concentration (Figure 8.4). Linearity equation was $y = 0.4484x - 0.0085$ with $R^2$ value 0.9987 for azathioprine determination for encapsulation efficiency.
Figure 8.3: UV spectrum of AZT at 281 nm

Figure 8.4: Calibration curve of AZT in methanol

**Drug Excipients Compatibility Study**

A comparison of the thermograms of pure drug, lipid and physical mixture has been displayed in Figure 8.5. In case of DSC thermogram of azathioprine in mixture, a sharp endothermic peak was observed at 268.77°C and an exothermic peak was observed at 263.39°C. It was quite similar with peak of pure azathioprine corresponding to melting point of drug (Figure 8.2). Thermogram of lipid exhibits three broad endothermic peaks characteristics of amorphous substances at 163.0°C, 190.5°C and 194.8°C. The DSC analysis of the physical mixture of the drug and the lipid showed 2 endothermic peaks (Figure 8.5). First peak at 169.13 °C represents
glass transition temperature of phospholipid and second peak revealed a negligible change in the melting point of Azathioprine in the presence of lipid mixture studied (263.39°C). It can be concluded from the comparison of the peak temperatures and endothermic transition contours of AZT, SPC, the physical mixture that no any chemical reactions and incompatibilities between ingredients and are chemically stable.

Figure 8.5: DSC spectra of mixture of Azathioprine and lipid

**Optimization of formulation**

Azathioprine transfersomes were successfully prepared by thin film hydration technique. Transfersomal suspension was like milky off white colored liquid because of off white colored drug (Figure 8.6).
Vesicle encapsulation efficiency

Surfactants are found in many existing therapeutic, cosmetic, and agro-chemical preparations. Surfactants have been employed to enhance the permeation rates of several drugs via transdermal route. For the surfactant molecules to interact with the deeper protein-rich regions in a normal corneum, they must diffuse through the lipid region. After binding to the proteins, surfactant causes the protein to denature, leading to the swelling of stratum corneum. Solubilization of fluid lipids and abstraction of calcium or other multivalent ions to reduce corneocyte adhesion enhances the accessibility of the proteins in the lower regions of the stratum corneum.  

From 9:1 to 1:9 ratios for PC: EA for both Span 80 (A1 to A9) and Tween 80 (A10 to A18) edge activators, % entrapment efficiency was determined (Table 8.3). %EE was plotted against %W/V of EA (Figure 8.7). Plot represents decrease in entrapment efficiency with the amount of EA increases. This is because of decreasing amount of lipid and so strengthening of bilayer of vesicles. For Span 80, it was decreased from 71.28±0.81% to 36.93±0.49%, whereas for Tween 80, it was decreased from 63.82±0.71% to 41.76±0.41%.

Table 8.3 demonstrates the effect of EA on the %EE of different transfersomal formulations. The % entrapment efficiency of deformable vesicles formulations were found to be in the range of 36.93±0.49% to 71.28±0.81%. The ratio 9:1 showed optimum %EE. Upon incorporation of EA in low concentration, growth in vesicle size occurred, whereas, further increase in the content of edge activator may have led to pore formation in the bilayers. When EA concentration exceeded 50%, mixed
micelles coexisted with the transfersomes, with the consequence of lower drug entrapment due to the rigidity and smaller size of mixed micelles. Patel et al. reported that, the effect of phospholipids and EA ratio in the lipid components of vesicles on the entrapment efficiency of lipophilic drug, curcumin, the efficiency decreased with increasing surfactant concentration\textsuperscript{30, 34}.

The effect of phospholipids and edge activator ratio in the lipid components of vesicles on the entrapment efficiency of lipophilic drug, azathioprine, efficiency decreased with increasing surfactant concentration and thus increased with increasing PC concentration but have certain limit of PC: EA\textsuperscript{30}. Up to ratio 5:5, %EE is acceptable (approx. 60%) for Span 80. For Tween 80, acceptable ratio is up to 3:7 (60% EE).

Table 8.3: Optimization results of AZT-TFS

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>PC:EA</th>
<th>%EE</th>
<th>DI</th>
<th>Particle size in nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>9:1</td>
<td>71.28±0.81</td>
<td>9.73±1.95</td>
<td>312.8</td>
</tr>
<tr>
<td>A2</td>
<td>8:2</td>
<td>64.47±0.44</td>
<td>10.98±0.95</td>
<td>287.4</td>
</tr>
<tr>
<td>A3</td>
<td>7:3</td>
<td>64.72±0.31</td>
<td>16.28±0.83</td>
<td>268.9</td>
</tr>
<tr>
<td>A4</td>
<td>6:4</td>
<td>62.98±0.60</td>
<td>24.81±0.92</td>
<td>244.9</td>
</tr>
<tr>
<td>A5</td>
<td>5:5</td>
<td>58.79±0.27</td>
<td>26.38±0.34</td>
<td>243.3</td>
</tr>
<tr>
<td>A6</td>
<td>4:6</td>
<td>42.71±0.62</td>
<td>25.80±0.91</td>
<td>245.7</td>
</tr>
<tr>
<td>A7</td>
<td>3:7</td>
<td>39.68±0.70</td>
<td>26.88±1.20</td>
<td>240.7</td>
</tr>
<tr>
<td>A8</td>
<td>2:8</td>
<td>37.13±0.31</td>
<td>27.49±1.46</td>
<td>241.4</td>
</tr>
<tr>
<td>A9</td>
<td>1:9</td>
<td>36.93±0.49</td>
<td>26.11±1.76</td>
<td>240.5</td>
</tr>
<tr>
<td>A10</td>
<td>9:1</td>
<td>63.82±0.71</td>
<td>9.18±2.10</td>
<td>371.8</td>
</tr>
<tr>
<td>A11</td>
<td>8:2</td>
<td>61.51±0.14</td>
<td>8.83±3.33</td>
<td>364.5</td>
</tr>
<tr>
<td>A12</td>
<td>7:3</td>
<td>59.14±0.71</td>
<td>17.15±0.68</td>
<td>342.3</td>
</tr>
<tr>
<td>A13</td>
<td>6:4</td>
<td>57.63±0.44</td>
<td>22.87±3.29</td>
<td>320.8</td>
</tr>
<tr>
<td>A14</td>
<td>5:5</td>
<td>55.57±0.52</td>
<td>20.61±1.76</td>
<td>291.8</td>
</tr>
<tr>
<td>A15</td>
<td>4:6</td>
<td>52.32±0.44</td>
<td>24.70±1.57</td>
<td>275.5</td>
</tr>
<tr>
<td>A16</td>
<td>3:7</td>
<td>48.16±0.99</td>
<td>21.89±2.93</td>
<td>255.6</td>
</tr>
<tr>
<td>A17</td>
<td>2:8</td>
<td>45.45±0.79</td>
<td>24.13±1.27</td>
<td>252.7</td>
</tr>
<tr>
<td>A18</td>
<td>1:9</td>
<td>41.76±0.41</td>
<td>28.18±3.00</td>
<td>256.9</td>
</tr>
</tbody>
</table>

\textit{Modi Chetna D.} 201 \textit{Ph. D. Thesis}
These results are related to the HLB values of these edge activators. They are 4.3 & 15 for Span 80 and Tween 80 respectively. Park et al. reported that the enhancers containing the ethylene oxide chain length of 2-5, HLB value of 7-9 and an alkyl chain length of C16 -C18 were effective promoters of ibuprofen flux. Graph shows rate of lowering %EE is high in Span 80 as compared to Tween 80.

In a study the parabolic relation between the piroxicam absorption and the polyoxyethylene length of nonionic surfactant was observed. Polyoxyethylene length from 5 to 15 was found to enhance percutaneous absorption to a greater extent. Based on these HLB values, the affinity for lipids was expected to be in the order of Span 80 > Tween 80. This consideration explains the higher EE% encountered with Span 80 compared to Tween 80. The entrapment efficiency of the Span 80 formulation was high because of the increase in the ratio of lipid volume in the vesicles as compared to the encapsulated aqueous volume.

**Deformability index**

Surfactants induce a concentration-dependent biphasic action with respect to alteration of skin permeability. Table 8.3 demonstrates the effect of EA on the deformability of different transfersomal formulations. The deformability index of
transfersomal formulations were found to be in the range of 8.83±3.33 to 28.18±3.00. At low concentrations, surfactants increase the permeability of the skin to many substances probably because they penetrate the skin and disrupt the skin barrier function. For the surfactant molecules to interact with the deeper protein-rich regions in a normal corneum, they must diffuse through the lipid region. Low concentrations of surfactant may emulsify stratum corneum lipids and improve permeability; however, higher concentrations promote the formation of micelles in the vehicle that trap permeate and decrease permeability.

Figure 8.8 demonstrate that deformability increases with increasing EA, but above 50%, deformability of vesicles being little constant. Span 80 (HLB 4.3) and Tween 80 (HLB 15) were found to interact with the human epidermis in degrees which were dependent on the polarity of the surfactant. Tween 80 which is more hydrophilic than Span 80 was found to be less effective in enhancing 5-fluorouracil skin penetration. Span 80 was found to affect the intercellular lipids by making them more fluid and enhancing the diffusivity.

Deformability of vesicles with Tween 80 was almost equal to Span 80 (Figure 8.8). Arellano reported that Tween 80 decreased diclofenac penetration rate due to a
decrease in thermodynamic activity as a result of micellar complexation. In contrast, the more hydrophobic sorbitans enhanced penetration due to changes in the barrier properties of skin. Diclofenac flux from the gel containing Span 80 was more than 10 times larger than that from the gel with Tween 80.\textsuperscript{34}

It is also proved that as the transition temperature of surfactants increase it leads to increase in the entrapment efficiency and decrease in the permeability. Spans with highest phase transition temperature provide the highest entrapment for the drug. The drug leaching from the vesicles can be reduced due to high phase transition temperature and low permeability.\textsuperscript{23}

Table 8.3 explains maximum concentration of Span 80 (A9) has low deformation than that of Tween 80 (A18). This is because being less bulky tween 80 showed higher deformations when compared to steroid like bulkier groups of spans. The less deformation indicates hydrophobicity. Hydrophilic hole formation can also be reduced and decreases the membrane fluidity. Bulkier ester surfactants being less flexible, less deformable.\textsuperscript{34}

**Particle size and surface charge**

Particle sizes of the formulations having vesicles were within range from 240.5 to 371.8 nm (Table 8.3). There exist a relationship with vesicle size and HLB. The use of surfactant with increased hydrophobicity results in decreased surface energy leading to the formation of vesicle with smaller size. Tween 80 is more hydrophilic than Span 80. Figure 8.8 clearly explains that smaller size of vesicles with Span 80. While considering hydrophilic surfactants their aqueous solubility does not allow them to get into a compact vesicular structure, resulting into an aggregated coalesced lamellar nature. But in case of elastic vesicles, the selection of surfactant to get smaller size vesicle may be irrelevant, as they are ultra deformable.\textsuperscript{23}
Vesicle size of AZT-TFS (A4) is reported in Figure 8.10. Results (Figure 8.9) revealed that concentration of EA has a significant effect on size of vesicles. From the observations, it was evident that changes in the concentration of EA significantly affect the particle size. The vesicle size was increased with increase in lipid concentration. This may be due to the rigid structure of lipid\(^{35}\). Results of polydispersity index (0.25–0.35) revealed that the deformable vesicles were uniformly distributed and homogenous mixture (Figure 8.10).
Figure 8.10: Particle size distribution of AZT-TFS (A4)

Figure 8.11: Zeta potential report of AZT-TFS (A4)
The surface-charge properties of the prepared formulation A4 was investigated and results showed negative charges on their surfaces with zeta value ranging from -24.9 mV (Figure 8.11). The zeta potential findings showed a good degree of stability for all the prepared formulations\textsuperscript{20}. Many non–ionic surfactants like tween 80 exhibit a low negative zeta potential, leading to repulsion between the bilayers. The reason behind this low zeta potential value may be due to the chemical structure. The disadvantage is that there is a significant increase in the size of vesicles\textsuperscript{23}.

From the optimization, Table 8.4 represents AZT-TFS batch with evaluation results.

Table 8.4: Optimized batch of AZT-TFS

<table>
<thead>
<tr>
<th>PC:EA</th>
<th>BATCH NO.</th>
<th>%EE</th>
<th>DI</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:4</td>
<td>A4</td>
<td>62.98±0.60</td>
<td>24.81±0.92</td>
<td>244.9</td>
</tr>
</tbody>
</table>

**Transmission electron microscopy**

Transmission electron microscopy was used to characterize AZT transfersomes. The morphology of the optimized formulation was observed using TEM. The TEM micrographs showed vesicles with uniform spherical shape of batch A4. The vesicles appeared non-aggregated characterized by smooth surface with narrow size distribution (Figure 8.12). These carriers invariably appeared as unilamellar vesicles.

![Figure 8.12: TEM image of AZT-TFS (A4)](image)

Modi Chetna D.
**FTIR study**

The Fourier transform infrared spectroscopy (FTIR) spectrum of the AZT-TFS was compared with pure drug AZT and lipid spectrums.

FTIR spectra of lipid phospholipon 90 G was provided by Lipoid, Germany. Peaks were observed at 3439.9 cm\(^{-1}\), 2060.0 cm\(^{-1}\), 1657.7 cm\(^{-1}\), 1118.0 cm\(^{-1}\) and 532.4 cm\(^{-1}\) (Figure 8.13).

From FTIR, the principal peaks of AZT depicted a number of characteristic peaks at 916, 1228, 1382, 1529, 1589, 3115 cm\(^{-1}\) which may be attributed to the C-H deformation, C-N stretch, CH\(_3\) bend, CNO\(_2\) stretch, C=N and N-H stretch respectively, confirming the purity of the drug (Figure 8.14).

In the FTIR spectra of the formulation (transfersomes) A4, the major peaks of drugs were observed and merged at wave numbers 3115 & 1589 cm\(^{-1}\), whereas, all peaks of lipid were observed at wave numbers 3450 cm\(^{-1}\), 2060 cm\(^{-1}\), 1650 cm\(^{-1}\) and 1118 cm\(^{-1}\). No additional peaks were observed with the formulation, possibly because of encapsulation of drug in vesicles (Figure 8.15).
Figure 8.13: IR spectra of lipid
Figure 8.14: FTIR spectra of AZT
Figure 8.15: FTIR spectra of AZT-TFS (A4)
This study reveals that all major lipid peaks were observed in spectra of azathioprine transfersomes and azathioprine peaks were merged and shifted in the same. So it was concluded that drug was completely incorporated in transfersomes.

**Physical stability**

Drug stability concerns about drug product safety, efficacy, and quality, found it to appropriate. The percentage drug loss from the formulations was used as a measure of storage stability. The initial entrapped drug in vesicular system was considered as 100%. The leakage of drug from transfersomes vesicles was not more significant at refrigerated condition. This fact can be justified as 98.57%, 98.24% & 98.17% drug was remaining in transfersomes after 1, 2 & 3 months respectively at 4±2°C. A significant loss of entrapped drug was found at the end of three month period when transfersomes dispersions stored at high temperature i.e. 25±2°C (Figure 8.16). The drug leakage at elevated temperatures may be related to the degradation of lipid in the bilayers resulting in defects in membrane packing them leaky. So there is a requirement of improve stability of transfersomal dispersion by incorporating them in semisolid form. It is feasible for transdermal application also. These properties help in breaking or bursting of vesicles after penetration to skin. It can be concluded that for better stability, the formulations should be stored at low temperature in refrigerator.

![Figure 8.16: Physical stability of AZT-TFS (A4)](image-url)
8.7. Conclusion

From the trial-and-error optimization design, azathioprine transfersomes were successfully evaluated. Preformulation study confirms purity of drug and compatibility of drug with excipients using DSC study. Effect of edge activators SPAN 80 and TWEEN 80 were found significant with the experimental results. It was confirmed that the increasing the concentration of Edge activator increases the deformability of transfersomes. But its %EE was decreased because of less concentration of lipid in vesicles. Ratio of PC: EA is important for %EE and Deformability character of vesicle, which will improve permeation. The prepared formulations were suitable for transdermal drug delivery. From characterization parameters of TEM, FTIR, and stability study, it was concluded that the formulation has acceptable morphology and particle size, no any chemical interaction and was stable at refrigerated condition respectively.

8.8. References


