Design of a Validated Stability Indicating HPLC Method for the analysis of Patrin-2 (Lomeguatrib)

Title

by

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A thesis submitted in partial fulfilment for the requirements for the degree of MSc (by Research) at the University of Central Lancashire

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DECLARATION

I declare that while registered as a candidate for this degree I have not been registered as a candidate for any other award from an academic institution. The work present in this thesis, except where otherwise stated, is based on my own research and has not been submitted previously for any other award in this or any other University.

Signed

Jane Chheda
ABSTRACT

Lomeguatrib or Patrin-2 has been formulated to work in combination with Temozolomide. It does so by blocking the action of ATase (MGMT), which repairs specific kinds of DNA damage and is particularly responsible for resistance to anti-cancer treatments. Patrin-2 acts as a pseudosubstrate inactivator of MGMT. It thus, helps Temozolomide proceed with it’s designed action, which now cannot be stopped by neither MGMT nor MMR. The degradation behaviour of Patrin-2 was studied by subjecting it to various stress conditions. A validated stability indicating high-performance liquid chromatography method was established for the analysis of the drug in the presence of its various degradation products. An acceptable separation of the drug and its degradation products was achieved on a C18 reverse phased column (Symmetry 300) using a mobile phase that consisted of 10mM TBAA, 10mM SDS and 25mM citric acid in 35:65 acetonitrile and HPLC grade water. 285nm was determined as the wavelength max for Patrin-2, however, three wavelengths (240nm, 254nm, 285nm) were selected for the study, so as to make sure, no degradation product of Patrin-2 goes undetected. The method was validated for linearity, accuracy, precision, selectivity, specificity and robustness. It proved to be linear over the range of concentration of 1-5mg/dL (n=2) with a correlation coefficient of 0.999. The RSD was found to be as low as 0.029 and 0.233 for its repeatability and reproducibility. The drug was found to be unstable when exposed to 0.4M of 30%H2O2. It was highly unstable in acidic condition, undergoing complete degradation in less than an hour when subjected to 0.1M H2SO4. It underwent high degradation when subjected to photolytic stress (UV radiations) or thermal stress (incubated at 50°C and 80°C). The drug was solubilised in n-methylpyrrolidone, one of the components used to tabulate it, but the results could not be confirmed, as no degradation product could be seen, but a change in shape of the peak was observed.
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Many things might have not gone the way they were expected to go or planned to go, but this has truly been a great learning experience for me. I can summarise it with Stuart Hill’s words,

“If you reach for the stars, you just might land on a decently sized hill.”
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4BTG</td>
<td>O$_6$-(4-bromothenyl) guanine (common name: Patrin-2)</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredients</td>
</tr>
<tr>
<td>ATase</td>
<td>Alkyltransferase</td>
</tr>
<tr>
<td>AZUR</td>
<td>A chromatography software’s name (French)</td>
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<tr>
<td>BER</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>gms</td>
<td>Grams</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>Sulphuric Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>MGMT</td>
<td>O$_6$-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mins</td>
<td>Minutes</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MPA</td>
<td>Mean Peak Area</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpyrrolidone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBAA</td>
<td>Tetrabutylammonium Acetate</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>UDP</td>
<td>Unidentified Degradation Product</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1
INTRODUCTION
1.1 Cancer: Glioma and treatments

Cancer is the uncontrolled growth of normal cells and their spread to other parts of the body (Trent et al., 2002). Various types of cancer are diagnosed: cervical, lung, brain, skin, blood and many others as cancer can affect almost any type of body tissue. The abnormal cancer cells invade the surrounding normal tissue and can thus spread on to distant sites in the body (Sawyer et al., 2006). Many cancers can be prevented by taking precautions when exposed to known common risk factors, such as tobacco smoke, radioactivity, UV radiations, as indicated by World Health Organisation (Kleihues et al., 2002). In addition, a significant proportion of cancers can be cured, by surgery, radiotherapy or chemotherapy, especially if they are detected early.

1.1.1 Gliomas

A glioma is the most widely affecting form of brain cancer. It is a medical term for a tumour that originates in the brain or spine. The name glioma is derived from the fact that it starts from glial cells (Chang et al., 2005).

Tumours can develop in any part of the brain or its nerves and covering tissues. The two major types of brain tumour are primary and secondary. Primary brain tumours start in the brain. Secondary brain tumours start in another part of the body, then spread to the brain (Maher et al., 2001). A glioma is a primary brain tumour, accounting for 45% of cancers that begin in brain cells.

The three main types of glioma include: astrocytoma, ependymoma, and oligodendroglioma. Each of these types can be assigned a grade, either low grade or high grade, with high grade being more malignant and aggressive (Bobola et al., 2004).

Astrocytomas are named for the cells where they originate, the astrocytes. These tumours can either show clear borders between normal brain tissue and the tumour (called focal) or no clear border (called diffuse). Focal astrocytomas are most common in children and are not often found in adults.
Ependymomas begin in cells called ependymal cells that are found lining certain areas of the brain and spinal cord. These cells help repair damaged nerve tissue. They usually occur in children and young adults.

Oligodendrogliomas form in oligodendrocyte cells, which produce a fatty substance called myelin that protects the nerve. More common in adults, these tumours may move to other parts of the brain or spinal cord.

1.1.2 Treatment: Chemotherapy

Chemotherapy is the major treatment technique for cancer. Various drugs have been formulated for the cancer treatment which works by altering or damaging the DNA or RNA. They treat cancer, if used at early stages, but again have their own side effects. These could sometimes be killing the healthy cells or sometimes altering the DNA to bring about a mutation. Likewise, the cancer cells regenerate after chemotherapy by activating their DNA repair system and so cancer resurfaces. At such times, damaging the DNA to an extent that their repair system is not reactivated is like boon and not a side effect. Scientists have not only been working on formulating drugs which could be given in combination with the chemotherapy drugs to cure or reverse or simply erase the adverse effects but also been working on those that can bring about a damage that would make the cure permanent.

Temozolomide

Temozolomide, a triazene compound, has been successfully used for the treatment of cancer for the past few years. Temozolomide is an alkylating agent that mediates its cytotoxic effects via $O^6$-methylguanine ($O^6$-meG) adducts in DNA and their recognition and processing by the postreplication mismatch repair system (MMR). (Kang et al., 1992). $O^6$-meG adducts can be repaired by the DNA repair protein $O^6$-alkylguanine-DNA-alkyltransferase (MGMT), which therefore constitutes a major resistance mechanism to the drug. Unlike many other chemotherapy drugs, it can reach the brain via the bloodstream. It slows or stops the growth of cancer cells, which causes them to die. The epigenetic silencing of the $MGMT$ ($O^6$-methylguanine–DNA methyltransferase) DNA-repair gene by promoter methylation compromises DNA repair and has been associated
with longer survival in patients with glioblastoma who receive alkylating agents (Westphal et al., 2003; Stupp et al., 2005).

However, resistance to Temozolomide can also be mediated by loss of MMR, which is frequently mediated by methylation of the *hMLH1* gene promoter. A summary of this can be seen in Figure 1.1.

![Figure 1.1](image)

**Figure 1.1** Figure showing resistance to the effect of Temozolomide by the MGMT, MMR and BER repair systems. Adapted from Kim, H et al, 2001. Pg 463

### 1.2 Lomeguatrib

Lomeguatrib or Patrin-2 is a drug recently formulated by the Paterson Cancer Institute, Manchester at the University of Manchester. The chemical structure of this drug is shown in Fig.1. It works by causing lethal DNA damage. If cancer cells are able to repair damaged DNA they can often survive the effects of treatment, so scientists aimed to develop drugs to inhibit DNA repair, in order to make Temozolomide chemotherapy more effective (Turrijziani et al., 2006).
Patrin-2 blocks the action of a molecule called ATase, which repairs specific kinds of DNA damage and is particularly important in resistance to anti-cancer treatments (Khan et al., 2008). Methylation of hMLH1 can be reversed by treatment of cells with 5-aza-2'-deoxycytidine, while the MGMT pseudosubstrate $O^6$-(4-bromothenyl) guanine (Patrin-2) can deplete MGMT activity. Using a drug-resistant cell line which expresses MGMT and has methylated hMLH1, it can be observed that while either of these treatments can individually sensitize cells to Temozolomide (Verbeek et al., 2008) the combined treatment leads to substantially greater sensitization. The increased sensitization is not observed in matched MMR proficient cells.

![Figure 1.2 Structure of Lomeguatrib.](image)

The biological effects of triazene compounds and cell resistance to them depend on at least three DNA repair systems, (a) $O^6$-alkylguanine-DNA-alkyltransferase, called also methyl-guanine methyl-transferase (MGMT); (b) mismatch repair (MMR), and (c) base excision repair (BER). (Marchesi et al., 2007)

MGMT is a small enzyme-like protein which is responsible for removing small alkyl adducts from the $O^6$ position of DNA guanine through a stoichiometric and auto-inactivating reaction (Verbeek et al., 2008). This reaction consists in a covalent transfer of the alkyl group from the alkylated site in DNA to an internal cysteine residue of MGMT protein. Normal and tumour cell start resisting triazenes due to high levels of MGMT. Pre-treatment with MGMT inhibitors such as $O^6$-benzylguanine or $O^6$-(4-bromoteny1)guanine (Lomeguatrib) is therefore recommended as it results in an increase in the activity of triazenes against target cells expressing high MGMT levels. (Shibata et al., 2006) MMR is represented
by a protein complex dedicated to the repair of biosynthetic errors generated during DNA replication. The MMR system recognizes base mismatches and insertion–deletion loops, cuts the nucleotide sequence containing the lesion, and restores the correct base sequence. Therefore, not only MGMT but also MMR is involved in target cell susceptibility to triazenes. However, the system does not suppress, but instead promotes the cytotoxic effects of triazenes. In fact, MMR is not able to repair the incorrect base pairing determined by treatment with triazenes and, according to a predominant hypothesis, it causes reiterated “futile” attempts of damage repair leading to the activation of cell cycle arrest and apoptosis. BER removes lesions due to cellular metabolism, or to physical or chemical agents. BER is able to repair $N^7$-methylguanine and $N^3$-methyladenine determined by treatment with triazenes (Marchesi et al., 2007). Therefore, triazene compounds can also kill tumour cells by a $N^3$-methyladenine-mediated mechanism if BER activity is inhibited by chemical agents (i.e. PARP inhibitors).

Temozolomide and Lomeguatrib are known to work best when given in combination. For any drug to be approved for usage, it has to undergo various clinical trials. Stability studies also have to be performed on them. HPLC is majorly used to conduct stability studies on drugs, to evaluate their metabolic by-products and to check them for toxicity levels. Temozolomide is an approved drug and so has been checked for stability earlier, the articles of which have been published. A few by-products like MIT (Kim et al., 2001) were even recognised and analysed. There aren’t articles representing the stability indicating studies for Patrin-2 available. Articles also haven’t been published to indicate the by-products of this drug. Designing a stability indicating method for Patrin-2 and find out its by-products on degradation and check their toxicity level, and hence concluding the feasibility of this drug for treatment of cancer without being dangerous to life will be of great interest. Also the by-products acquired when the two drugs are used in combination will be an interesting study.
1.3 Drug Stability Studies

Stability testing of drugs requires an accurate analytical method that quantifies active pharmaceutical ingredients (API) without interference from degradation products, process impurities and other potential impurities. Stability-indicating HPLC methods are designed for drugs. Stability-indicating analytical method is validated, specific, and quantitative analytical method, which is capable of accurately testing API, degradation products, and other components of interest without interference. The stability-indicating method is used to detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product. The quality of a drug product depends on the quality of API. The changes in API itself and related impurities have to be examined throughout the drug life. An ideal situation is that the API and all the impurities can be accurately measured with one method. Nevertheless, secondary validated complemented method may also be used when a single method is unattainable to examine all of the components at the same time. Stability information is needed for regulatory submissions such as IND (Investigational New Drug Application) and NDA (New Drug Applications) and to set expiration dates for the API or drug product.

1.3.1 Forced Degradation

The forced degradation studies are another very important part of the validation of the stability indicating method. In forced degradation studies, samples are stored under extreme conditions (acid, base, peroxide, heat, light, humidity etc) in order to rapidly screen drug product stabilities. Stability-indicating methods are traditionally performed using gradient elution, in order to ensure that degradants of various chemical compositions are all detected.

1.3.2 Methods of Degradation:

In a typical study, relevant stress conditions are light, heat, humidity, hydrolysis (acid / base influence) and oxidation or even a combination of described parameters. If it is necessary to form degradation products, the strength of stress
conditions can vary due to the chemical structure of the drug substance, the kind of drug product, and product specific storage requirements (Alsante, 2011). A typical study design should be able to cover different stress conditions using different time periods in order to assess the degradation kinetics.

In our study we are going to use H$_2$O$_2$ to carry out the oxidation. The compound will be subjected to UV light, the correlation of a spectrum peak of which will be tested at maximum and at beginning and end of a peak. Heat stress or thermal stress and acidic stress will also be applied.

1.4 **HPLC**

1.4.1 Working of HPLC

High Performance Liquid Chromatography (HPLC) is one of the most used analytical techniques. Chromatographic process is a separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture (Lindsay and Barnes, 1992). The stationary phase can be a liquid or a solid phase. Reversed phase chromatography is commonly used to carry out stability studies. They are called reversed phase because the stationary phase is a tightly packed hydrophobic material. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components (Skoog and Leary, 1992). The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases (Lindsay and Barnes, 1992). As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures. HPLC is a dynamic adsorption process (Snyder, 1996). Analyte molecules, while moving through the porous packing beads, tend to interact with the surface adsorption sites as they are competing with the eluent.
molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface (Narola et al., 2010).

Figure 1.3 Typical HPLC system. Adapted from Narola et al., 2010

1.4.2 Validation of HPLC

Method development involves a series of sample steps; based on what is known about the sample, a column and detector are chosen; the sample is dissolved, extracted, purified and filtered as required; an eluent survey (isocratic or gradient) is run; the type of final separation (isocratic or gradient) is determined from the survey; preliminary conditions are determined for the final separation; retention efficiency and selectivity are optimized as required for the purpose of the separation (quantitative, qualitative or preparation); the method is validated using ICH guidelines (Jain, 2013). The validated method and data can then be documented.
For stability studies, a stability indicating method is vital. This is for separating, detecting, and quantitating possible degradants that could be generated during storage conditions. The method should be sensitive to the reportable impurity levels. There should also be linearity in the results being obtained (Narola et al., 2010). Aspects such as the repeatability and reproducibility can be assured on the validation of the techniques. The following is the significance of all aspects:

**Linearity and range:** The linearity of a test procedure is its ability (within a given range) to produce results that are directly proportional to the concentration of analyte in the sample. The range is the interval between the upper and lower levels of the analyte that have been determined with precision, accuracy and linearity using the method as written. ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is 80–120% of the theoretical content of active. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. The regression coefficient \((r^2)\) is .998 and is generally considered as evidence of acceptable fit of the data to the regression line. The percent relative standard deviation (RSD), intercept and slope should be calculated.

**Accuracy:** A method is said to be accurate if it gives the correct numerical answer for the analyte. The method should be able to determine whether the material in question conforms to its specification. However, the exact amount present is unknown, which is why a test method is used to estimate the accuracy. Furthermore, it is rare that the results of several replicate tests all give the same answer, so the mean or average value is taken as the estimate of the accurate answer.

**Specificity:** Developing a separation method for HPLC involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the
analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products and process impurities) is compared with the response of a solution containing only the analyte.

**Precision:** Precision means that all measurements of an analyte should be very close together. All quantitative results should be of high precision - there should be no more than a ±2% variation in the assay system. A useful criterion is the relative standard deviation (RSD) or coefficient of variation (CV), which is an indication of the imprecision of the system.

**Limits of detection and quantitation:** The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

**Robustness:** Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters. It also provides some indication of the reliability of an analytical method during normal usage. Parameters that should be investigated are per cent organic content in the mobile phase or gradient ramp; pH of the mobile phase; buffer concentration; temperature; and injection volume. These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment.
1.5 Research questions and specific aims

The main question to be answered by this study is,

Is it possible to design a validated Stability Indicating Method for Lomeguatrib using HPLC?

Other questions that could arise out of this study are,

Is it possible to quantify and identify the products formed on degradation of Lomeguatrib under different conditions?

1.5.1 The working hypothesis

A validated Stability Indicating Method can be designed for the analysis of Lomeguatrib by subjecting it to accelerated or forced degradation.

1.5.2 Specific Aims

1. Design a method where Lomeguatrib is subjected to varied stress conditions and thus observe the difference in the degradation pattern.

2. To observe the drug lability under various stress conditions.

3. To recognise conditions in which the drugs is stable and thus, design a method that can indicate the shelf-life of the drug.

4. To locate degradation products of Lomeguatrib on the HPLC Chromatogram and try and identify them, if possible.

5. To validate the method designed.
CHAPTER 2
MATERIALS AND METHODS
2.1 Materials and Reagents

The chemicals, reagents and materials used in this study are listed below. They were handled and stored as instructed by the suppliers or based on the manuals provided with them. Chemicals and reagents were of HPLC grade and suitable for analysis. They have been summarised in tabular format and listed alongside is the name of their supplier.

Table 2.1 List of materials and reagents used in this study.

<table>
<thead>
<tr>
<th>Chemicals/Materials/Reagents</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patrin-2 (Lomeguatrib) (2gms)</td>
<td>Sequoia Research Products (SeqChem)</td>
</tr>
<tr>
<td>HPLC grade Acetonitrile 98%</td>
<td>Fisher Scientific (UK)</td>
</tr>
<tr>
<td>HPLC grade Methanol 99+%</td>
<td>Fisher Scientific (UK)</td>
</tr>
<tr>
<td>Hydrogen Peroxide 30%</td>
<td>Sigma Aldrich (UK)</td>
</tr>
<tr>
<td>1-Methyl-2-pyrrolidinone; anhydrous, 99.5% or n-methylpyrrolidone</td>
<td>Sigma Aldrich (UK)</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide (DMSO) 99+%</td>
<td>Fisher Scientific (UK)</td>
</tr>
<tr>
<td>HPLC grade Water</td>
<td>Fisher Scientific (UK)</td>
</tr>
<tr>
<td>Citric acid ≥99.5%</td>
<td>Sigma Aldrich (UK)</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate (SDS) ≥99.0%</td>
<td>Sigma Aldrich (UK)</td>
</tr>
<tr>
<td>Tetrabutylammonium acetate (TBAA) 97%</td>
<td>Sigma Aldrich (UK)</td>
</tr>
<tr>
<td>pH meter buffers</td>
<td>Sigma Aldrich (UK)</td>
</tr>
</tbody>
</table>
2.2 Laboratory Instruments and Equipment

The instruments and equipment used in this study are as listed below. Listed alongside is the name of the company they were purchased from.

Table 2.2 List of instruments and equipment used in this study.

<table>
<thead>
<tr>
<th>Instrument/ Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC Pump (Jasco PU-1580)</td>
<td>Jasco (UK)</td>
</tr>
<tr>
<td>HPLC Detector (Jasco UV-970M; 4-λ intelligent)</td>
<td>Jasco (UK)</td>
</tr>
<tr>
<td>HPLC column 1 (Symmetry Shield rp18 5μm 5.6*250mm Column)</td>
<td>Waters Limited (UK)</td>
</tr>
<tr>
<td>HPLC column 2 (Symmetry300 C18 5μm 4.6*250 Column)</td>
<td>Waters Limited (UK)</td>
</tr>
<tr>
<td>HPLC column guard (SymmetryShield RP18 5um 3.9*20 Guard 2pk)</td>
<td>Waters Limited (UK)</td>
</tr>
<tr>
<td>Water bath (Nuve water bath nb 5)</td>
<td>Biotech lab (Bulgaria)</td>
</tr>
<tr>
<td>Magnetic Stirrer (RCT basic)</td>
<td>IKA RCT basic (Germany)</td>
</tr>
<tr>
<td>Gilson Micropipettes</td>
<td>Thermo Scientific (UK)</td>
</tr>
</tbody>
</table>

Besides these, glassware like glass bottles with screw cap (1L and 500ml), vials with screw cap (2ml, 3ml, 5ml, 10ml), beakers (500ml and 1L) were purchased from Fisher Scientific (UK). UV Lamp Reflector chamber and Quartz cuvettes with cover from AlphaCure were used for photolysis. Spectrophotometer, incubators and oven from Thermo Scientific were used. Glass pipettes, weighing scale, pH meter, round bottomed flasks with the mark of 10ml, 20ml, 50ml, 100ml, 1L and measuring cylinders of 50ml, 100ml, 500ml and 1L markings were also from Thermo Scientific, UK. Paper labels, cellotape, parafilm, aluminium foil and other stationery was obtained from the Maudland Stores.
2.3 Stock Solution for Patrin-2

Patrin-2 is not easily soluble in organic or inorganic solvents. So, based on initial studies, we tried dissolving it in HPLC grade Acetonitrile and Methanol solution prepared in (80:20) ratio. A stock solution of concentration 5mg/dL was attempted to be prepared for this study. Patrin-2 was sparsely soluble in this combination and had to be stirrer on a magnetic stirrer for at least half an hour. Also the peak quality obtained by running this solution was superior and symmetrical.

2.3.1 Selection of solvent to form a stock solution of Patrin-2

Patrin-2 is known to be readily soluble in DMSO. Hence a solvent with a low concentration of DMSO was formed. A solvent with 5% DMSO and 25% Acetonitrile was made up in HPLC grade water. Water diluted the concentration of both DMSO and Acetonitrile to avoid them from bringing about degradation in Patrin-2 as that could have lead to false results when this solution was subject to stress. A stock solution of 5mg/dL is to be formed by accurately weighing out 5 mgs of Patrin-2 powder and solubilising it in 1L of the solvent.

2.3.2 N-methylpyrrolidone as solvent

N-methylpyrrolidone is one of the drug components tested to solubilise Patrin-2 when formulating it’s drug form. We will hence prepare some samples of Patrin-2 in NMP of 5mg/dL conc.

2.3.3 Maximum wavelength selection for Patrin-2

A small amount from the stock solution was taken to run it on a standardised spectrophotometer to determine the maximum wavelength for Patrin-2. It was done so by keeping the solvent (5% DMSO and 25% Acetonitrile in HPLC grade water) without any Patrin-2 added to it as a blank.

The wavelength max was determined to be 285nm. A high peak was also seen at 254nm and 240nm.
2.4 HPLC

2.4.1 HPLC System

Jasco PU-1580 pump attached to a Jasco UV-970M (4-λ.intelligent) detector and a Viglen Pentium-4 was used in the HPLC set-up. AZUR version 5.0.10.0 software was used for the recording and analysis of the chromatogram of the solutions. Figure 2.1 represents a typical HPLC set-up. The set-up includes a solvent (mobile phase) which is run through the high pressure pump generating and measuring the flow rate of the mobile phase in millilitres per minute. The sample to be analysed is introduced in the injector from where it is introduced in the mobile phase flowing through the column consisting of chromatographic packing material (C18) also termed as the stationary phase. From the column the flow is directed to the detector. The components are then analyzed by the detector from where the chromatographic image is produced in the computer. The chromatogram produced displays the retention time and the peak area of the expected compound on the monitor with the help of AZUR 5.0.0.

![HPLC set-up](image)

**Figure 2.1** HPLC set-up used for the analysis.
The chromatographic experiments were performed at three wavelengths 240nm, 254nm and 285nm. The flow rate was kept constant at 1mL/min. A mobile phase was selected from a previous work (Shervington, L.A. et al, 2005). This was prepared by adding 10mM SDS, 10mM TBAA and 25mM citric acid to 35% Acetonitrile in HPLC grade Water. For a litre of this mobile phase, 2.88gms of SDS, 4.88gms of citric acid and 3.0273gms of TBAA is weighed and solubilised in 350ml of acetonitrile and then water is added to fill it up to the 1L mark.

2.4.2 HPLC column:

We had selected a reverse phase chromatography system. The columns in this set up usually have silica as the main packing material bonded with carbon chains. The longer the chains, the more is the retentive capacity. The sample size that can be used in a column with C\textsubscript{18} packing is twice the size of the sample that can be used on the column with C\textsubscript{4} packing. We looked for a column that matched our needs and gave us a broader range of usage. Symmetry300 column with a column guard from Waters’, UK was used for the analysis. This is a reversed-phase C\textsubscript{18} column. This particular one was selected so we could obtain sharp and superior peak.

2.5 Validation of HPLC

The HPLC system had to be validated before it could be used to analyse the products formed by subjecting the Patrin-2 solution to stress.

This was done by considering the aspects as per the guidelines described before. The following tests were carried out.
2.5.1 Linearity

To measure the linearity of the system, a stock solution of 5mg/dL was made and diluted to 4mg/dL, 3mg/dL, 2mg/dL and 1mg/dL. It was done by adding the stock solution and the solvent mix (DMSO, acetonitrile and water mix) in appropriate ratios. These samples were then run on the HPLC system as specified earlier. Each sample was run twice and a mean of the peak area was recorded. Based on this, we calculated the slope and the regression co-efficient. The values for these have been listed in the results section.

2.5.2 Repeatability (Intraday precision study)

To check the repeatability of the system, the system was injected 5 times with the same sample, to check the peak area obtained after every run. 2mg/dL was the working concentration. The sample prepared of this concentration was injected five times to the system and was let to run till the solution came out of the column. The mean of the peak areas obtained after the five runs was taken. The peak area values were used to obtained the standard deviation and relative standard deviation for this method.

2.5.3 Reproducibility

Reproducibility of the method was checked by preparing five samples of a particular concentration of Patrin-2 in a similar manner and running them on the system twice. A mean peak area of the two runs of every sample was considered. A mean these MPAs was then taken and checked for standard deviation and relation standard deviation for this method. 2mg/dL was the working concentration for this method.

2.5.4 Limit of Detection

Limit of detection was obtained by serially diluting the Patrin-2 solution to its half. We started with 1mg/dL and went down until the peak area of Patrin-2 was not visible on the chromatogram. Each sample at every concentration was run twice. We stopped when the sample at a particular concentration wasn’t repeatable. It was found to be at 0.0039mg/dL for Patrin-2.
2.5.5 Limit of Quantification

Limit of quantification was determined by serially diluting the Patrin-2 solution to the last concentration which was repeatable. It was found to be 0.0078mg/dL. We stopped when the concentration of the solution could not be accurately repeated and selected the last repeatable concentration as the limit of quantification. We then injected the sample five times to check if it can be quantified. We recorded the peak area values each time at this concentration and conducted tests for standard deviation and relative standard deviation.

Chromatographs for these tests have been recorded in the appendix section of this thesis.

After obtaining a validated system for the study, we move on to stability studies.

2.6 Stability Study of Patrin-2

Stability studies were conducted by subjecting the Patrin-2 solution (5mg/dL) to various stress conditions:

Thermal stress: The stock solution was filled up in vials with screw caps and 60-70 samples were incubated at 40°C, 50°C and 80°C, keeping it loosely covered with screw caps. These were temperatures higher than the human body temperature. 80°C is a high temperature for storing drugs and is known to degrade chemicals when incubated at that high a temperature. This was selected to pass the drug through forced degradation. 40°C and 50°C were comparatively lower temperatures, but fast enough to bring about some degradation in the drug. This could be compared to check how temperature affects the drug stability, the rate of degradation and it’s relation with the temperature and also could help determine a shelf life at that particular temperature. Along with these, nitrogen treated samples were prepared by sealing them under nitrogen and covering them right after. These samples were incubated under these conditions as well. The ones at 40°C and 50°C were analysed every three days, whereas the ones at 80°C were analysed on daily basis, until the compound degraded completely.
**Oxidative stress:** Two concentrations of \( \text{H}_2\text{O}_2 \) (30% purity) were prepared, 0.4M and 0.8M. These were added to labelled vials and the solution was added in the same quantity thus keeping the proportion of \( \text{H}_2\text{O}_2 \) and stock solution to 1:1. The final solution thus obtained is 2.5mg/dL. These samples were incubated at RT, 40\(^\circ\)C and 50\(^\circ\)C. This being an accelerated reaction, it was analysed in the interval of every half hour.

**Photolytic Stress:** Photolysis is breakdown of products with strong light conditions. Photolytic stress was applied to the stock solution of Patrin-2 (5mg/dL) by setting up a UV lamp reflector (330nm) chamber and filling up glass vessel in the lamp with the stock solution (180ml). The set up was placed in a UV hood and was covered with aluminium foil, so as to avoid contact with the UV radiations. The system was carefully turned off every half an hour, and a little amount of the solution was taken from the tap below to analyse.

A few samples were left to be subjected to direct sunlight at the room temperature.

**Acidic and Basic solutions:** 0.1M acid and base solutions were obtained. They were then added in different apparatus to the stock solution of 5mg/dL in 1:1 ratio and were then incubated in vials at RT and 40\(^\circ\)C. The acid reaction was to be an accelerated one, so had to be analysed every 15mins. The base samples were analysed everyday initially but was then shifted to once a week analysis. The final concentration of these samples is 2.5mg/dL

**Patrin-2 in NMP:** 5mg of Patrin-2 was weighed out accurately and solubilised in 100ml of N-methylpyrrolidone. The samples were then analysed every three days.

Solid form of the compound was only incubated at the three temperatures. All other stress conditions were applied to the liquid form of the compound. The solid form, however, was difficult to analyse later, as weighing out small quantity and form up solution of a quantity that could be detected within the limits of the software to give precise area were both difficult.
2.7 Statistical Analysis

The data from the HPLC was recorded in chromatographs using AZUR version 5.0. AZUR is a chromatography software developed in Montreal, France. It graphically records the column elusions based on the detection in the HPLC detector. This gives a graph with peaks when an elute comes out, the size of which is based on it’s concentration in the sample injected. The chromatograph draws a straight line for the mobile phase elution as the set up is standardised to let it run out without giving peaks.

The chromatograms were then analysed to obtain the peak areas of Patrin-2 and also some of the unidentified by-products so as to compared the rate of degradation. This data was recorded in graphic form or tabular form, wherever required. The data obtained from the chromatograms was statistically analysed on MS Excel and SPSS. Standard tests like standard deviation and average were performed. Also the regression coefficient and slope were found wherever required.
3.1 Validation of HPLC system for Patrin-2 analysis

As it can be seen in the initial chromatographs in the appendix section, sharp peak symmetry was achieved for Patrin-2 using the mobile phase made up by adding 10mM SDS, 10mM TBAA and 25mM citric acid in a solution of 35% Acetonitrile and 65% HPLC grade Water. The pH was maintained at 3.4. The validation of the HPLC system for Patrin-2 was done at 285nm ($\lambda_{\text{max}}$). The retention time for Patrin-2 was noted to be approximately 10.7mins. The analysis was carried out on the HPLC column Symmetry Shield (Waters, UK) and the flow rate of the system was maintained at 1ml/min. For the analysis, Patrin-2 was dissolved in a solution of 5% DMSO and 25% Acetonitrile in 70% HPLC grade Water to form a stock solution.

This method was validated by analysing it for linearity, repeatability, reproducibility, limit of detection (LOD) and limit of quantification (LOQ). A summary of the results is tabulated below.

3.1.1 Linearity

For checking the linearity, Patrin-2 solutions were prepared in the range of 1mg/dL to 5mg/dL, keeping the interval 1 (mg %). Duplicates of each concentration were made and run on the HPLC system. A mean of the two results, which were more or less identical, was considered.

Table 3.1 Linearity test of Patrin-2. $n=2$, where $n$ is the number of samples of the same concentration analysed and averaged.

<table>
<thead>
<tr>
<th>Concentration (mg %)</th>
<th>Mean Peak Area (MPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2015.31</td>
</tr>
<tr>
<td>4</td>
<td>1616.73</td>
</tr>
<tr>
<td>3</td>
<td>1193.065</td>
</tr>
<tr>
<td>2</td>
<td>779.02</td>
</tr>
<tr>
<td>1</td>
<td>393.895</td>
</tr>
</tbody>
</table>
A calibration curve was plotted with the mean peak areas against their respective concentrations. The range was of concentration was again 1mg/dL to 5mg/dL. The regression coefficient ($R^2$) value was determined using this plot.

![Linearity for Patrin-2](image)

**Figure 3.1:** Calibration curve for Patrin-2 with samples ranging from concentrations 1mg/dL to 5 mg/dL against their mean peak area (n=2). The regression coefficient ($R^2$) = 0.999.
3.1.2 Repeatability

The repeatability of this method was checked using the solution of concentration 2mg/dL. The standard deviation (SD) of the results was 0.2307 and relative standard deviation (RSD) was found to be 0.0296. Since the RSD value was lies between the ranges of 0 to 1, the method was determined as repeatable.

**Table 3.2** Repeatability test for Patrin-2. Solution of 2mg/dL concentration was injected 5 times and a mean of the peak areas was taken to determine the SD and RSD values.

<table>
<thead>
<tr>
<th>Injection number</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>779.02</td>
</tr>
<tr>
<td>2</td>
<td>778.97</td>
</tr>
<tr>
<td>3</td>
<td>778.56</td>
</tr>
<tr>
<td>4</td>
<td>779.14</td>
</tr>
<tr>
<td>5</td>
<td>779.09</td>
</tr>
<tr>
<td>Mean Peak Area</td>
<td>778.956</td>
</tr>
<tr>
<td>SD</td>
<td>0.230716</td>
</tr>
<tr>
<td>RSD</td>
<td>0.029618</td>
</tr>
</tbody>
</table>
3.1.3 Reproducibility

The reproducibility of this method was checked by making 5 solution of concentration 2mg/dL. The standard deviation (SD) of the results was 1.8204 and relative standard deviation (RSD ) was found to be 0.2333. Since the RSD value lies between the range of 0 to 1, the method was determined as repeatable.

Table 3.3 Reproducibility test for Patrin-2. Five samples of 2mg/dL concentration were made and injected (n=2). A mean of their peak areas was taken to determine the SD and RSD values.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>779.02</td>
</tr>
<tr>
<td>2</td>
<td>781.86</td>
</tr>
<tr>
<td>3</td>
<td>782.01</td>
</tr>
<tr>
<td>4</td>
<td>778.46</td>
</tr>
<tr>
<td>5</td>
<td>778.44</td>
</tr>
<tr>
<td>Mean Peak Area</td>
<td>779.958</td>
</tr>
<tr>
<td>SD</td>
<td>1.8204</td>
</tr>
<tr>
<td>RSD</td>
<td>0.2333</td>
</tr>
</tbody>
</table>
3.1.4 Limit of Detection

The limit of detection for the Patrin-2 solution was found to be 0.003925 mg/dL. This was obtained by serially diluting the 1mg/dL solution to half its concentration, until the limit of detection was achieved. At this concentration, a small peak could be detected, but was not repeatable when it came to the area. Thus, the peak detection was qualitative and not quantitative.

Table 3.4 Limit of detection for Patrin-2 solution. \( n=2 \).

<table>
<thead>
<tr>
<th>Concentration (mg %)</th>
<th>Mean Peak Area (MPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>240.30</td>
</tr>
<tr>
<td>0.25</td>
<td>131.79</td>
</tr>
<tr>
<td>0.125</td>
<td>76.645</td>
</tr>
<tr>
<td>0.0625</td>
<td>38.76</td>
</tr>
<tr>
<td>0.0312</td>
<td>17.54</td>
</tr>
<tr>
<td>0.0156</td>
<td>8.53</td>
</tr>
<tr>
<td>0.0078</td>
<td>5.73</td>
</tr>
<tr>
<td>0.0039</td>
<td>2.94</td>
</tr>
</tbody>
</table>
3.1.5 Limit of Quantification

The limit of quantification for Patrin-2 solution was 0.00785mg/dL. This was achieved by serially diluting the 1mg/dL solution of Patrin-2 to half its concentration. 0.00785mg/dL was the last concentration, the peak of which could be quantified. The analysis of this concentration was repeated 5 times and the SD for the results was 0.1247 and the RSD was calculated to be 2.10. The RSD value is comparatively low, and so the result was accepted.

Table 3.5 Limit of quantification for Patrin-2. The sample of the concentration 0.00785mg/dL was injected 5 times and a mean of the peak areas was calculated and the SD and RSD values were thus derived.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.73</td>
</tr>
<tr>
<td>2</td>
<td>5.54</td>
</tr>
<tr>
<td>3</td>
<td>5.81</td>
</tr>
<tr>
<td>4</td>
<td>5.52</td>
</tr>
<tr>
<td>5</td>
<td>5.61</td>
</tr>
</tbody>
</table>

Mean Peak Area 5.642
SD 0.1247
RSD 2.10
A summary of the results for every parameter analysed to validate the HPLC method is mentioned in the table below. This validated the HPLC system and it could be used for further analysis.

**Table 3.6** Summary table for validation of the HPLC method for Patrin-2.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical wavelength (nm)</td>
<td>285</td>
</tr>
<tr>
<td>Stock Solution (Conc.)</td>
<td>5mg/dL</td>
</tr>
<tr>
<td>Linearity Range (mg %)</td>
<td>1 to 5</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$Y = 406.054x - 14.558$</td>
</tr>
<tr>
<td>Regression coefficient ($R^2$)</td>
<td>0.999</td>
</tr>
<tr>
<td>RSD (Repeatability)</td>
<td>0.0296</td>
</tr>
<tr>
<td>RSD (Reproducibility)</td>
<td>0.2333</td>
</tr>
<tr>
<td>RSD (LOQ)</td>
<td>2.10</td>
</tr>
<tr>
<td>LOD</td>
<td>0.0039mg/dL</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.0078mg/dL</td>
</tr>
</tbody>
</table>
3.2 Stability indicating method for Patrin-2

A stock solution of Patrin-2 of 5 mg/dL was prepared in 5% DMSO, 25% Acetonitrile and 70% HPLC grade Water. It was incubated in vials or appropriate apparatus under various conditions as described in the methods. By doing this, it was subjected to forced degradation and under all conditions, there were degradation products formed. In some condition, the degradation was accelerated, while in others, the compound was relatively stable and took longer to disintegrate. The HPLC analysis was carried out on three different wavelengths, but the results were summarised from one (254nm). The stability of Patrin-2 under various conditions is summarised for every condition.

3.2.1 Stability of Patrin-2 at 40°C, 50 °C and 80 °C

Samples were incubated at the three temperatures in vials, sealed loosely from above with a plastic screw cap. The nitrogen treated sample vials were incubated at the three temperatures too. A standard was made of the same concentration as the samples (5mg/dL) and incubated in the refrigerator so that we could accurately get the original peak size of Patrin-2 when we run it on the chromatogram before running the other samples that have been incubated in the refrigerator. This helps in monitoring the extent of degradation each day. The peak area for the standard was 1502.02 as can be seen in Appendix Figure 6.32. The samples were then analysed on HPLC after a specific time interval. The results have been summarised in tables that will follow.

Table 3.7 The results for the stability of Patrin-2 (5mg %) incubated at 40°C, with and without treatment with nitrogen. The results are the ones obtained at 254nm. 

(n=2)
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Time (days)</th>
<th>Retention Time (min)</th>
<th>MPA</th>
<th>Compound</th>
<th>% of Patrin-2 left after degradation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>3.49</td>
<td>436.16</td>
<td>UDP Patrin-2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12.98</td>
<td>1468.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>3.42</td>
<td>517.99</td>
<td>UDP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.82</td>
<td>38.15</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.28</td>
<td>39.55</td>
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</tr>
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<td>15.06</td>
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</tr>
<tr>
<td></td>
<td>12.38</td>
<td>1230.33</td>
<td>Patrin-2</td>
<td>81.91%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>3.42</td>
<td>672.14</td>
<td>UDP</td>
<td>-</td>
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<td></td>
<td>6.81</td>
<td>94.26</td>
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<td>80.97</td>
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<td>74.58</td>
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<td>8.35</td>
<td>22.23</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>8.72</td>
<td>25.71</td>
<td></td>
<td></td>
<td>-</td>
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<tr>
<td></td>
<td>12.30</td>
<td>950.91</td>
<td>Patrin-2</td>
<td>63.30%</td>
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</tr>
<tr>
<td>Nitrogen-treated</td>
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<td>3.50</td>
<td>395.70</td>
<td>UDP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>13.03</td>
<td>1474.66</td>
<td>Patrin-2</td>
<td>98.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>3.42</td>
<td>427.18</td>
<td>UDP</td>
<td>-</td>
</tr>
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<td></td>
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<td>1310.01</td>
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<td>87.21%</td>
<td></td>
</tr>
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<td>3.42</td>
<td>371.99</td>
<td>UDP</td>
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</tr>
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<td>95.46</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>8.72</td>
<td>8.99</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9.67</td>
<td>13.72</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12.32</td>
<td>1147.50</td>
<td>Patrin-2</td>
<td>76.39%</td>
<td></td>
</tr>
</tbody>
</table>
The percentage values in the table corresponding the Patrin-2 concentration are taken with comparison to the peak area of Patrin-2 for the standard which was 1502.02. This means 1502.02 is considered as 100%. It was observed that the nitrogen-treated samples degraded slower than the ones not subjected to nitrogen. Very little and very slow degradation of Patrin-2 is seen at 40°C.

Table 3.8 The results for the stability of Patrin-2 (5mg %) incubated at 50°C, with and without treatment with nitrogen. The results are the ones obtained at 254nm. 

\( (n=2) \)

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Time (days)</th>
<th>Retention Time (min)</th>
<th>MPA</th>
<th>Compound</th>
<th>% of Patrin-2 left after degradation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>3.50</td>
<td>538.72</td>
<td>UDP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.85</td>
<td>31.28</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.59</td>
<td>34.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>9.98</td>
<td>11.23</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>13.00</td>
<td>1268.25</td>
<td>Patrin-2</td>
<td>84.43%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>3.42</td>
<td>716.39</td>
<td>UDP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6.82</td>
<td>102.50</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>7.26</td>
<td>117.41</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.75</td>
<td>93.34</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.37</td>
<td>30.75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.76</td>
<td>39.07</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12.36</td>
<td>858.19</td>
<td>Patrin-2</td>
<td>57.02%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.41</td>
<td>111.29</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>1194.06</td>
<td>UDP</td>
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<tr>
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<td>6.83</td>
<td>237.44</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>7.29</td>
<td>235.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.79</td>
<td>222.21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.38</td>
<td>67.64</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrogen-treated</td>
<td>3</td>
<td>3.50</td>
<td>480.07</td>
<td>UDP</td>
<td>-</td>
</tr>
<tr>
<td>----------------</td>
<td>----</td>
<td>------</td>
<td>--------</td>
<td>-----</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>6.85</td>
<td>26.30</td>
<td>UDP</td>
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<td>-</td>
</tr>
<tr>
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<td>45.60</td>
<td>UDP</td>
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<td>UDP</td>
<td>-</td>
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<td>1357.05</td>
<td>Patrin-2</td>
<td>90.34%</td>
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</tr>
<tr>
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<td>15.18</td>
<td>30.37</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>3.42</td>
<td>624.24</td>
<td>UDP</td>
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<td>-</td>
</tr>
<tr>
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<td>6.81</td>
<td>87.65</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.25</td>
<td>140.09</td>
<td>UDP</td>
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<tr>
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<td>99.53</td>
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<td>8.36</td>
<td>18.87</td>
<td>UDP</td>
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</tr>
<tr>
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<td>27.04</td>
<td>UDP</td>
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<tr>
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<td>9.68</td>
<td>24.80</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12.33</td>
<td>912.28</td>
<td>Patrin-2</td>
<td>60.73%</td>
<td>-</td>
</tr>
<tr>
<td>115</td>
<td>3.42</td>
<td>950.41</td>
<td>UDP</td>
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<td>-</td>
</tr>
<tr>
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<td>6.81</td>
<td>160.39</td>
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</tr>
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<td>7.25</td>
<td>302.63</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.74</td>
<td>229.76</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.36</td>
<td>85.68</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.76</td>
<td>75.56</td>
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</tr>
<tr>
<td></td>
<td>12.33</td>
<td>449.36</td>
<td>Patrin-2</td>
<td>29.91%</td>
<td>-</td>
</tr>
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<td>14.36</td>
<td>201.62</td>
<td>UDP</td>
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<td>15.98</td>
<td>68.02</td>
<td>UDP</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As can be seen in Table 3.7 and Table 3.8, Patrin-2 disintegrated to a number to unidentified products on being subjected to degradation. It degraded faster, the more the temperature was increased. At 50°C, the degradation seen with or without the treatment of nitrogen is more or less the same. The percentage of
Patrin-2 left as compared to the original amount is lesser in the 50°C incubated samples than the 40°C incubated samples.

Table 3.9 The results for the stability of Patrin-2 (5mg %) incubated at 80°C, with and without treatment with nitrogen. The results are the ones obtained at 254nm. (n=2)

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Time (hrs)</th>
<th>Retention Time (min)</th>
<th>MPA</th>
<th>% of Patrin-2 left after degradation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24</td>
<td>12.27</td>
<td>688.78</td>
<td>45.85%</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>11.61</td>
<td>539.84</td>
<td>35.94%</td>
</tr>
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<td></td>
<td>168</td>
<td>13.01</td>
<td>17.08</td>
<td>1.13%</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>12.74</td>
<td>10.46</td>
<td>0.69%</td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>-</td>
<td>Undetected/totally degraded</td>
<td>-</td>
</tr>
<tr>
<td>Nitrogen-treated</td>
<td>24</td>
<td>12.17</td>
<td>318.97</td>
<td>21.23%</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>11.95</td>
<td>13.24</td>
<td>0.88%</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>11.96</td>
<td>10.95</td>
<td>0.72%</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>-</td>
<td>Totally degraded</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>-</td>
<td>Totally degraded</td>
<td>-</td>
</tr>
</tbody>
</table>

At 80°C, the samples treated with nitrogen degraded at a very high rate and much faster than the ones that weren’t treated with nitrogen. The rate of degradation was, in general quite high as compared to the other temperatures.

The decrease in percentage of the Patrin-2 present after degradation has been shown in the last column of every table. It can be observed that the rate at which the Patrin-2 is decreasing in the samples is higher at 80°C, whereas its lower at 50°C and the lowest at 40°C.
3.2.2 Oxidation of Patrin-2 using H$_2$O$_2$

H$_2$O$_2$ was added to the 5mg/dL stock solution of Patrin-2 in (1:1). Thus, the final concentration of the solution incubated was 2.5mg/dL. H$_2$O$_2$ was added in two different concentrations to different stock solutions, 0.4M and 0.8M.

The solution prepared in 0.4M H$_2$O$_2$, was incubated at 37°C (in a water bath), 40°C and 50°C, whereas the one prepared in 0.8M H$_2$O$_2$, was incubated at 37°C (in a water bath) and 40°C.

There was a high degradation and the compound completely degraded in a few hours. The figures below summarise the reaction.

![Graph](image1.png)

(a.)

![Graph](image2.png)

(b.)
Figure 3.2 Oxidized Patrin-2 at (a.) 240nm, (b.) 254nm, (c.) 285nm. The complete degradation of Patrin-2 took 390 mins when 0.4M hydrogen peroxide solution of Patrin-2 (2.5mg/dL) was incubated at RT (37°C in water bath).

This was the slowest oxidation at 390 mins. At other temperatures and for the other concentration (0.8M), it took as little as 190 to 240 mins. However, this was an accelerated reaction. Patrin-2 level decreased drastically in this reaction and the compound probably degraded in a different manner, giving different by products on reacting with H₂O₂. The by-products which increased prominently during this procedure were seen at 2.86mins and 3.45mins. There were minor quantities of by-products also seen at 6.80mins, 9.78mins and 14.64mins. The major by-products of this reaction were highly non-polar, as this is a reverse phase chromatography.
Figure 3.3 Effect of H$_2$O$_2$ (0.4M) on Patrin-2. Patrin-2 gradually degraded in the presence of hydrogen peroxide of the lower concentration, when no heat was exerted over it. (n=2)

Peak area of Patrin-2 decreased with time when incubated at RT, in 0.4M H$_2$O$_2$. It decrease is linear as can be seen in Figure3.3 and so the decrease in peak area or rather concentration of Patrin-2 and increase in the concentration of by-products is directly proportional to the time of the reaction. Even though H$_2$O$_2$ reaction with the sample is a quick reaction, the one under these conditions mentioned above is comparatively lower than the higher incubation temperatures and higher H$_2$O$_2$ concentration samples. A summary table can be made to compare the rate at which Patrin-2 degraded when subjected to 0.8M H$_2$O$_2$ and incubated at RT and 40 °C and subjected to 0.4M H$_2$O$_2$ and incubated at 40 °C and 50 °C. Table 3.10 shows a summary of the rate at which the compound degraded at approximately the same time intervals. It also shows how the concentration of H$_2$O$_2$, the temperature incubation or both could accelerate the reaction.
Table 3.10 Effect of H$_2$O$_2$ on Patrin-2 solution, when (a) 0.4M of it is added to it (1:1 ratio) and incubated at 40°C and 50°C, (b) 0.8M of it is added to it (1:1 ratio) and incubated at RT and 40°C. values derived from the chromatogram at 254nm.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>MPA (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4M H$_2$O$_2$</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
</tr>
<tr>
<td>15</td>
<td>647.31</td>
</tr>
<tr>
<td>30</td>
<td>432.86</td>
</tr>
<tr>
<td>60</td>
<td>231.77</td>
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<td>120</td>
<td>86.78</td>
</tr>
<tr>
<td>180</td>
<td>18.13</td>
</tr>
<tr>
<td>240</td>
<td>-</td>
</tr>
</tbody>
</table>

a.)
**Figure : 3.4** The HPLC chromatogram showing (a) effect of 0.4M H$_2$O$_2$ at 50°C at 180min, (b) effect of 0.4M H$_2$O$_2$ at 40°C at 240min, (c) effect of 0.8M H$_2$O$_2$ at 40°C at 120min and (d) effect of 0.8M H$_2$O$_2$ at 37°C at 180min @254nm

**3.2.3 Stability of Patrin-2 on exposure to UV light:**

UV Lamp Reflector Chamber was set-up and 180ml of the 5mg/dL stock solution of Patrin-2 was poured in the glass vessel of the chamber. It was carefully taken out from the tap at the base of the chamber after a certain interval. The results of the reaction are summarised below.

**Figure 3.5** Effect of UV light on Patrin-2 solution (5mg/dL) solution after 5 hours of exposure, as seen on a HPLC chromatogram @254nm.

The figure 3.5 indicates the presence of non-polar by-products when Patrin-2 solution is exposed to UV light. By-products are seen at 2.47, 2.82, 3.02 and 6.74mins. There are minor traces seen at 4.24, 4.54, 8.58 and 15.55mins too. These are the identified peaks seen on photolysis of Patrin-2.
Table 3.11 Gradual degradation of Patrin-2 in its solution form (5mg/dL) when exposed to UV light. (Abs. 254nm) n=2.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Mean Peak Area (MPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>802.88</td>
</tr>
<tr>
<td>60</td>
<td>278.19</td>
</tr>
<tr>
<td>90</td>
<td>71.93</td>
</tr>
<tr>
<td>120</td>
<td>33.33</td>
</tr>
<tr>
<td>150</td>
<td>26.57</td>
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<tr>
<td>180</td>
<td>20.85</td>
</tr>
<tr>
<td>210</td>
<td>14.46</td>
</tr>
<tr>
<td>240</td>
<td>9.54</td>
</tr>
<tr>
<td>270</td>
<td>6.18</td>
</tr>
<tr>
<td>300</td>
<td>4.22</td>
</tr>
</tbody>
</table>

Figure 3.6 Graph indicating the rate of degradation of Patrin-2 (5mg%) when exposed to UV light

Graph indicating the rate of degradation of Patrin-2 (5mg%) when exposed to UV light

Figure 3.6 Graph indicating the rate of degradation of Patrin-2 with increase in the time. n=2 for MPA.
3.2.4 Stability of Patrin-2 when incubated in acidic and basic solutions:

**Acid:** 0.1M H$_2$SO$_4$ was prepared and added to the stock solution of Patrin-2 (5mg/dL) in the ratio of 1:1 to give a final sample of concentration 2.5mg/dL. This was incubated at 40°C and RT. Rapid degradation was seen and the end results were as follows.

![Figure 3.7](image1.png)  
**Figure 3.7** Patrin-2 solution in 0.1M H$_2$SO$_4$, (1:1) and was incubated at 40 °C. Patrin-2 completely degraded in 45mins. (Abs. 254nm)

![Figure 3.8](image2.png)  
**Figure 3.8** Patrin-2 solution in 0.1M H$_2$SO$_4$, (1:1) and was incubated at RT. Patrin-2 completely degraded in 50mins. (Abs. 254nm)

57
Patrin-2 quickly degraded under both conditions when acid of as low as 0.1M concentration was added to it. It took just about the same time to degrade completely in both the conditions. Similar by-products to those in the reaction of the sample with H₂O₂ were seen during the acid reaction of the sample too. There non-polar by-products seen in this reaction. By-products were detected at 2.83, 3.15, 3.50, 6.81 and 14.48mins. Small traces of by-products were seen at 7.85, 16.10 and 20.32mins.

**Base:** 0.1M NaOH was prepared and added to the stock solution of Patrin-2 (5mg/dL) in 1:1 ratio to give 2.5mg/dL concentration samples. This sample was incubated at 40°C. The results are as below.

![HPLC chromatogram](image)

**Figure 3.9** HPLC chromatogram at 254nm showing the degradation in Patrin-2 15 days after the base was added to it. The Patrin-2 peak can be seen at 12.18mins.
Figure 3.10 HPLC chromatogram at 254nm showing the degradation in Patrin-2 45 days after the base was added to it. The Patrin-2 peak can be seen at 12.52mins.

As can be clearly seen, Patrin-2 has a better stability towards base as compared to acid. The same degradation products were formed every time it was subjected to degradation. There were a number of both polar and non-polar by-products to this reaction. Even though the quantity of the by-products increased, it was gradual and slow and the quantity of Patrin-2 went down slowly. Some by-products that were observed eluted out at 2.10, 2.64, 5.40, 6.75, 7.15, 7.53, 8.34, 14.53 and 15.73mins.
3.2.5 Stability of Patrin-2 in N-methylpyrrolidone

Patrin-2 was dissolved in N-methylpyrrolidone to give samples of 5mg/dL conc. These samples were left at RT and at 40°C. Figures below show the effect NMP had on Patrin-2.

**Figure 3.11** A standard of 5mg/dL conc. of Patrin-2 in NMP.

**Figure 3.12** HPLC chromatogram of Patrin-2 in NMP (5mg/dL) kept at RT. After a month, no degradation products can be seen, but there is a change in the peak shape.
Figure 3.13 HPLC chromatogram of Patrin-2 in NMP (5mg/dL) kept at 40°C. After a month, no degradation products can be seen, but there is a change in the peak shape.

The change in peak shape suggests some degradation activity in Patrin-2 when dissolved in NMP, but the excipients or the degradation products have about the same run time in this particular HPLC system. Also, the peak shapes are wide so it’s hard to distinguish how many different by-products have been detected. This system does not manage to separate the by-products of Patrin-2 solution and N-methylpyrrolidone.
CHAPTER 4
DISCUSSION
4.1 Validation of HPLC System

Validation of the HPLC system was carried out following standard procedures for validation. The system was checked for factors like precision, specificity, robustness. To do so, linearity test, repeatability and reproducibility tests, determination of LOD and LOQ were done. The results were positive in all case. They resembled those obtained in the previous works by Dr Leroy Shervington on the similar study of Patrin-2. The regression coefficient of the linearity test was calculated to be 0.999 which suggested a strong correlation between the concentration and the area. This suggested that the dilutions had been carried out with precision, accuracy and was specific too. The Relative Standard Deviation (RSD) for repeatability and reproducibility were found to be as low as 0.029 and 0.233 respectively. This suggested that the protocol was reliable and repeatable. Various buffers were made and run on the system before selecting the one that was used. The buffer recipe was adopted from a previous work done by (Shervington, L.A et al, 2005). HPLC column (Symmetry 300) was ordered from Waters Ltd. This particular column was selected for the study based on the fact that it gives a superior and sharp peak. This was owed to the packing material in the column which was C_{18} chains bonded to the silica. Longer chains produce packing that are more retentive and allow use of larger samples, the maximum sample size of column type C18 is roughly twice of that of C4 under similar conditions (Skoog et al., 2007).

Patrin-2 is does not have a good solubility. DMSO is the only listed solvent, that Patrin-2 is known to dissolve in. This is not an ideal solvent for stability studies. A solvent had to be formulated in a way that the concentration DMSO, if it’s required at all, had to be kept low. A mix of DMSO (5%), Acetonitrile (25%) in HPLC grade water proved to work efficiently.

4.2 Degradation of Patrin-2 at the three temperatures

Three temperatures were selected to carry out forced degradation; samples were incubated at 40°C, 50°C and 80°C. The 5mg/dL Patrin-2 samples reacted in varied manner under all the conditions.
The compound tends to degrade very slowly when stored at 40°C and analysed. The samples treated with nitrogen should a slower degradation as compared to samples that aren’t treated with nitrogen. Also, solid samples had been incubated and tested for degradation and didn’t display degradation. This shows the compound is far more stable at 40°C and didn’t show signs of degradation for about a month after incubation. Little degradation was seen after 45 days of incubation.

The compound got unstable when incubated at 50°C. The nitrogen-treated and non-treated samples, both showed rapid degradation. This helped in determining the degradation products formed. On the HPLC chromatogram acquired by running the sample on the validated HPLC system, many degradation products peaks were seen at 3.42 min, 6.82min, 7.28min, 7.78min, and 8.38min respectively on the chromatogram. These were some common peaks seen in all chromatograms acquired of degraded Patrin-2 in different conditions. These were observed at all the three working absorbance’s and could be quantified comparatively better on the 254nm wavelength.

The drug showed rapid and accelerated degradation when incubated in the 80°C oven. The drug degraded completely after 15days of incubation. The rate of degradation, in this case, was higher in nitrogen treated samples than the ones that weren’t treated with nitrogen.

Forced degradation was achieved with the photolytic tests. Also, the drugs lability to each temperature condition could be checked, and helped us determine that Patrin-2 is highly unstable when subjected to photolytic stress. The more the thermal stress, the faster the compound degrades.

4.3 Effect of H₂O₂ on Patrin-2

0.4M and 0.8M solution was made out of 30% H₂O₂ and this was added to the 5mg/dL stock solution of Patrin-2 in the ratio (1:1). These samples of 2.5mg/dL thus formed, were stored in varied temperatures and caused oxidative stress to the compound.

They were accelerated degradations. The samples added with 0.4M H₂O₂ and stored at 50°C completely degraded in approximately 180mins.
The samples incubated at 40°C showed a slight difference with the change in concentration of H₂O₂. The ones in which 0.4M of H₂O₂ was added, took 180mins before the compound completely degraded, whereas the ones which were formed with 0.8M H₂O₂ displayed a quicker degradation rate and it was 120mins before the drug completely degraded.

At body temperature, however, the rate of degradation was slightly slow. The solution made of 0.4M H₂O₂ degraded completely around 390mins and the one made in 0.8M H₂O₂ degraded completely around 240mins when incubated in a 37°C water bath. This was to maintain the temperature similar to the human body temperature.

The results clearly indicated that the compound is labile to oxidative stress. When oxidative stress, combined with thermal stress is applied, the compound’s degradation accelerates.

### 4.4 Effect of light: UV and direct sunlight

Exposure to direct sunlight had negligible effect on the 5mg/dL Patrin-2 solution. The slight change in the peak shape was probably due to the degradation caused by DMSO and acetonitrile present in the solvent, even though in a small, in which Patrin-2 was solubilised. Over the time, they could be slowly bringing out the degradation.

The exposure to UV radiation, however gave rise to an accelerated forced degradation in the compound. The compound displayed a very high degradation rate for the initial one hour. Once the quantity of Patrin-2 decreased tremendously and the degradation products started forming, the rate of further degradation of Patrin-2 effectively slowed down a bit. The compound was nearly degraded completely after 5hours of exposure.

The drug is clearly labile to photolytic stress.

The drug is subjected to a high temperature, every time its put through photolytic stress, or acid stress, or direct temperature stress. If we can accurately determine the temperature during each stability profile, we can determine a rate constant for that profile based on the rate of degradation at that temperature. As the
temperatures have been recorded during our studies, we can determine the rate constant.

4.5 Effect of pH on Patrin-2

The compound was incubated in acidic and basic conditions. It was stored in 0.1M acid in 1:1 ratio and so also with 0.1M base. There was a huge difference is the reactions that took place after in both the cases.

Patrin-2 with acid: Patrin-2 is highly unstable when subjected to acidic stress. The acidic samples were incubated at RT and at 40°C, both of which degraded completely within 45mins of incubation. The acidic stress brings about highly accelerated degradation in Patrin-2 and the compound is labile to acidic stress and this stress is enough by itself to bring about accelerated degradation.

Patrin-2 with base: The compound was labile in basic surrounding too. But the rate of degradation was very slow as compared to the degradation rate seen on its incubation in acid. It showed gradual degradation and took about 45 days before it showed considerable degradation in base.

4.6 Patrin-2 in NMP

N-methylpyrrolidone was one of the solvent used to form Patrin-2 tablets. We, therefore, decided to check for the stability of Patrin-2 in this solvent. Patrin-2 readily solubilised in this solvent, however when it was run on HPLC, it showed a wide peak, even on a column that was designed for superior peak quality. After incubating the compound in NMP for a month, no degradation product seemed to have formed in the solution. However, there was seen a change in the peak shape. There was, therefore, a possibility that the compound had formed a degradation product or maybe products in this solvent which had the retention time nearly similar to that of Patrin-2. The wideness of the peak also makes it unsuitable for usage in stability indication. There are, however, chances it could give a superior peak on some other HPLC system. This could interestingly be used for further HPLC studies and analysis on Patrin-2.
CONCLUSION

A validated stability indicating method was achieved using HPLC. The validation of the system was carried out effectively indicating the method to be linear, precise, accurate, specific and robust. The stability studies on the drug were carried out successfully. The drug when subjected to thermal, photolytic, oxidative and acidic stress degraded into many degradation products. In most of the cases, the degradation rate was seen to be directly proportional to the amount of stress applied. The more the thermal stress was increased by increasing the incubation temperature, the faster the degradation took place. The more the concentration of H₂O₂, the faster the drug degraded. This further accelerated with the increase in temperature. It however, displayed a uniform rate of degradation when acidic stress was applied. Even an increase in temperature didn’t seem to affect it further. It showed a very high degradation rate when photolysed using UV radiations. However, the rate of degradation dropped after the compound level in the solution dropped to a very low amount. The drug was seen to be highly unstable in most of the conditions it was subjected to. However, it stayed stable at RT without any external stress. Even exposure to sunlight had very little effect on it. It gradually degraded in alkaline environment too, making it labile to acid and base both. It degraded to a number of degradation products. Since this drug is designed for usage in treatment for glioma, and has to work in combination with Temozolomide, it is highly recommended to try and identify the degradation products. This could be done using a mass spectrometer linked to the HPLC unit. Also, studies of Patrin-2 have been carried out in combination with temozolomide in the past and the drug is withdrawn from usage because of it’s highly unstable nature. Most of the degradation was carried out in the solution form, and it can be concluded that Patrin-2 is highly unstable when formed into a solution. It’s lability to almost all condition makes it difficult to store it if formed into a solution.

This study could also be used to determine the shelf-life of the compound in the drug form.
FUTURE PROSPECTS

The study carried out as described above has clearly shown Patrin-2 to be highly unstable in solution form. It degraded under all stressful conditions it was subjected to, some at a high rate and some at slow. It would be interesting, though, to analyse the compound in solid form. It could be subjected to various stress conditions (thermal, photolytic etc) and analysed check for it’s lability. The drug being difficult to solubilise, it would also be interested to check the effect humidity has over the solid form.

A number of unidentified degradation products were formed on degradation of this compound. Not enough work has been done on indentifying these products. The study could be taken to it’s next stage by identifying some of these products and checking for their toxicity. This can be done by running the solutions on chromatographic units linked to a mass spectrometer (LC-MS or GC-MS).

A similar method could be designed in future, keep similar parameters, if the monologue of a compound with similar configuration or properties is to be tested for stability and has to be subjected to stress conditions to check it’s lability.


Appendix 1:

Validation of HPLC system for Patrin-2:

Figure 6.1 HPLC chromatogram for Patrin-2 (5mg/dL) n=2.

Figure 6.2 HPLC chromatogram for Patrin-2 (4mg/dL) n=2
Figure 6.3 HPLC chromatogram for Patrin-2 (3mg/dL) $n=2$.

Figure 6.4 HPLC chromatogram for Patrin-2 (2mg/dL) $n=2$
Figure 6.5 HPLC chromatogram for Patrin-2 (2mg/dL) $n=5$. Repeatability.

Figure 6.6 HPLC chromatogram for Patrin-2 (2mg/dL) $n=5$. Reproducibility.
**Figure 6.7** HPLC chromatogram for Patrin-2 (1mg/dL) $n=2$

**Figure 6.8** HPLC chromatogram for Patrin-2 (0.5mg/dL) $n=2$
Figure 6.9 HPLC chromatogram for Patrin-2 (0.25mg/dL) n=2

Figure 6.10 HPLC chromatogram for Patrin-2 (0.125mg/dL) n=2
Figure 6.11 HPLC chromatogram for Patrin-2 (0.0625 mg/dL) \( n=2 \)

Figure 6.12 HPLC chromatogram for Patrin-2 (0.03125 mg/dL) \( n=2 \)
Figure 6.13 HPLC chromatogram for Patrin-2 (0.0156mg/dL) n=2

Figure 6.14 HPLC chromatogram for Patrin-2 (0.0078mg/dL) n=2. Limit of quantification.
Figure 6.15 HPLC chromatogram for Patrin-2 (0.0039mg/dL) \( n=2 \). Limit of Detection.

Appendix 2:

Formulae for Relative Standard Deviation (RSD):

\[
RSD = \frac{SD}{MPA} \times 100
\]

Where,

SD= Standard Deviation

MPA= Mean Peak Area.
Appendix 3

HPLC chromatograms for degradation:

Figure 6.16 Degradation at 40°C on day 3 (240nm)

Figure 6.17 Degradation at 40°C on day 3 (254nm)
Figure 6.18 Degradation at 40°C on day 3 (285nm)

Figure 6.19 Degradation at 40°C on day 115 (240nm)
Figure 6.20 Degradation at 40°C on day 115 (254nm)

Figure 6.21 Degradation at 40°C on day 115 (285nm)
Figure 6.22 Degradation at 50°C on day 3 (240nm)

Figure 6.23 Degradation at 50°C on day 3 (254nm)
Figure 6.24 Degradation at 50°C on day 3 (285nm)

Figure 6.25 Degradation at 40°C on day 115 (240nm)
Figure 6.26 Degradation at 50°C on day 115 (254nm)

Figure 6.27 Degradation at 50°C on day 115 (285nm)
Figure 6.28 Total degradation of Patrin-2 @ 80°C in 408 hrs (seen at 240nm, 254nm and 285nm, respectively)
Figure 6.29 NMP run by itself with the working mobile phase at (a) 240nm, (b) 254nm and (c) 285nm
Figure 6.30 Patrin-2 solution (5mg/dL) exposed to direct sunlight at RT. Chromatogram for the changes after one month of exposure (in summertime)

Figure 6.31 Patrin-2 solution (5mg/dL) exposed to direct sunlight at RT. Chromatogram for the changes after two month of exposure (in summertime)
Figure 6.32 Patrin-2 solution (5mg/dL) standard solution. Peak Area of Patrin-2 is 1502.02