Drug delivery systems to include liposomes and microparticles in order to aid treatment of glioma

By
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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

Neha Parkar

ABSTRACT

Targeted drug delivery is achieved by enhancing drug availability at the response site while minimising its availability at other sites, especially those that manifest toxicity. The effects of liposomes and polymeric nanoparticles in cancer chemotherapy have been investigated in recent years and there have been some interesting outcomes resulting in improved survival rates of the patients. Thus, the present study was carried out to investigate the effect of encapsulating TMZ and Patrin-2 into liposomes and microparticles separately in order to enhance the delivery.

A validated HPLC system was used for the analysis of both TMZ and Patrin-2. First phase of our study involved the preparation of a delivery system of TMZ and Patrin-2 through liposomes in order to enhance the treatment of glioma. It involved the application of freeze-thaw and dehydration-rehydration methods to encapsulate the TMZ into liposomes. However, the desired encapsulation efficiency (EE %) of TMZ was not achieved using these methods, the maximum entrapment achieved with freeze-thaw method was 11.54 ± 0.70 % while for dehydration-rehydration method was 26.69 ± 0.34 %. Therefore the second phase focussed on preparing polymeric microparticles for continuous delivery of intact TMZ and Patrin-2 using the spray dry method. The maximum entrapment achieved using this method for TMZ was 64.32 ± 2.58 % while the maximum entrapment achieved for Patrin-2 was 68.47 ± 1.47 %, thus, this technique was found to be successful for the preparation of both TMZ and Patrin-2 loaded PLGA microparticles. Furthermore the release study of both TMZ and Patrin-2 was investigated using dispersion and dialysis methods. TMZ and Patrin-2 showed an initial burst release, however TMZ later showed decrease in concentration over the period of time while Patrin-2 showed a slow release with an increase in concentration.

This novel study aids in comparing various methods used for investigating the preparation of TMZ loaded liposomes, and TMZ and Patrin-2 loaded microparticles. In preparation of liposomes based on this research dehydration-rehydration was found to be a more efficient method than the freeze-thaw method for encapsulating TMZ, while the spray dry method was found to be more effective than the emulsifying solvent evaporation method in obtaining the maximum EE % for TMZ and Patrin-2 for preparation of microparticles. The release profiles of TMZ and Patrin-2 from the microparticles was studied using the dispersion method and the dialysis bag diffusion technique however due to tome being the limited factor the technique could not be explored completely.

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"If A equals success, then the formula is: A = X + Y + Z, X is work. Y is play. Z is keep your mouth shut." ***Albert Einstein***

LIST OF ABBREVIATIONS

4BTG	O^6-(4-bromothenyl) guanine (Patrin-2)
AIC	5-aminoimidazole-4-carboxamide
AP	Apurinic/Apyrimidinic
ATase	Alkyltransferase
BCNU	Carmustine
BER	Base Excision Repair
CCNU	Lomustine
Da	Dalton
DMPC	1,2-distearoyl-*sn*-glycerol-3-phosphocholine
DNA	Deoxyribonucleic acid
DSPC	1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine
EE %	Encapsulation Efficiency
GA	Glycolide
GUVs	Giant Unilamellar Vesicles
HPLC	High Performance Liquid Chromatography
hr	Hour
LOD	Limit of Detection
LOQ	Limit of Quantification
LUVs	Large Unilamellar Vesicles
MGMT	O^6-methylguanine-DNA methylltransferase
min	Minute
MLVs	Multilamellar Vesicles
MMR	Mismatch Repair

MPA	Mean Peak Area
MPG	Methylpurine glycosylase
MTIC	Methyltriazen-1-yl imidazole-4-carboxamide
MVVs	Multivesicular Vesicles
MW	Molecular Weight
MX	Methoxyamine
N^7-meG	N^7-methylguanine
N^7-meGDNA	N^7-methylguanine DNA
N/A	Not Applicable
O^6-BG	O^6-benzylguanine
O^6-meG	O^6-methylguanine
O^6-meGDNA	O^6-methylguanine DNA
PBS	Phosphate Buffer Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PLA	Polylactide
PLGA	Poly(d,l-lactide-*co*-glycolide)
PVA	Polyvinyl Alcohol
rpm	Rotations Per Minute
RSD	Relative Standard Deviation
RT	Retention Time
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy

SUVs	Small Unilamellar Vesicles
TBAA	Tetrabutylammonium Acetate
TMZ	Temozolomide
UDP	Unidentified Product
ULVs	Unilamellar Vesicles
USP	United States Pharmacopoeia
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Glioma

Gliomas are the most frequent primary brain tumours, derived from glial cells of astrocytic, oligodendroglial and ependymal origin. Glial cells are the supporting cells of central nervous system, providing the support and nourishment to the neural cells in the brain (Trent *et al.,* 2002). Glial cells show an ability to undergo cell division and on loss or uncontrolled cell division giving rise to tumour known as glioma. Gliomas start in the brain or spinal cord tissue and do not spread to other areas of the body but can spread within the nervous system and can be either benign or malignant (Sawyer *et al.,* 2006). Since there are mainly three types of glial cells, glioma is also of three types namely- astrocytoma, ependymoma and oligodendroglioma. A fourth type of glioma is also present, mixed glioma, and is a mixture of the other types. Gliomas have been defined pathologically as tumours that display histological, immunohistochemical, and ultrastructural features of glial differentiation (Chang *et al.,* 2005). Based on pathological and histological appearance, glioma can be classified into different grades. The most widely used classification of human gliomas is that of World Health Organisation (WHO), revised in year 2000 (Kleihues *et al.,* 2002). According to the WHO classification of brain tumours gliomas are divided in low-grade (grades I and II) and high-grade (grades III and IV) tumours (Scott *et al.,* 1998). Low grade tumours are well differentiated, nonanaplastic, benign tumour showing better prognosis, where as high grade tumours are undifferentiated, highly aggressive, anaplastic and malignant tumour showing very poor prognosis (Maher *et al.,* 2001). Surgery, radiotherapy and chemotherapy are the most commonly used treatments for gliomas, however none of these procedures have been able to cure the disease due to the diffuse nature of gliomas (Bobola *et al.,* 2004). These procedures are sometimes used in a combination to improve the survival and quality of life (Cher *et al*., 2008). Several randomized clinical trials have showed that median survival to

be longer with radiation and chemotherapy (Sandberg-Wollheim *et al*., 1991). The most commonly used drugs in chemotherapy include Temozolomide (TMZ), Carmustine (BCNU), and a three drug combination of Procarbazine, Lomustine (CCNU) and Vincristine, together abbreviated as (PCV) (Westphal *et al.*, 2003; Stupp *et al*., 2005).

1.2 Temozolomide

TMZ is an oral alkylating agent which has a single highly reactive site (Fig. 1.1). It can readily cross the blood brain barrier (Newlands *et al.*, 1992; Darkes *et al.*, 2002), and has proved to be active against variety of tumour type *in vitro* as well as *in vivo*. Due to its acid-stability and lipophilic character, TMZ taken orally has a bioavailability of ≈100% (Darkes *et al.*, 2002).

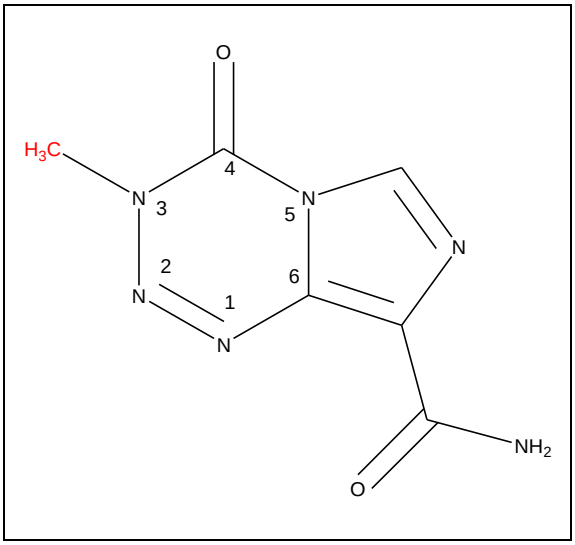


Figure 1.1 Chemical structure of Temozolomide with a single highly reactive site (The chemical structure was drawn using the chemdraw ultra 12.0 software).

TMZ is a modified form of imidazotetrazinones and is also chemically associated to dacarbazine and is the 3-methyl derivative of an anticancer drug mitozolomide (Newlands *et al.*, 1997). TMZ is found to be unstable above pH 7 and is instantaneously hydrolysed to methyltriazen-1-yl imidazole-4-carboxamide (MTIC) (Lowe *et al.*, 1992). Assimilation in the intestine results in the electropositive carbonyl at C4 position located in the tetrazinone ring in TMZ being disposed to nucleophilic attack by water

(Denny *et al.*, 1994). The resulting ring cleavage with the loss of carbon dioxide leads to the development of methyltriazen-1-yl imidazole-4-carboxamide (MTIC) which then undergoes further break down to an inactive carboxylic acid derivative, 5-aminoimidazole-4-carboxamide (AIC), and a highly reactive methyldiazonium ion (Fig. 1.2) (Clark *et al.*, 1995). The methyldiazonium ion methylates guanine residues in guanine-rich regions (Friedman *et al.*, 1998). Methylation at the O^6 position of guanine in genomic DNA is an important step as damage caused by methylation at guanine O^6 are usually repaired by the enzyme O^6-methylguanine-DNA methyltransferase (MGMT) (Fig. 1.3). Each time a DNA lesion is repaired one molecule of the MGMT enzyme goes into a non-functional state (Margison *et al.*, 2005). If TMZ is taken on a regular basis the repair protein is eradicated each time and more damaging DNA lesions will be amassed (Friedman *et al.*, 1998).

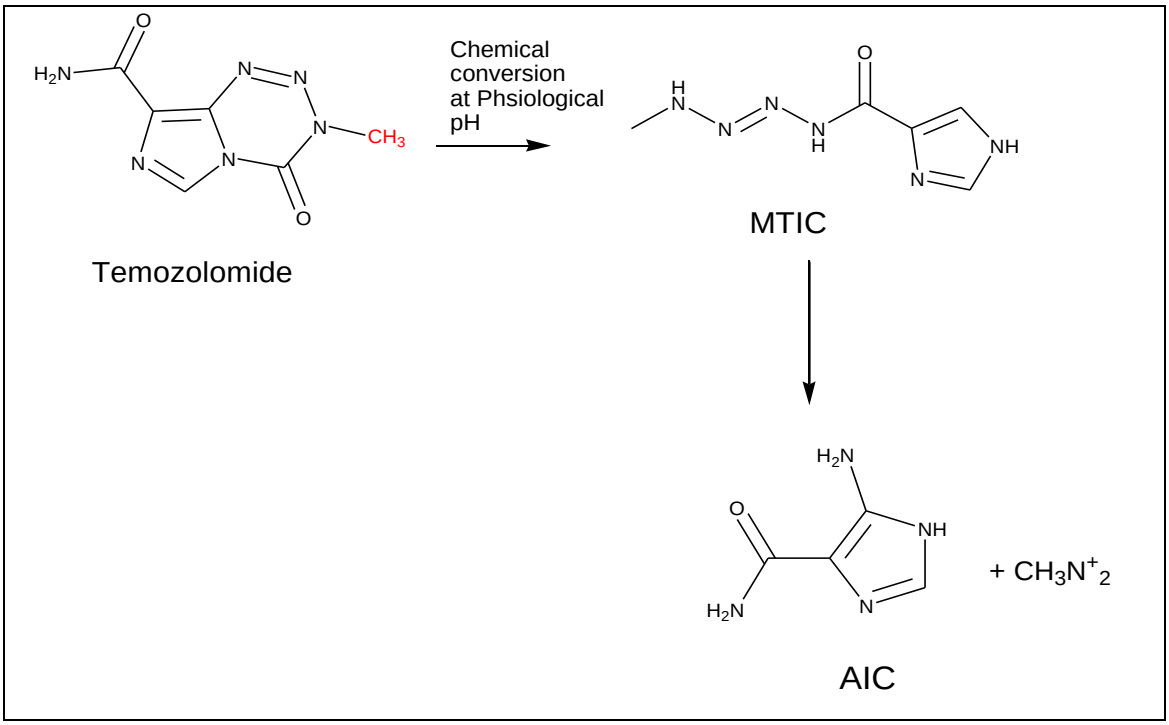


Figure 1.2 Mechanism of action of TMZ above pH 7 (The chemical structure was drawn using the chemdraw ultra 12.0 software).

Many brain cancer types do not express the MGMT enzyme (Friedman *et al.,* 1998). However, certain types of cell lines and xenografts express high concentrations of the MGMT enzyme and show resistance to TMZ exposure (Brada, 2002). Damage occurs due to mutation and those that cannot be repaired by MGMT are identified by mismatch repair (MMR) enzymes, which recover the mismatch caused in the daughter strand by removing the thymine base from O^6-methylguanine (O^6meg): thymine base pairs. However, MMR enzymes are not always successful in performing their task and eventually cause breaks and nicks in the daughter strands (Hirose *et al.,* 2001). This event leads cell arrest at the G2/M phase prior to apoptosis. TMZ, similar to other alkylating agents have an effective antitumour activity for the replicating cells (D'Atri *et al.,* 1998; Darkes *et al.,* 2002).

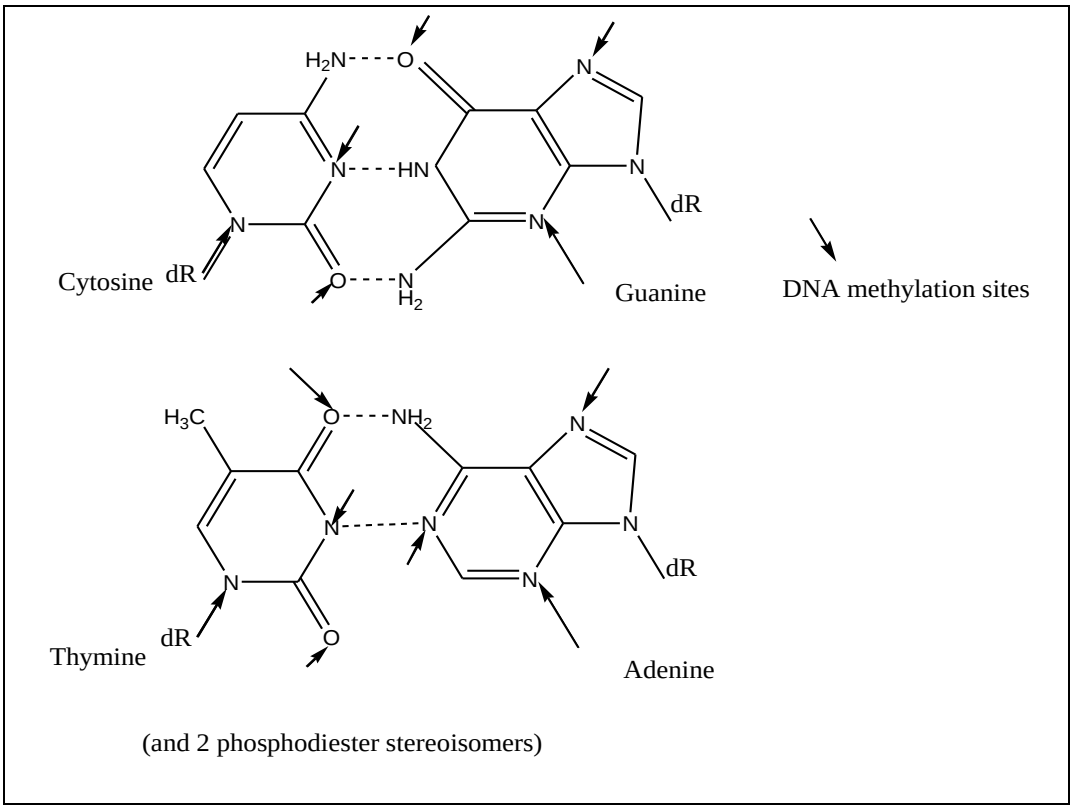


Figure 1.3: DNA methylation sites (The chemical structure was drawn using the chemdraw ultra 12.0 software).

The three main DNA repair mechanisms responsible for TMZ resistance are: elevated intracellular levels of MGMT; a deficient mismatch repair process (MMR); and base excision repair pathway (BER) with activation of the poly (ADP)-ribose polymerase protein (Tentori *et al.*, 1997). Primary resistance to TMZ is directly correlated to high MGMT levels but in MGMT-deficient cells, low or deficient MMR protein levels become important in conferring resistance (Darkes *et al.*, 2002). One of the ways of enhancement of TMZ activity is by direct inactivation of ATase. From previous studies, ATase inactivator O^6-(4-bromothenyl) guanine (4BTG, also known as Patrin-2) used in *in vivo* studies as a pre-treatment to the mouse melanoma model has shown to improve the antitumor effect of TMZ without any additional toxicity (Brock *et al.*, 1998).

TMZ, despite its advantages also shows limitations in its use as an anticancer reagent. TMZ is found to develop unusual cardiomyopathy, acute toxicity which leads to myelosuppression and oral ulcerations when it binds to the site other than the target site (Huang *et al.*, 2008). In order to prevent these consequences and to obtain better efficacy, it is important to develop a treatment that enables the drug to reach directly to the site of action (Yang *et al.*, 1999). Studies have shown that the use of colloidal substances such as microemulsions, liposomes, niosomes and polymeric nanoparticles as drug carriers have significantly helped to overcome these drawbacks such as acute and chronic toxicity or low stability by decreasing the number of dosage of the drug (Muller *et al.*, 2000; Huang *et al.*, 2008). Furthermore, liposome and microparticles also help to attain controlled release of TMZ (Allen and Cullis, 2004). Thus one of the main aims of this study is to prepare a delivery system of TMZ in order to decrease its toxicity and side effects (Fig. 1.4) (Menei *et al.*, 1996; Menei *et al.*, 1997). TMZ is usually required in high doses to attain the therapeutic brain levels because of its short half life that is 1.8 hr in plasma (Reni *et al.*, 2004; Zhang and Gao, 2007). Thus,

resulting insufficient doses reaching the target site. These problems could be overcome to some degree by use of liposomes or microparticles for the drug delivery as their use reduces the high amount of temozolomide intake in the body which is the reason of side effects (Ostro, 1983; Moghimi and Davis, 1994).

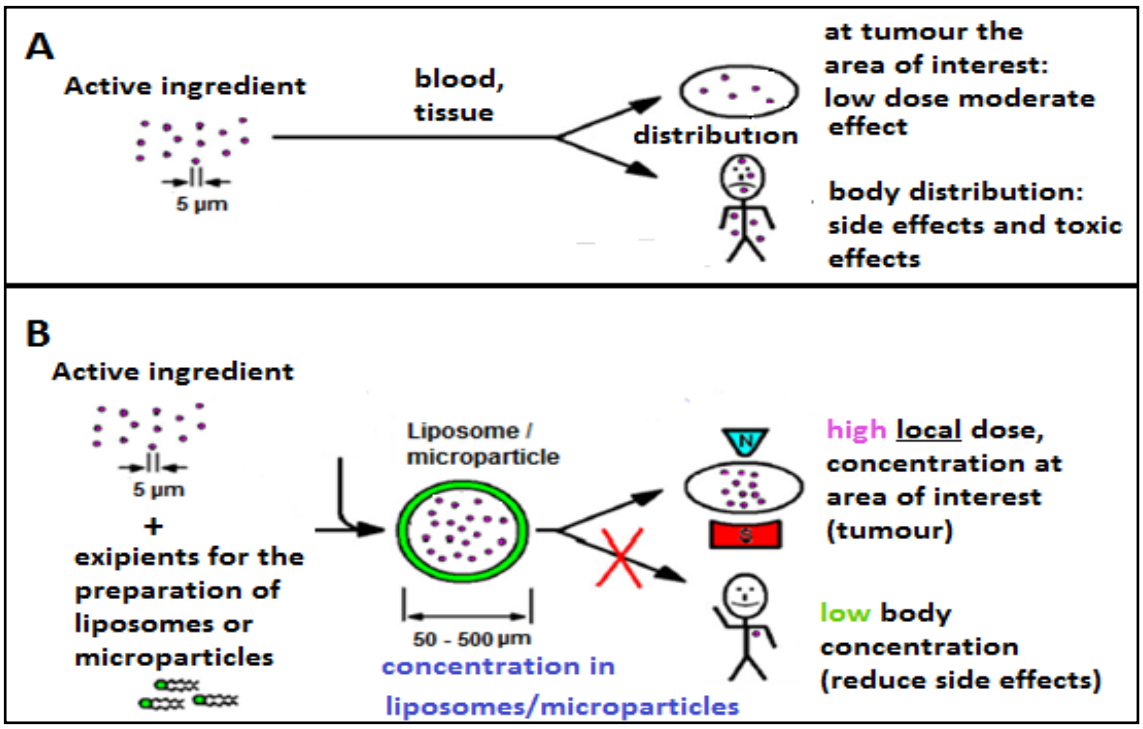


Figure 1.4 Use of liposome and microparticles as drug carriers improves the effect of molecular active substances (Modified from http://ubm.opus.hbz-nrw.de/volltexte/2009/1880/pdf/diss.pdf).

1.3 Patrin-2

In order to further enhance the TMZ activity there is a need to use a drug/substrate which would support its mechanism of action and increase the sensitivity of chemotherapy. Patrin-2 is a compound that has recently been introduced into clinical studies (Turriziani *et al.*, 2006). Patrin-2 is a non-hazardous pseudo substrate with low-molecular weight and has the ability of inactivating MGMT (Fig. 1.5) (Khan *et al.*, 2008). Patrin-2 has also proved to be better tolerated than O^6-benzylguanine (O^6-BG) derivative (Bobola *et al.*, 2005; Woolford *et al.*, 2006).

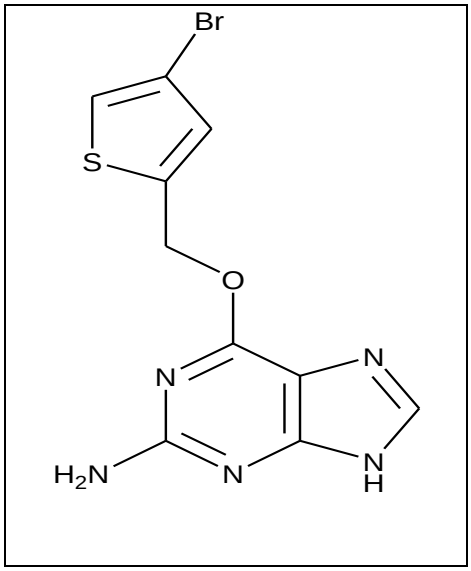


Figure 1.5 Chemical structures of Patrin-2 (The chemical structure was drawn using the chemdraw ultra 12.0 software).

It has mainly been used in combination with triazene compounds to enhance their anti-tumour efficacy against different types of tumour cells endowed with elevated MGMT activity (Barvaux *et al.,* 2004 a and b; Clemons *et al.,* 2005). When a combination of Patrin-2 and TMZ was used, Patrin-2 showed sensitisation of human tumour xenografts of the methylating compounds (Middleton *et al.,* 2002).

The DNA damage caused by TMZ or other methylating therapeutic drug in human cells is usually repaired by MGMT and other DNA repair mechanisms (Papouli *et al.,* 2004). Such methylating therapeutic drugs cause possible DNA lesions such as O^6-meG and N^7-methylguanine (N^7-meG) which are cytotoxic in nature (Fig. 1.6). MGMT by means of covalent transfer of the alkyl group to cysteine the conserved active site, removes the O^6-alkylguanine DNA adduct and thus restoring the guanine to normal. In this process MGMT is inactivated by receiving the methyl group from O^6-meG and is subjected to ubiquitin-mediated degradation. Similarly when MGMT transfers and accepts an alkyl group from Patrin-2 or O^6-BG it becomes inactivated and is degraded thus, MGMT is also called as suicidal enzyme (Pearson *et al.,* 2005). When

an O^6-meG DNA adducts escape from MGMT repair, during DNA replication O^6-meG DNA forms a base pair with thymine. This base pairing is recognised by the MMR pathway causing futile cycles of repair which leads to cell death. N^7-meG DNA adducts formed by TMZ (>70% of total DNA adducts) are successfully repaired by the BER pathway. The BER pathway contribution to the cytotoxicity of TMZ is only minimum. In this pathway, methylpurine glycosylase (MPG) binds methoxyamine (MX) to AP (apurinic/apyrimidinic), sites which are refractory to AP endonuclease cleavage, causing blockage of the BER pathway (Fig. 1.6). This causes disrupted replication, strand breakage and increased cytotoxicity of TMZ (Bobola *et al.*, 2004; Liu and Gerson, 2006).

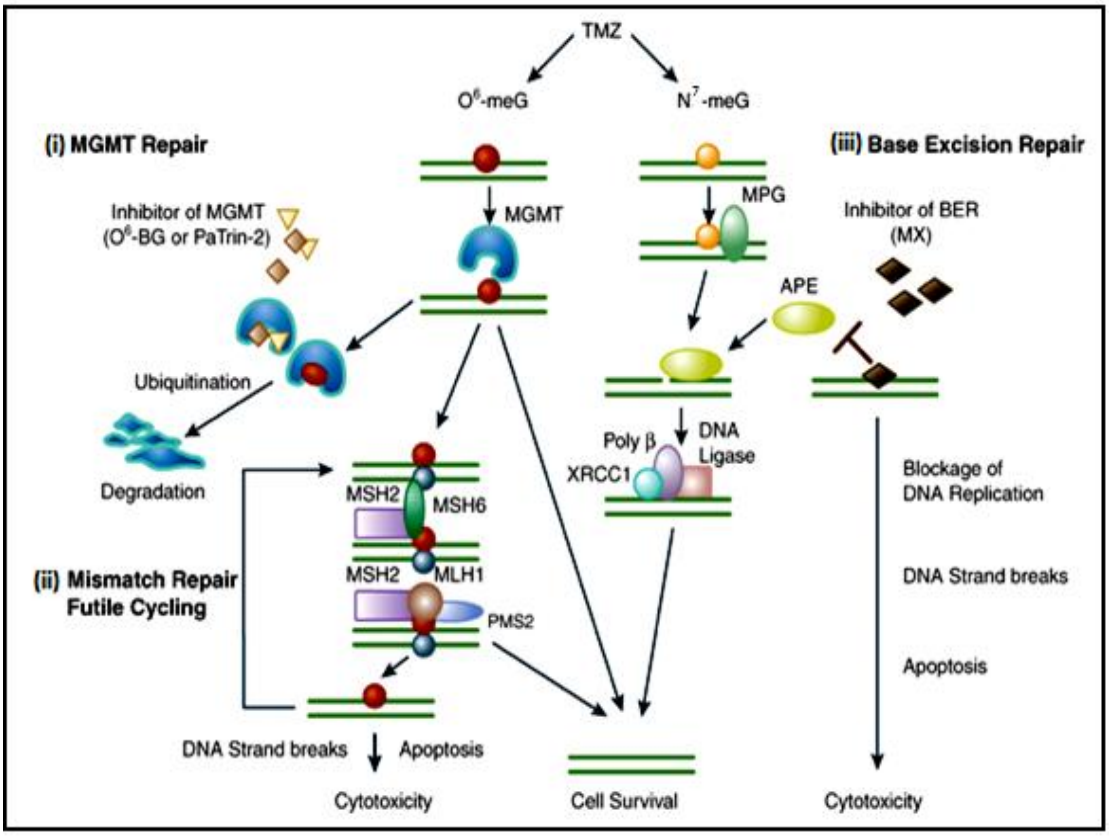


Figure 1.6 Action of TMZ and Patrin-2 causing MGMT and other DNA repair mechanisms to deal with DNA damage caused due to methylation of the TMZ, in human cells (Liu and Gerson, 2006).

Thus, the anti-tumour activity of methyl-triazene compounds such as TMZ mainly depends on the production of methyl adducts to O^6 or N^3 or N^7 of purine bases of DNA

(Philip *et al.,* 1996; Tentori & Graiziani *et al.,* 2002; Bodell *et al.,* 2003). The two DNA repair enzyme systems, MGMT and MMR, play crucial roles in circumstancing the cytotoxic effects of methyl-triazene compounds (Pegg *et al.,* 1983; Dolan *et al.,* 1990; Pegg, 2000; Gerson, 2004; Trivedi *et al.,* 2005; Morimoto *et al.,* 2005; Turriziani *et al.,* 2006). Resistance to triazenes is attributed to the high levels of MGMT related with target cells, while MMR aids the cytotoxic effect of these agents (Esteller *et al.,* 2000; Fedier and fink, 2004). Resistance to the triazenes such as TMZ caused by MGMT can markedly be decreased *in vitro* and *in vivo* by use of the pseudo substrate Patrin-2 (Middleton *et al.,* 2000; Kaina and Christmann, 2002). Based on these studies, the present study was carried out to investigate the effect of encapsulating TMZ and Patrin-2 into liposomes and microparticles separately and in combination in order to enhance the cytotoxicity of TMZ. Some work has been carried out to investigate the effects of liposome and polymeric nanoparticles on cancer chemotherapy and there have been some interesting outcomes regarding reduced side effects and can result in improved survival rates of the patients (Kitamura *et al.,* 1996; Ranson *et al.,* 2007).

1.4 Liposomes

Liposomes were discovered by A. Bangham and his team about 45 years ago and ever since then they developed into versatile tools in biology, medicine and biochemistry. Liposomes are the smallest artificial vesicles of spherical shape that can be produced from natural untoxic phospholipids and cholesterol (Bangham *et al.,* 1965). Liposomes can be defined as a particular kind of vesicles or any structure formed by lipid bilayers which are capable to enclose a volume of the substance (Ostro, 1983; Connor & Huang, 1986). These amphipathic molecules constitute one or more hydrophobic chains, one hydrophilic region where hydrophilic region forms the head group while the hydrophobic region form the tail. When suspended in an aqueous medium they

instinctively form bilayer vesicles (Gomez-hens and Fernandez-Romero, 2006). The basic structure of liposomes comprises of hydrocarbon chains which lean towards each other while the polar head groups are in contact with encapsulated and surrounding aqueous medium (Fig. 1.7) (Storm and Crommelin, 1998; Gomez-hens and Fernandez-Romero, 2005). The structure of the liposomes can be determined by the number of bilayers present. Single bilayers (lamella) liposomes are called unilamellar vesicles (ULVs), while liposomes containing more than one bilayer are multilamellar vesicles (MLVs). Other liposomes consisting of small vesicles incorporating large vesicle are known a multivesicular vesicles (MVVs) (Gomez-hens and Fernandez-Romero, 2005). The entrapment of active compounds also depends on the different sizes of liposomes ranging from nanometre (nm) to micrometre (µm), such as small unilamellar vesicles (SUVs, 25-50 nm), large unilamellar vesicles (LUVs, 100 nm-1 µm), giant unilamellar vesicles (GUVs, 1.0-200 µm), multilamellar vesicles (MLVs, 0.1-15 µm), and multi-vesicular vesicles (MVVs, 1.6-10.5 µm) (Silva *et al.*, 2010). Liposomes can usually be stored in a buffer at pH 7.4 and at temperature approximately 4 °C for 5-7 days. However, storage time depends on a number of factors which include temperature, pH and medium (Oberholzer *et al.*, 1999).

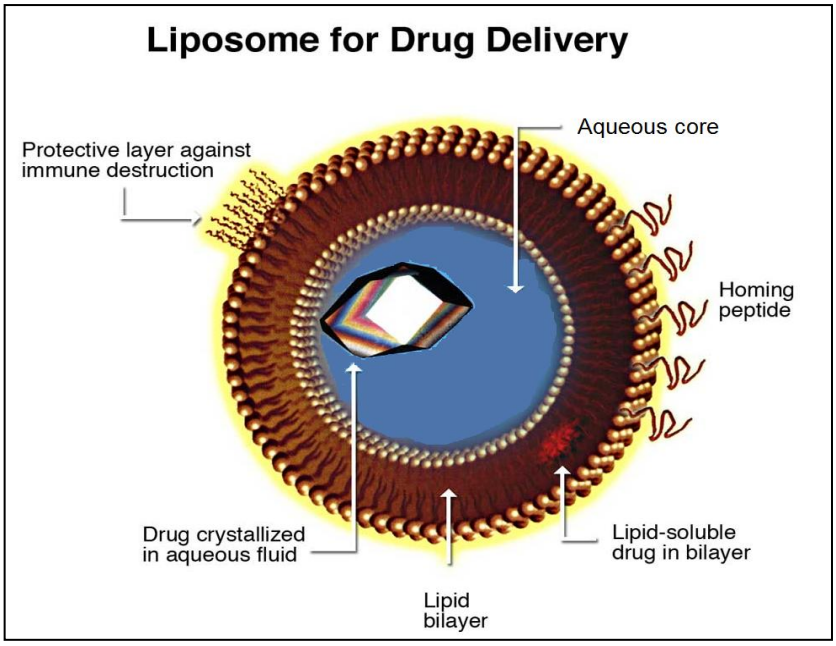


Figure 1.7: Representation of the structure of liposome encapsulating a drug (Modified from http://en.wikipedia.org/wiki/File:Liposome.jpg).

1.4.1 Applications of liposomes

Liposomes are mainly used as delivery systems for many substances which can easily be entrapped or act as anchor to various substituents (Gregoriadis, 1974). Liposomal delivery systems are well-known procedures used for encapsulation of anticancer and antimicrobial drugs, enzymes and antigens (Felnerova *et al.*, 2004). The application of liposomes in the delivery systems aid in processes such as transport, distribution, controlled release, protection and localization of encapsulated drugs (Gregoriadis, 1974; Egger *et al.*, 2001). In order to improve the performance of the enclosed drug, it is useful to consider the basic reasons for applying liposome as drug carriers (Storm and Crommelin, 1998).

Some of the features of liposomes:

Targeting- Liposomes when modified can enhance the therapeutic efficacy (drug targeting, site-specific delivery) of the drug by directing it to the target site in the body. Similarly, liposomes may also prevent the drug to target other body sites that are

particularly sensitive to the toxic action of the drug (site-avoidance delivery) (Cagnoni, 2002).

Duration- The material encapsulated in liposomes is slowly liberated. Such prolong processes help to maintain the therapeutic drug levels in the bloodstream and decreases the frequency of administration (Storm and Crommelin, 1998; Grant *et al.*, 2001; Yowell & Blackwell, 2002).

Protection- Liposomes protect the encapsulated compounds against other unfavourable aspects such as degradative enzymes existing in the host. Also the liposomes protect the body from toxic effects of the drugs (Hattori *et al.*, 2004).

Internalisation- One of the most beneficial aspects is the interaction of liposomes with target cells enhancing the delivery of the drug intracellularly which is critical for the non-encapsulated drugs due to the incompatible physiochemical characteristics (e.g. DNA molecules) (Vail *et al.*, 2004).

The cavity of the liposomes can be filled with drugs or other therapeutic agents. One of the functions of liposomes is the efficient delivery of biologically active compounds into the cells (Esposito *et al.*, 2003). Endocytosis appears to be the principal pathway by which liposomes enter cells to release the encapsulated drug and it is interesting to note that the pH of endocytotic vesicles is slightly acidic (Xu & Szoka, 1996).

1.4.2 Drug interaction with liposome

Liposomes, because of their biphasic character, can act as carriers for both lipophilic and hydrophilic drugs. Depending upon their solubility and partitioning characteristics, the drug molecules are located differently in the liposomal environment and exhibit different entrapment and release properties (Fig. 1.7). Based on the solubility, the drugs can be divided into four classes (1) highly hydrophilic, (2) highly lipophilic, (3) amphiphilic drugs that exhibit good biphasic solubility, and (4) drugs that exhibit

biphasic insolubility (Gulati *et al.*, 1998). Thus, different drugs depend on different types of methods in order to be encapsulated as shown in Fig.1.8. The main feature of the method is the medium in which the compound is to be dissolved (Gomez-hens and Fernandez-Romero, 2006). Highly lipophilic drugs can be encapsulated almost entirely in the lipid bilayer of the liposomes (Vadiei *et al.*, 1989). Since they are very weakly soluble in water, problems such as leakage of entrapped drug on storage are negligible. The most problematic compounds for liposomal entrapment are drug molecules that possess poor biphasic solubility. Being insoluble in both aqueous or lipid phases results in insufficient inadequate uptake by the liposomes (Goyal *et al.*, 2005). The encapsulation efficiency of hydrophilic or other drugs that show weak encapsulation can be enhanced by making them lipophilic through the addition of hydrophobic side chains or by the addition of external compounds such as lactose or sodium chloride (Gulati *et al.*, 1998).

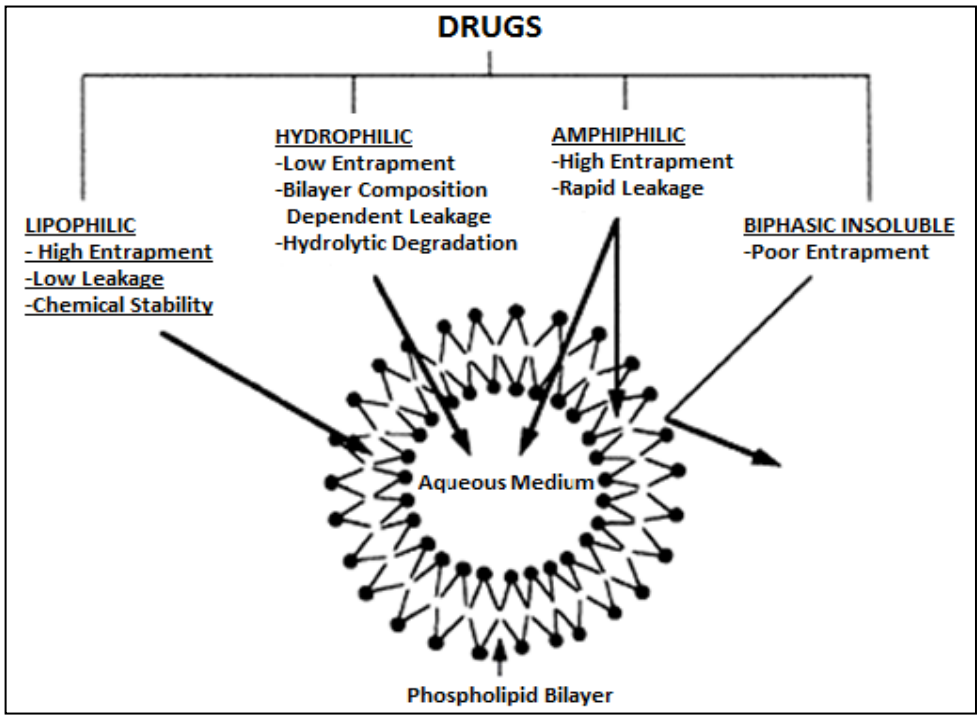


Figure 1.8 Types of the drugs and site of their incorporation into liposomal vesicles (Gulati *et al.*, 1998).

1.4.3 Phospholipids

The phospholipid composition and the capability of binding to other chemical species is one of the main features of their use in liposome formation (Rachev *et al.*, 2001; Gomez-hens and Fernandez-Romero, 2005). Phospholipids are part of all cell membranes and play an important role in the signal transduction mechanism. The phospholipid structure consists of phosphoric residue, hydrophilic head and hydrophobic tail (Fig. 1.9). The hydrophilic head consists of the phosphate group, glycerol back bone, choline, ethanolamine and inositol, with different chemical properties. When exposed to an aqueous environment, phospholipids tend to form unique assemblies called "bilayers". The hydrophilic heads of the phospholipids turn towards the water molecules while the hydrophobic tails hide from water molecules.

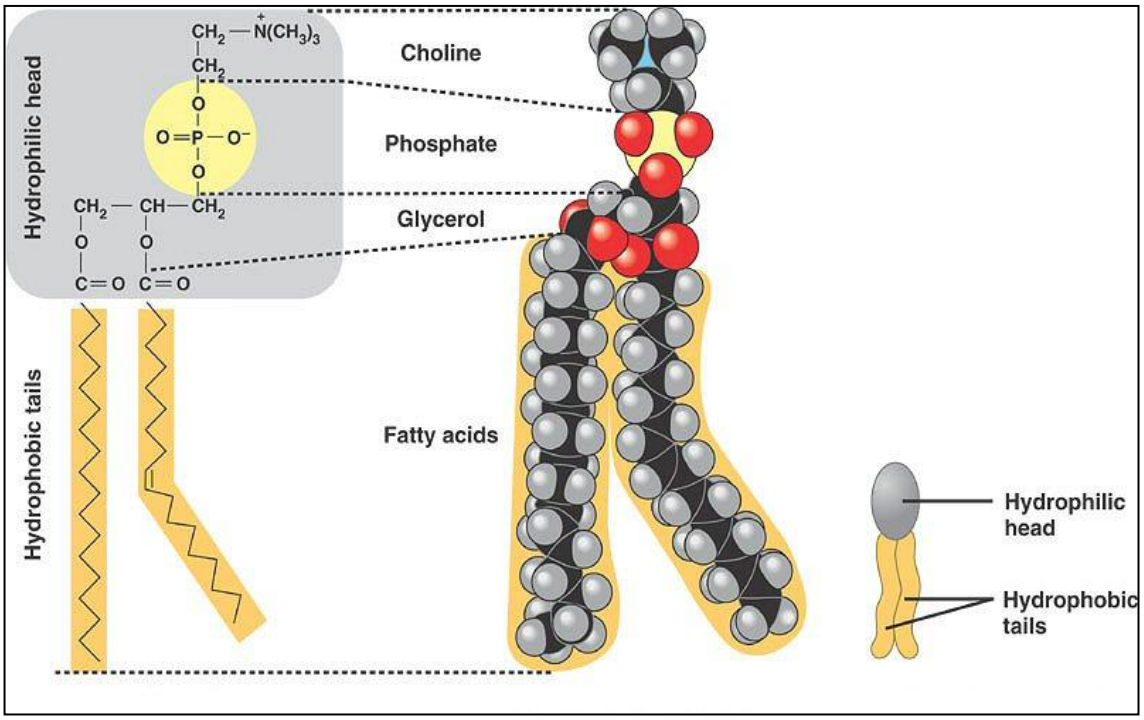


Figure 1.9 Chemical structure of Phospholipid (Adapted from http://kvhs.nbed.nb.ca/gallant/biology/phospholipid_structure.jpg).

There are two basic sources of phospholipids: synthetic and natural. Natural lipids are generally either egg-derived or bovine-derived. Regardless of the regulatory issues, animal-derived products do not offer any advantages to synthetic lipids. They are inherently less stable due to the polyunsaturated fatty acids, and in most cases the synthetic counterpart costs the same or less than the tissue-derived product (Lasic, 1992). Synthetic lipids from different sources are not necessarily equal either. Synthetic lipids can be prepared from glycerol or glycero-3-phosphocholine (GPC) derived from a plant or animal source (Perrie and Gregoriadis, 2000). The latter is sometimes referred to as semi-synthetic lipid because a portion of the molecule is derived from a natural source (Harrington *et al.*, 2002). Lipids derived from glycerol require the chiral centre be synthetically prepared which may lead to stereochemical impurities present in the final product (Gregoriadis, 1974). The typical plant source for GPC is soybean lecithin. This type of compound is synthesized by converting a natural PC into an acyl group through a chemical reaction. A highly pure, high-quality hydrogenated soy phospholipids that can assist you in developing new products is generally obtained from carefully selected, non-genetically modified soybeans as a source of raw materials (Lasic, 1992).

Phospholipids derived from natural sources are mostly used as the parental liposome formation. Such phospholipids include phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Harrington *et al.*, 2002). These phospholipids contain certain acyl chains which differ with source of origin, for example acyl chain found in egg PC is different to acyl chain in soy bean PC. Phospholipids also possess lyso-phospholipids where the acyl chain is located at C1-position of glycerophosphocholine and the other is eliminated by hydrolysis (Sessa and Weissmann, 1968). In addition, peroxidation can occur if unsaturated bonds are present

in the acyl chains. In the early 1980s the quality of lipids of several suppliers differed both qualitatively as well as quantitatively (Zuidam, 1993; Zuidam, 1996). However, the supplies nowadays are more consistent with high quality products. An interesting feature of liposomes is that their mechanical properties can be changed by incorporating a wide range of substances into their structure such as cholesterol and stearic acid (Rachev *et al.*, 2001). The phospholipids used in this study are lα-phosphatidylcholine, 1,2-distearoyl-*sn*-glycerol-3-phosphocholine (DSPC), 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine (DMPC), lecithin and phospholipon 90H. These phospholipids can also be classified as natural and synthetic phospholipids where lα-phosphatidylcholine and lecithin are obtained naturally while DSPC, DMPC and phospholipon 90H are derived synthetically.

1.4.3.1 Lα-phosphatidylcholine

Lα-phosphatidylcholine has an important function in the maintenance of integrity of the cell-membrane. It has also been recognized as an important signalling molecule (Exton, 1994). Lα-phosphatidylcholine comprises of phosphatidylcholine as its major component, it is a pure form of soyabean phospholipids. Its structure comprises of saturated as well as unsaturated fatty acids. Its phase transition temperature was reported to be unstable ranging between 55-65 °C and fatty acid composition was provided by the supplier Sigma Aldrich, UK. It comprises of 13 % palmitic acid, 4-6 % stearic acid, 10 % oleic acid, 64 % linoleic acid and 6-8 % linolenic acid.

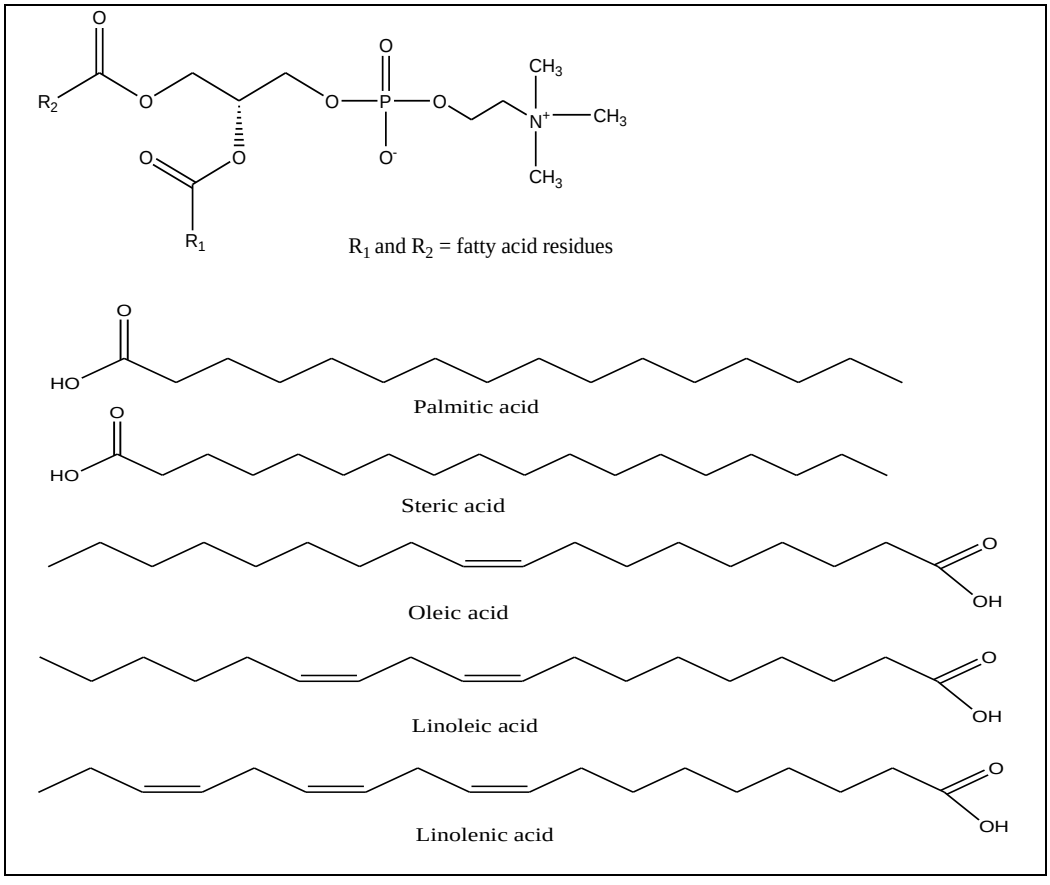


Figure 1.10 The structure of Lα-phosphatidylcholine along with its fatty acid composition (The chemical structure was drawn using the chemdraw ultra 12.0 software).

1.4.3.2 1,2-distearoyl-*sn*-glycerol-3- phosphocholine (DSPC) and 1,2- dimyristoyl-*sn*-glycerol-3-phosphocholine (DMPC)

DSPC and DMPC are like chain phosphatidylcholine induced by variety of additives including glycerol, methanol, ethylene glycol, benzyl alcohol, chlorpromazine, tetracaine, ethanol, thiocyanate ion (Fig. 1.11) (McDaniel *et al.,* 1983; McIntosh *et al.,* 1983; Cunningham & Lis, 1986; Slater & Huang, 1988). The phase transition temperature of DSPC was 53 °C and of DMPC was 23 °C.

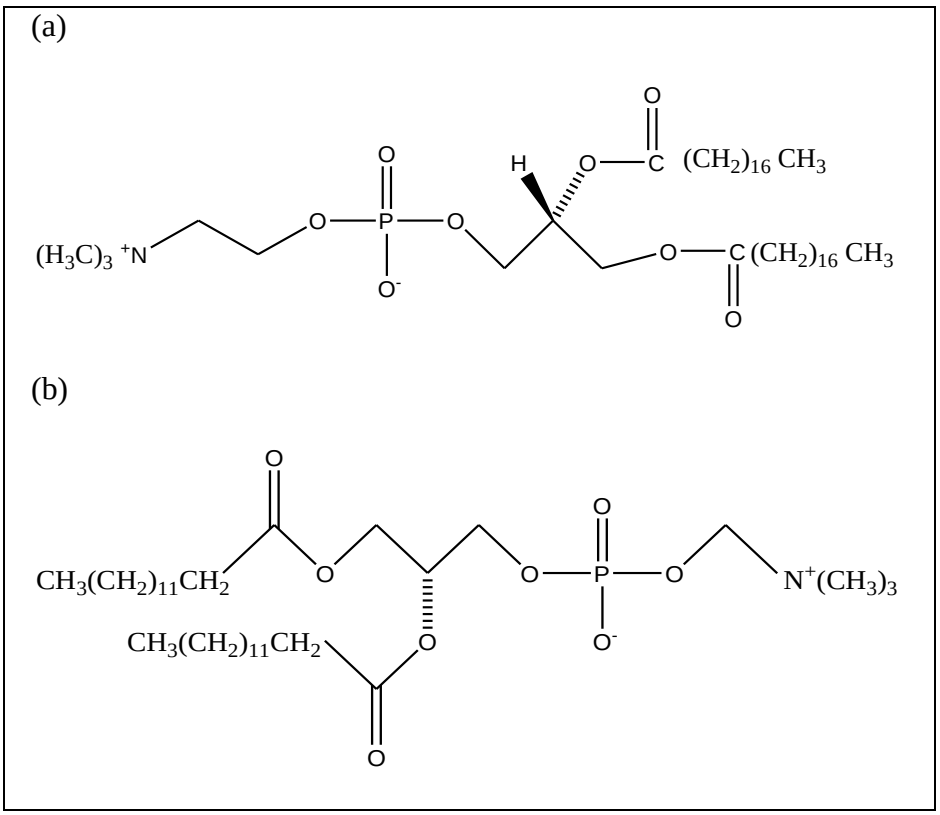


Figure 1.11 The structure of DSPC and DMPC (The chemical structure was drawn using the chemdraw ultra 12.0 software).

1.4.3.3 Lecithin derived from Soybean

Lecithin is composed of a mixture of phospholipids: phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, n-acyl-phosphatidylethanol- amine and phosphatidylinositol. Its fatty acid composition comprises of 12-17 % palmitic acid, 2-5 % stearic acid, 11-15 % oleic acid, 59-70 % linoleic acid and 3-7 % linolenic acid (Fig. 1.12). Its phase transition temperature was reported to be 65 °C and fatty acid composition was provided by the supplier Sigma Aldrich, UK.

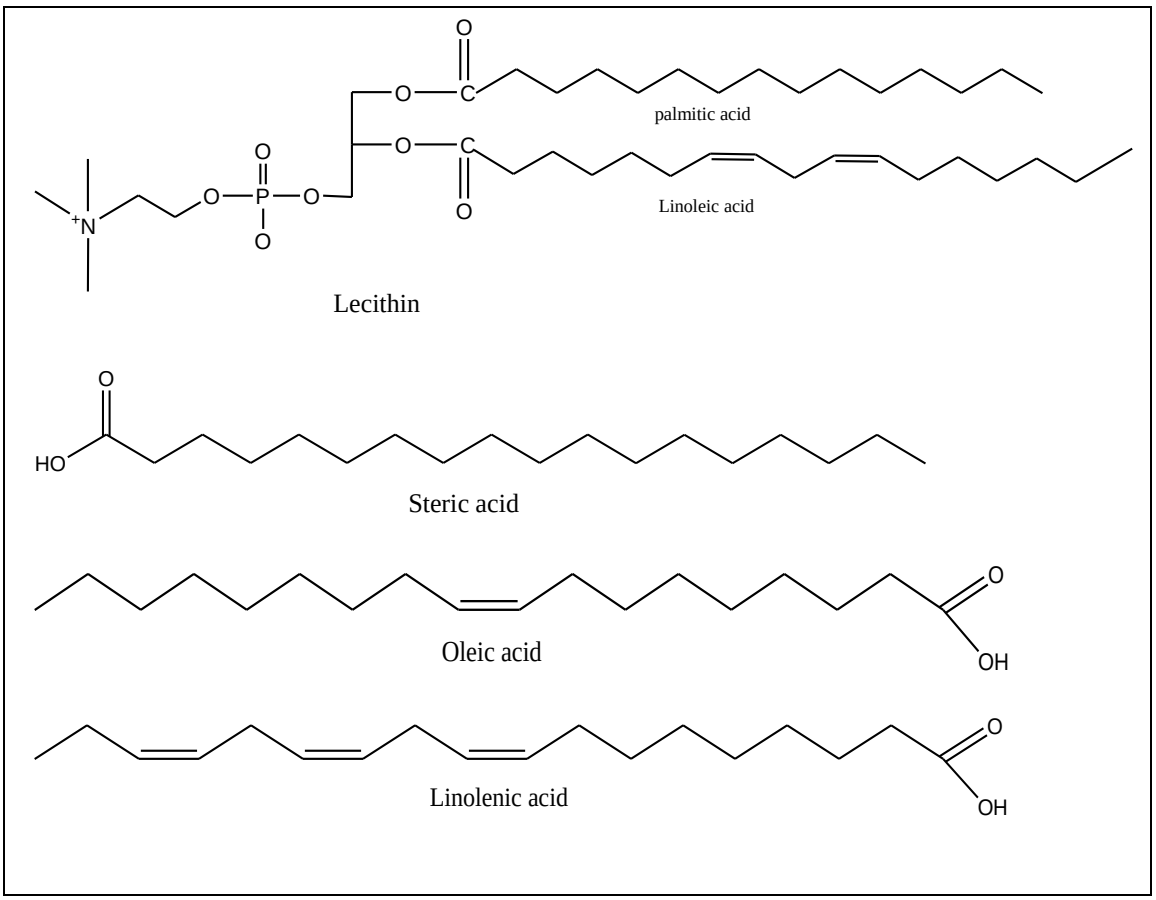


Figure 1.12 The structure of Lecithin with palmitic and linoleic acid chain attached to it along with its fatty acid composition (The chemical structure was drawn using the chemdraw ultra 12.0 software).

1.4.3.4 Phospholipon® 90 H

Phospholipon® 90H is hydrogenated form of lecithin product derived from soybean. Phospholipon®90H comprises of phospholipids such as hydrogenated phosphatidylcholine and hydrogenated lysophosphatidylcholine. Its fatty acid composition comprises of 15 % palmitic acid and 85 % stearic acid (Fig. 1.13). Due to its composition phospholipon 90H is found to be more stable than phosphatidylcholine and is also been previously used as for application of liposomes (Glavas-Dodov *et al.*, 2005; Parmar *et al.*, 2010). Its phase transition temperature was reported to be 54 °C and fatty acid composition was provided by the supplier Phospholipid GmbH, Germany.

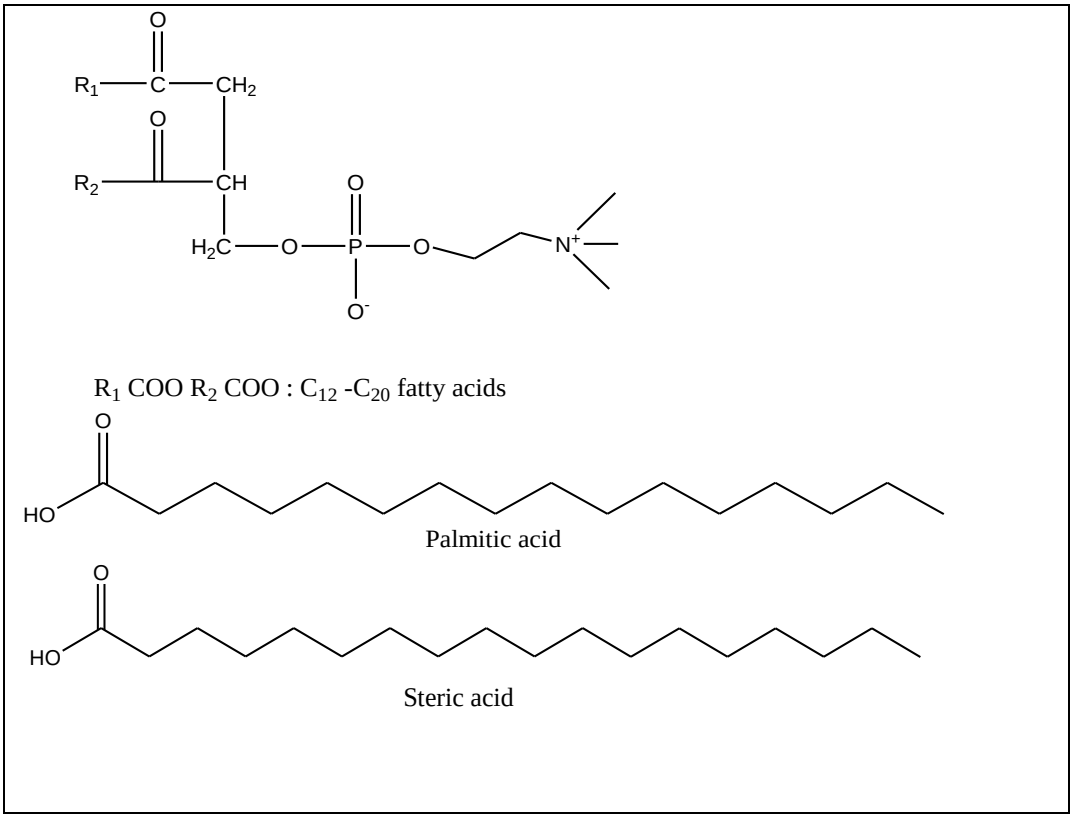


Figure 1.13 The structure of Phospholipon® 90H along with its fatty acid composition (The chemical structure was drawn using the chemdraw ultra 12.0 software).

1.4.3.5 Cholesterol and Stearic acid

Cholesterol, is an alternative habitual component of the liposome structure and a natural constituent of the biological membranes, whereas stearic acid is a saturated form of fatty acid, cholesterol and stearic acid play an important role in controlling the fluidity and the permeability of the artificial vesicle i.e. it makes the membranes more rigid thus avoiding leakage of the encapsulated compound (Fig. 1.14) (Gomez-hens and Fernandez-Romero, 2005; Perrie and Gregoriadis, 2000). In order to improve the rigidity of the liposome, cholesterol and stearic acid were combined with other phospholipids to form liposome formulations.

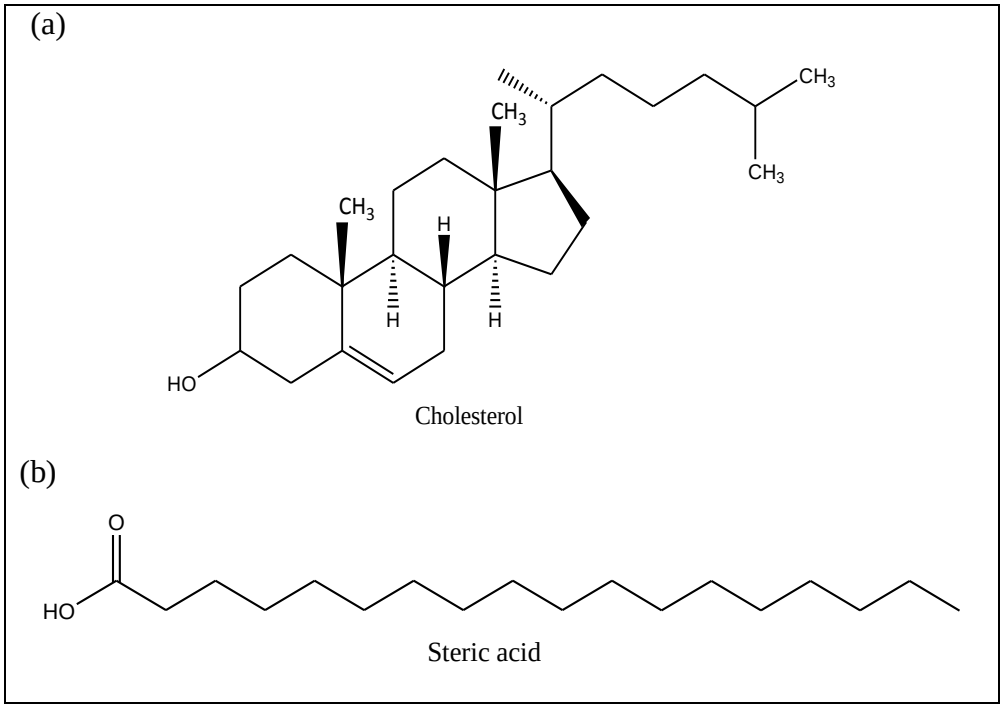


Figure 1.14 The structures of (a) cholesterol and (b) stearic acid (The chemical structure was drawn using the chemdraw ultra 12.0 software).

1.4.4 Techniques used in the current study for formation of Liposomes

Methods such as freeze-thaw and dehydration-rehydration form liposomes that are multilamellar vesicle (MLVs). These MLVs possess greater encapsulating capacity, in addition to variation in vesicle size, size dispersion and lamellarity (Gabriels, 2003; Moscho, 1996). These factors are based on the type of the lipids used and their distribution efficiency. Repetition of these mechanical cycles such as freeze-thaw cycles or dehydration-rehydration cycles converts the initial MLV dispersion into other liposomes of different sizes (Gomez-hens and Fernandez-Romero, 2005). The freezing process aids in the encapsulation of TMZ inside the liposomal vesicle while the thawing breaks the multilamellar vesicles and promotes mixing of the encapsulated content on release (Shazly *et al.*, 2008). Similarly dehydration causes fusion of the vesicle, liposomes are concentrated by the drying process and fuse to form multilamellar planes forming a sandwich of the drug between the resulting lamellae. To further disperse the vesicles, the addition of PBS and vortexing is carried out (Ostro, 1983). The TMZ loaded liposomes were then analysed for encapsulation by the application of HPLC.

Particle size analysis

The performance of laser diffraction depends on the particles passing through a laser beam scattering light at an angle that is inversely proportional to their size (large particles scatter light at low angles, whereas, small particles scatter light at high angles). Thus, to calculate particle size distribution, the intensity of light scattered from a sample is measured as a function of the angle (Fig. 1.15) (Ingebrigtsen and Brandl, 2002). The laser diffraction analyser was used in this study to measure the size of the TMZ loaded liposomes formed.

Fundamental principle

Laser diffraction is based on the principle that particles scatter light in all directions with an intensity pattern depending on the particle size. A light scattering instrument was used consisting of a light beam (usually a laser), a detector to measure the scattering pattern, a small volume dispersion unit and a computer for both control of the instrument and calculation/computation of the particle size distribution (Chu, 1991). Advantages in using this method are that it is easy to use and less time consuming. It is reproducible and an extremely wide size range is covered straddling almost five orders of magnitude ranging from nanometres to millimetres (Ruf *et al.*, 1989).

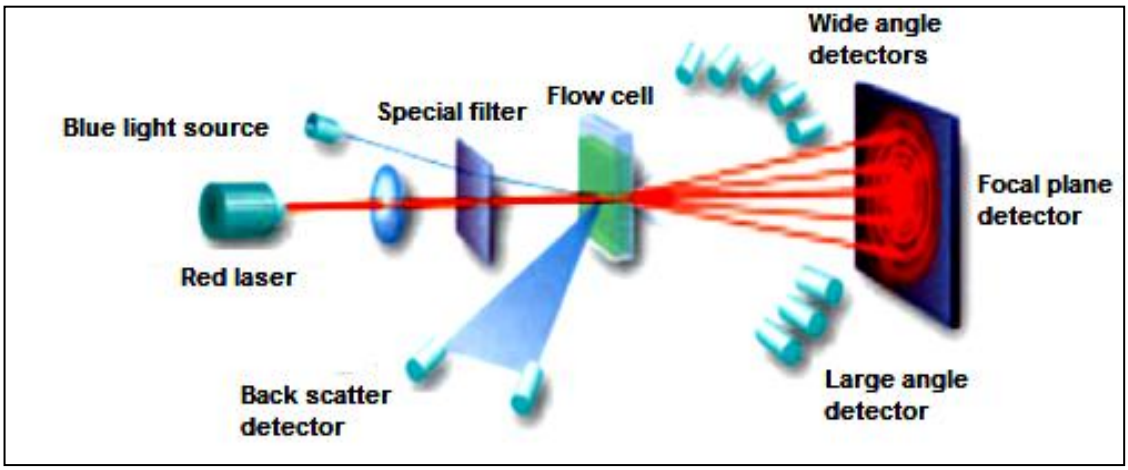


Figure 1.15 An illustration of particle size analyser (Adapted from http://www.malvern.com/ LabEng/products/Mastersizer/MS2000/mastersizer2000.htm).

1.5 Microparticles

The most widely used clinical drugs are often found to be of low molecular weight exhibiting a short half-life in the blood stream and an overall high clearance rate (Haag and Kratz, 2006). Such small molecules diffuse into the healthy tissues in a rapid manner and are distributed throughout the body. Based on their size such molecules are known as either microparticles or nanoparticles. The advantage of nanoparticles over microparticles is different as their size opens the potential for crossing the various biological barriers within the body. From a positive viewpoint, especially the potential

to cross the blood brain barrier may open new ways for drug delivery into the brain. In addition, the nanosize also allows for access into the cell and various cellular compartments including the nucleus (De Jong and Borm, 2008). Microparticles on other hand also carry these advantages but at certain aspects require surface modification to pass through various cellular compartments.

In order to optimize the biodistribution of drugs to target sites a number of nanoparticulate delivery systems for disease targeting and site-selective drug release have been developed as described by Dr. Paul Ehrlich over a century ago who described the concept of drug delivery at a disease site without harming the healthy tissue as the "magic bullet" (Witkop, 1999). Drug delivery systems are classified as either polymer-drug conjugates (polymer therapeutics) or as nanoparticle therapeutics both approaches aim at increasing the therapeutic index maximum quantity of the drug molecules available at the target site with reduction of systemic drug exposure. (Davis *et al.,* 2008; Caliceti and Veronese, 2003).

In developing a drug delivery system certain aspects are taken in to consideration as the system should positively impact on the rate of metabolism, distribution absorption and excretion of the drug or other related chemical substances in the body. The structure of the polymeric nanoparticles enables the drug to bind to its target receptor and influence receptor's signalling and activity (Fig. 1.16). The substance used for the development of the microparticles should be degradable into fragments after completion of their role and be either metabolised or excreted (Niidome *et al.,* 2006; De Jong and Borm, 2008). Thus, for effective and safe drug delivery systems, biodegradable nanoparticle formulations are favoured for the purpose transportation and release of the drug in order to be effective (Gupta and Gupta, 2005). The use of nanotechnology in drug delivery

systems and in medicine is spreading rapidly. Pharmaceutical sciences have been using this technology to reduce toxicity and side effects of the drugs. Numerous substances have been considered to use for drug delivery systems and more specifically in cancer therapy (Ferrari, 2005).

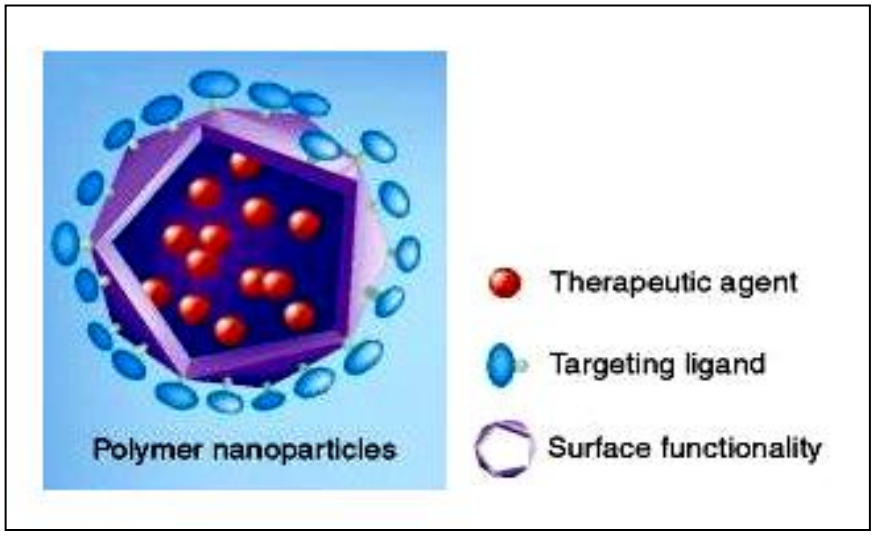


Figure 1.16 Representation of the structure of PLGA nanaoparticle encapsulating a drug (Modified from Mamo *et al.,* 2010).

1.5.1 Applications of microparticles

Drug delivery and other pharmaceutical developments related to nanomedicine pose a special function related to treatment, prevention and diagnosis of the disease. In order for appropriate selection of carriers to be used in drug delivery systems certain parameters needs to be considered such as drug incorporation and release, biocompatibility and biodistribution, stability and shelf life related to formulation and site targeting (De Jong and Borm, 2008). A major issue of limiting intracranial therapeutic levels for chemotherapeutic agents' administration is the blood brain barrier. TMZ is one of the most effective anticancer agents to cross the blood brain barrier, however, in high systemic doses it is unable to achieve the therapeutic brain levels due to its short half-life and also causes certain side effects (Rongy *et al.,* 2005). Thus,

according to the literature reports it is proved that implantation of polymeric devices have extended the release period of the drug (Zhang and Gao, 2007).

Drug delivery can be localized or systemic. Parenteral drug delivery is typically achieved using liposomal or polymeric carriers (Langer, 1998). There is no universal platform, as each system has its advantages and disadvantages. While liposomes have a high drug-carrying capacity, their release profiles are more difficult to regulate. In contrast, polymeric drug-delivery systems can be synthesized to generate specific molecular weights and compositions, but their drug-carrying capacity is relatively low (Duncan, 2003). While an ideal general drug-delivery platform may never be realized, hybrid drug-delivery systems that incorporate the benefits of various approaches will be tailored to address the needs of specific applications. In addition to the standard advantages of polymeric and liposomal drug-delivery systems, including sustained and controlled release, as well as the potential for targeted delivery, nano-scale dimensions confer improved bioavailability by increasing the payload's solubility and stability. Smaller particle sizes facilitate deeper penetration into capillaries and through fenestrations and, ultimately, enhanced cellular uptake. Indeed, 100 nm size particles exhibit *in situ* uptake efficiencies 15–250-fold of those of small microparticles (1 and 10 μm) (Desai et al., 1996). Nanoparticles have even been shown to cross the blood–brain barrier.

1.5.2 Poly(d,l-lactide-*co*-glycolide) (PLGA)

In order to meet the requirements of a sustained release system a biodegradable polymer such as polylactide (PLA) and its copolymers (PLGA) with glycolide (GA) are commonly used. PLGA has proved to be a much more effective polymer than PLA because of its polymer properties i.e. manipulation of the co-monomer ratio, molar mass and polymer crystallinity (Fig. 1.17). In addition, drug release rates can be controlled

through alteration in the ratio, use of different molecular weight PLGA and also in the physical characteristics of the resulting microparticles. Degradation of the PLGA plays a crucial role in the drug release mechanism (Porjazoska *et al.,* 2004). Furthermore, previous studies have reported the biocompatibility and biodegradability of blank PLGA (i.e. without any active ingredient encapsulated in it) in brain cells when implanted in brain tissue (Emerich *et al.,* 1999; Menei *et al.,* 1993). The blood brain barrier consists of tight junctions of blood vessels of brain endothelial cells, thus the application of prodrugs and drug carriers have been used to overcome this limitation. The surface modified carriers have drawn the attention due to their ability for site specific delivery of the drug (Zhang and Gao, 2007). Because of their small size and modified surface these polymeric nanoparticles can also be targeted to specific cells and locations in the body. The factors such as drug encapsulation and release profile depend on the type of polymer used and its physiochemical characteristics such as particle size and morphology (Torchilin, 2006a). The melting point of PLGA is found to be amorphous and the glass transition temperature is reported to be 45-50 °C.

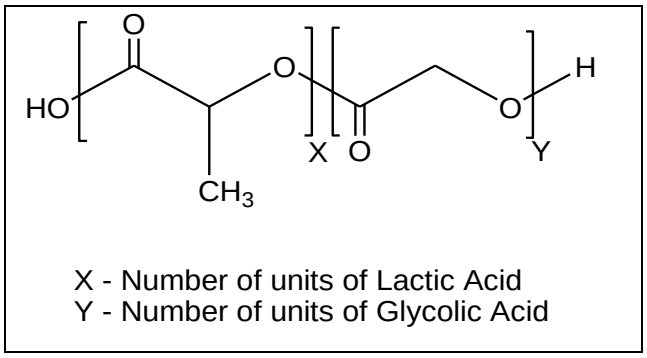


Figure 1.17 Representation of the structure of PLGA (The chemical structure was drawn using the chemdraw ultra 12.0 software).

1.5.3 Techniques used in the current study for formation of Microparticles

The preparation of the PLGA microparticles involved both emulsifying-solvent evaporation and spray dry techniques. The emulsifying-solvent evaporation consists of dissolving the polymer with the drug in a solvent and mixing it with aqueous medium containing surfactant. The mixture stirred overnight during which time diffusion takes place between the water-soluble solvent and the water-insoluble phase, resulting in an interfacial turbulence which results in the formation of microparticles (Torchilin, 2006b). Spray dry technique is performed by using a Mini Spray Dryer. Polymer together with the drug was dissolved in a solvent which is subjected to spray drying where the matrix material and the active ingredients are heated above the melting point (Wang and Wang, 2003). The microparticles are formed based on the principle of a co-current air and product stream, i.e. the sprayed product and hot air have the same flow direction (Fig. 1.18). Compressed air is used for the dispersion of the liquid body into fine droplets which are subsequently dried in the cylinder. These droplets are solidified into particles and can thus be separated.

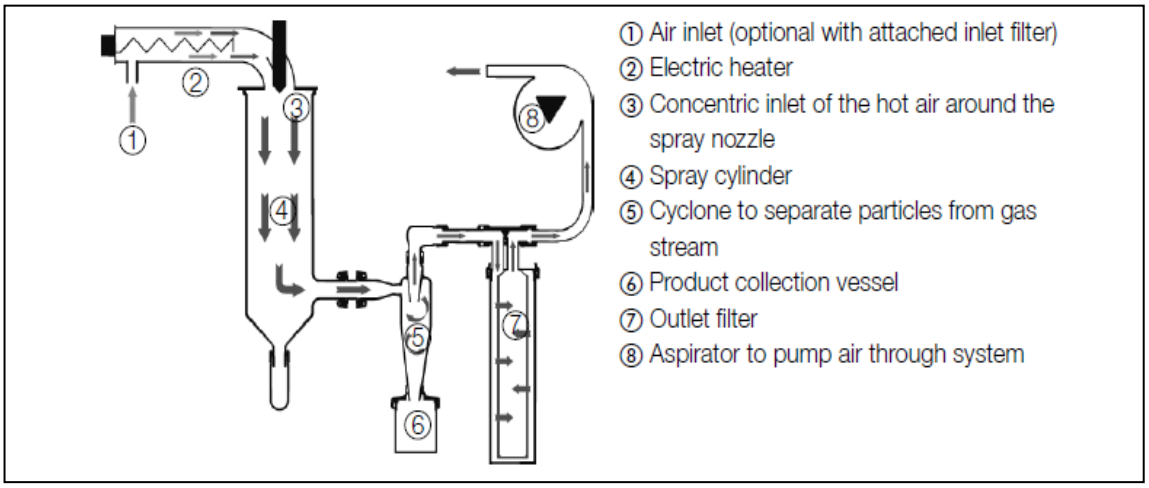


Figure 1.18 Functional principle of the spray dry method. (Adapted from http://www.mybuchi.com/Mini_Spray_Dryer_B-290.179.0.html).

The spray drying technique converts the drug–polymer suspension into fine particles by spraying through an atomization nozzle into hot air. The solvent present within the atomized droplets evaporates quickly and solid microparticles are formed. This technique has been used previously to encapsulate anticancer drugs such as paclitaxel and etanidazole respectively into PLGA microparticles (Mu and Feng, 2001; Wang and Wang, 2003).

1.5.4 Dissolution techniques used for determination of drug release from the microparticles.

Two dissolution techniques used to monitor the release of drugs from liposomes and microparticles are dispersion and dialysis bag diffusion methods. The release process of drugs from microparticles is based on the physiochemical characteristics of the drug molecules and the matrix of the polymer (Hutchison and Furr, 1990). Certain aspects in which the release of the drug depends include the mode of drug attachment or encapsulation involving factors such as surface adsorption, covalent conjugation and dispersion homogeneity of the drug molecules in the polymer matrix, in addition to this the factors determining the degradation and the control of matrix hydration are also involved (Allemann *et al.,* 1993; Niwa *et al.,* 1993; Soppimath *et al.,* 2001).

The release of the drug from the polymer can be described in different phases, rapid release of the drug occurs due to desorption this can be due to the drug being bonded weakly to the polymer surface and can be expressed as the initial burst release phase which can sometimes takes place as result of bulk degradation of the polymer (Allemann *et al.,* 1993; Polakovic *et al.,* 1999; Panyam *et al.,* 2004; Liu *et al.,* 2005; Tamber *et al.,* 2005). If the bulk degradation of the polymer is made to exhibit surface degradation or layer-by-layer degradation, the polymer could present a promising control release of the drug. These phenomena is termed as sustained release (Conte *et*

al., 1993; Abdul and Poddar, 2004; Chen *et al.,* 2005; Loo *et al.,* 2005; Frank *et al.,* 2005; Loo *et al.,* 2010). If the drug is distributed uniformly in the matrix of the polymer, the release takes place by diffusion (i.e. if the drug is in a crystalline form it is first dissolved locally and then diffusion takes place). Sometimes the release takes place by the erosion of the matrix or a combination of both mechanisms causes the release (Torchilin, 2006a). The stage where no drug release is observed is termed as the lag phase, where the drug is not diffusing out of the polymer, one of the mode by which this could be overcome by treating the sample by radiation (e-beam) which modifies the physical properties of the polymer and thereby reduces the lag phase (Miao *et al.,* 2009; Loo *et al.,* 2010). Some of the parameters that should be taken into consideration for monitoring the release mechanism include polymer molecular weight distribution, melting and glass transition temperature, crystallinity and hydrophobicity or hydrophilicity. It is also observed from previous studies that degradation time of the polymer is shorter for low molecular weight polymers and greater for amorphous, hydrophilic and polymers with high glycolide content (Gallia et al., 2005; Torchilin, 2006a).

1.5.5 Particle size analysis

The scanning electron microscope (SEM) was used for analysing the particle size and morphology of the TMZ and Patrin-2 loaded PLGA microparticles. In order to generate a range of signals at the surface of the microparticles SEM uses a focussed beam of high energy electrons (Fig. 1.19). The signals generated is attributed to electron and sample interactions and provides details on the sample related to its external morphology, size, chemical composition and orientation of materials used in making of the sample (Painbeni *et al.,* 1998). Data is collected from the selected surface area of the sample

and a two dimensional image is produced displaying the spatial differences in these properties.

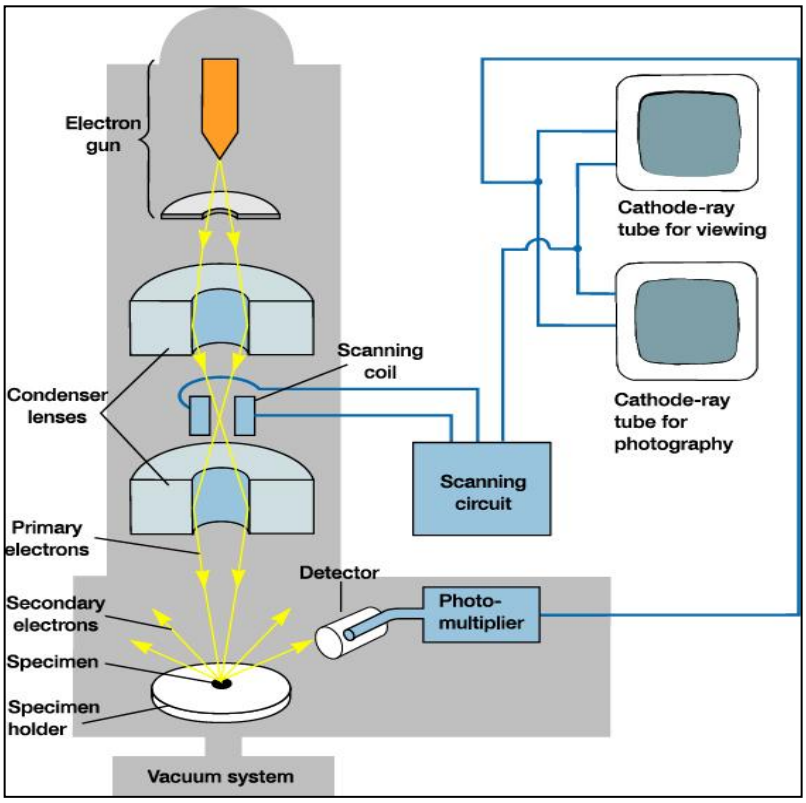


Figure 1.19 Schematic diagram of scanning electron microscopy. (Adapted from http://cms.daegu.ac.kr/sgpark/microbiology/SEM.jpg).

Fundamental principle

The signals generated by electron-sample interactions include secondary electron (that are responsible for producing the SEM image), backscattered electrons, diffracted backscattered electrons (used in determination of the crystalline structure of the sample), photons, visible light and heat. The essential components of SEM includes the electron source, electron lenses, sample stage (where the sample is placed for analysis), detectors for analysing all possible signals, and the display or data output device (Bodmeier *et al.*, 1989).

1.6 High Performance Liquid Chromatography (HPLC)

All quantitative analysis of the actives encapsulated in both liposomes and microparticles were performed using HPLC. HPLC is a form of column chromatography used frequently in pharmaceutical analysis to separate, identify and quantify various compounds (Lindsay and Barnes, 1992). Though there are two modes one being isocratic and the other gradient the separation of the compounds, in this study was carried out using isocratic mode in which the mobile phase composition remains constant throughout the procedure. The HPLC system consists of a stationary phase (column), mobile phase, injector, pump and a detector (Fig. 1.20).

Most widely two type of HPLC are used that are normal and reverse phase chromatography. These two types can be distinguished on the basis of relative polarities of the mobile and stationary phase (Lindsay and Barnes, 1992). Normal phase chromatography consists of highly polar stationary phase such as water or triethylene glycol and the mobile phase is a relatively non-polar solvent such as hexane or i-propyl ether while the reverse phase chromatography consists of the typical stationary phase which are non-polar hydrocarbons, waxy liquids or bonded hydrocarbons such as C_{18}, C_8 or C_4 while its mobile phase is a relatively polar solvent such as methanol, water, acetonitrile, or tetrahydrofuran (Skoog and Leary, 1992). The C_{18}, C_8 and the phenyl bonded phases are most often used in the reverse phase mode (Skoog *et al.,* 2007). Bonded phases are made by bonding of a molecule covalently on to a solid stationary phase which is intended to prepare liquid coatings which will be permanent (Skoog and Leary, 1992). Silica is a reactive substrate used, to which the functionalities such as the alkyl (C_{18} and C_8), cyano, aromatic phenyl and amino groups can be attached or bonded. In normal phase, the least polar component is eluted first thereby increasing the polarity of the mobile phase and decreasing the elution time (Skoog and

Leary, 1992). However, in reverse phase chromatography the most polar component is eluted first, increasing the polarity of the mobile phase and increasing the elution time (Lindsay and Barnes, 1992). One of the many advantages of reverse phase chromatography is the ability to use water as the mobile phase which is an inexpensive and UV-transparent solvent compatible with biological solutes, also it is found that mass transfer as well as solvent equilibration is rapid with non-polar stationary phase (Skoog *et al.*, 2007). The R group of the siloxane used in these coatings is a C_{18} chain (*n*-octyldecyl) or C_8 chain (*n*-octyl). The effect of the alkyl groups on performance depends on the chain length (Skoog and Leary, 1992). Longer chains produce packings that are more retentive and allow use of larger samples, the maximum sample size of column type C_{18} is roughly twice of that of C_4 under similar conditions (Skoog *et al.*, 2007).

The technique involves separation of the components in a mixture pushed through the column usually at a constant flow rate is carried out using a liquid mobile phase. The solvent (mobile phase) is run through the high pressure pump which generates and measures 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 (C_{18}) 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 detector normally used is a UV lamp. The chromatogram produced displays the retention time and the peak area of the expected compound. The retention time is a characteristic of the sample for a particular set of conditions and helps in identification of the compound. The peak area of the compound indicates the concentration of the compound used in the analysis (Lindsay and Barnes, 1992).

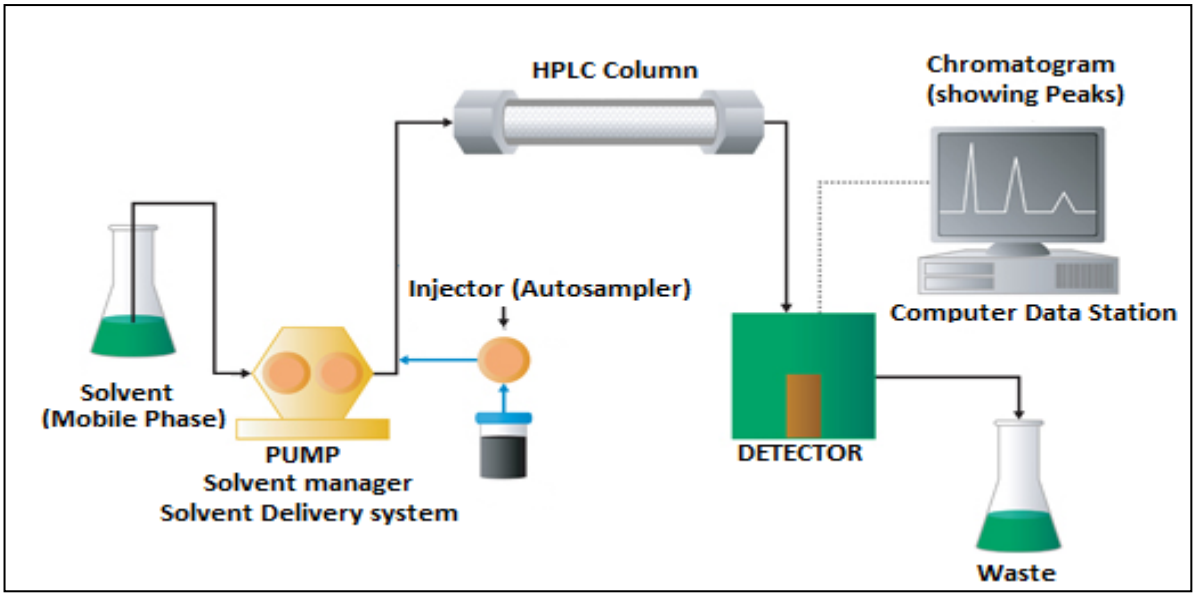


Figure 1.20 Schematic diagram of HPLC (Adapted from http://www.waters.com/waters/nav.htm?cid=10049055&locale=en_GB).

1.7 The main research questions & aims

Questions to be addressed concerning the investigation

Can the preparations of liposomes and microparticles be effectively used for encapsulation of TMZ and Patrin-2?

Can their release from the formed liposomes and microparticles be efficiently monitored using the dissolution techniques in order to enhance the treatment of glioma?

Hypothesis

TMZ & Patrin-2 could be encapsulated separately into liposomes and microparticles using established or improvised methods.

1.7.1 Main aim

The main aim of our investigation was to prepare TMZ loaded liposomes (by using different ratios of phospholipid), TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles.

TMZ has proved to be effective in patients that lack MGMT activity since MGMT counteracts the effect of TMZ by removing the methyl group (originally added by TMZ) (Rimel *et al.*, 2009). Patrin-2 is used to counteract the effect of MGMT and enhances the effective activity of TMZ. Because TMZ and Patrin-2 are not compatible due to their differences in stability at different pH's they cannot be encapsulated together in the same vehicle, thus the microparticles formulations prepared for both drugs were prepared separately.

1.7.2 Specific aims of the research:

1. To establish a method for the preparation of TMZ loaded liposomes with maximum encapsulation efficiency monitored by HPLC and to determine particle size.
2. To establish a method for preparing TMZ loaded microparticles resulting in maximum encapsulation monitored by HPLC.
3. To use different molecular weights of PLGA for the preparation of TMZ and Patrin-2 loaded microparticles.
4. To measure the particle size and morphology of the TMZ and Patrin-2 loaded microparticles using scanning electron microscopy.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Materials used in this study are detailed in Table 2.1. All chemical reagents used were of analytical grade**.** All materials used were handled and stored according to the instructions given by the suppliers.

Table 2.1 Materials used in this study.

Chemicals	**Molecular weight (Da)**	**Supplier**
TMZ	194.15	Sigma Aldrich (UK) and Paterson institute (UK)
Patrin-2 (Lomeguatrib)	326.17	Zhejiang Wonderful Pharma & Chem Co. (China)
Lα-phosphatidylcholine	760.09	Sigma Aldrich (UK)
1,2-distearoyl-*sn*-glycerol-3-phosphocholine	677.93	Sigma Aldrich (UK)
1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine	734.10	Sigma Aldrich (UK)
Lipoid S-100 Lecithin from soy	790.00	Lipoid GmbH (Germany)
Stearic acid	284.48	Sigma Aldrich (UK)
Cholesterol	386.67	Sigma Aldrich (UK)
Phospholipon® 90H	784.00	Phospholipid GmbH (Cologne)
Poly (DL-lactide-co-glycolide) (75: 25)	$1.7x10^4$	Purac biomaterials (Netherlands)
Poly (DL-lactide-co-glycolide) (75: 25)	$6.6x10^4$ - $1x10^4$	Sigma Aldrich (UK)

Poly (DL-lactide-co-glycolide) (50: 50)	$4x10^4 – 7.5x10^4$	Sigma Aldrich (UK)
Poly (DL-lactide-co-glycolide) (50: 50)	$1.5\ x10^4$- $4\ x10^4$	Sigma Aldrich (UK)
Dialysis tubing, benzolated	MW cut off 1200-2000	Sigma Aldrich (UK)
Polyvinyl alcohol	20000 - 30000	Fisher Scientific (UK)
Sodium Dodecyl Sulphate (SDS)	288.38	Sigma Aldrich (UK)
Citric acid	192.12	Sigma Aldrich (UK)
Tetrabutylammonium acetate (TBAA)	301.51	Sigma Aldrich (UK)
Phosphate buffered saline (PBS 1x)	N/A	Autogen Bioclear (UK)
HPLC grade chloroform	N/A	Fisher Scientific (UK)
HPLC grade dichloromethane	N/A	Fisher Scientific (UK)
HPLC grade acetonitrile	N/A	Fisher Scientific (UK)
HPLC grade acetone	N/A	Fisher Scientific (UK)
HPLC grade ethanol	N/A	Fisher Scientific (UK)
HPLC grade methanol	N/A	Fisher Scientific (UK)
HPLC grade water	N/A	Fisher Scientific (UK)
Acetic Acid	N/A	Fisher Scientific (UK)
Triton X-100	N/A	VWR International (UK)

2.2. Equipments

All the instruments used to carry out the experiments are detailed in table 2.2.

Table 2.2 Equipment used in this study

Equipment	**Type**	**Supplier**
HPLC pump	Jasco PU-1580	Jasco (UK)
HPLC detector	Jasco UV-970M (4-λ intelligent)	Jasco (UK)
HPLC Column	Shandon C18	Thermo scientific (UK)
Mini spray dryer	Buchi mini spray dryer B-290	Buchi (UK)
Particle size analyser	Mastersizer 2000	Malvern instruments ltd. (UK)
Scanning electron microscopy (SEM)	Scanning electron microscopy	Edax-ametex (UK)
Water bath	Nuve water bath *nb* 5	Biotech lab (Bulgaria)
Centrifuge	Centrikon T-324	Kontrons instruments (UK)
Rotary evaporator	Buchi RE111 and Buchi 461 water bath	Buchi (UK)
Gold coater	Sputter coater	Emitech ltd. (UK)
Shaker and incubator	Shaker and incubator	Gallenkamp (UK)
Microcentrifuge	Microcentrifuge	MSE Sanyo (UK)
Tube rotator	Blood tube rotator SBI	Jencons scientific (UK)
Magnetic stirrer	RCT basic	IKA RCT basic (Germany)

Sonicator	Ultra sonicator- ULT065	Ultra wave (UK)
Analytical Balance	Balance P/PI-114	Denver instruments (UK)
Micropipettes	Gilson	Thermo Scientific (UK)
Vortex	Whirl mixer	Fisons scientific equipment (UK)

2.3 HPLC methods

2.3.1 HPLC instrumentation

HPLC system used in the study consisted of Jasco PU-1580 pump attached to a Jasco UV-970M (4-λ intelligent) and a Viglen Pentium-4 contender with an AZUR version 5.0.10.0 software package for analysis of TMZ and Patrin-2 (Fig. 2.1).

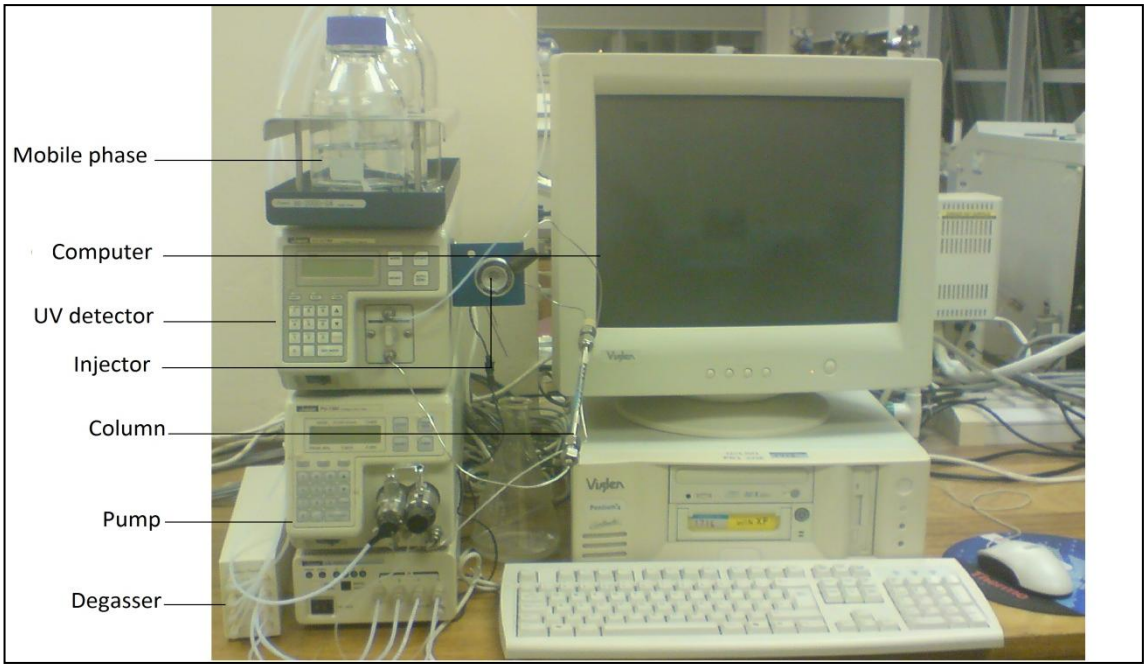


Figure 2.1 High Performance Liquid Chromatography coupled with UV detector used for sample analysis.

2.3.2 HPLC column

The chromatography was performed using a Shandon C18 (150 × 4.60 mm) column consisting of particle sizes equivalent to 5 µm. Manual injections were carried out using a Rheodyne model 7725i injector with a 20 µl loop. The mobile phase used for TMZ was 0.5 % acetic acid:methanol in the ratio of 9:1 (pH 2.8) (Moffat *et al.*, 2004) and the mobile phase used for Patrin-2 was 35 % acetonitrile consisting of 10 mM tetrabutylammonium acetate (TBAA), 10 mM sodium dodecyl sulphate (SDS) and 25 mM citric acid (pH 3.4) (Shervington *et al.*, 2005). Analysis was performed only after the column had reached equilibrium (approximately 30-60 min at a flow rate of 1 ml/min). The mobile phase was always filtered and degassed before use. The final flow rate was set to 1 ml/min and the detector was set to monitor at wavelengths 330 nm and 287 nm. All the analyses were carried out at ambient temperature (25 ^{o}C).

2.3.3 Validation of HPLC system

The validation of HPLC system for TMZ and Patrin-2 was carried out following standard procedures described in United States Pharmacopoeia (USP). This included linearity, repeatability and reproducibility, limit of detection and limit of quantification.

2.3.3.1 Selection of Mobile phase:

Based on the literature review the following two mobile phases were used for TMZ and Patrin-2.

1) For the analysis of TMZ: 0.5% acetic acid:methanol in the ratio of 9:1(v/v).

This mobile phase was prepared by mixing 100 ml of methanol to 900 ml of 0.5 % acetic acid. It was then sonicated and filtered before running it on the HPLC system. The pH of the mobile phase was found to be 2.8 (Moffat *et al.*, 2004).

2) For the analysis Patrin-2: 35 % acetonitrile consisting of 10 mM TBAA, 10 mM SDS and 25 mM citric acid was used.

This mobile phase was prepared by mixing 175 ml of HPLC grade acetonitrile with 325 ml of HPLC grade water to which 1.5076 g of TBAA, 1.4419 g of SDS and 2.4015 g of citric acid was added and dissolved. It was then sonicated and filtered before running it on the HPLC system. The mobile phase pH was measured to be 3.4 (Shervington *et al.*, 2005).

2.3.3.2 Stock solution:

Stock solution for TMZ was prepared by adding 10 mg of the TMZ to a 100 ml volumetric flask dissolving it with mobile phase and then making up to the mark with the mobile phase, thus giving a final concentration of 0.1 mg/ml. Similarly, 10 mg of Patrin-2 was added to a 100 ml volumetric flask dissolving it with acetonitrile and making up to the mark with acetonitrile to give a final concentration of 0.1 mg/ml. The stock solutions were stored in a refrigerator at 4 °C.

2.3.3.3 Calibration curve (linearity testing of the HPLC system):

For linearity testing of TMZ and Patrin-2, aliquots of concentration 0.08, 0.06, 0.04, 0.02 and 0.01 mg/ml were prepared from stock solutions and the corresponding peak areas were determined and the calibration curves were plotted.

2.3.3.4 Repeatability (Intraday analysis):

Intraday analysis was carried out for TMZ and Patrin-2 by analysing the sample at a concentration of 0.02 mg/ml six times on the same day and the SD and RSD were determined.

2.3.3.5 Reproducibility:

Reproducibility for TMZ and Patrin-2 were carried out by analysing six samples of both TMZ and Patrin-2 at a concentration of 0.02 mg/ml individually on the same day. Each sample was analysed twice and the SD and RSD were determined.

2.3.3.6 Limit of detection (LOD) and Limit of quantification (LOQ):

The limit of detection (LOD) is the minimum concentration at which an analyte can be detected. LOD for TMZ and Patrin-2 was carried out by diluting the samples from an initial concentration of 0.01 mg/ml and gradually decreasing the concentration down to a point where the detector was just able to distinguish between the signals to noise ratio. Based on the limit of detection of TMZ and Patrin-2, the limit of quantification was carried out by determining the lowest quantifiable concentration that could be achieved in a repeated manner with an acceptable RSD.

2.4 Stability test of TMZ and Patrin-2:

In order to determine whether TMZ and Patrin-2 were compatible in PBS at a pH of 7.4 following tests were carried out.

2.4.1 Standard solution:

Standard solution for TMZ was prepared by dissolving 5 mg of the TMZ into a 100 ml volumetric flask and then making up to the mark with the mobile phase, thus giving a final concentration of 0.05 mg/ml. Similarly, two different solutions were prepared one consisting of 5 mg of Patrin-2 and other consisting of a combination of 5 mg of each TMZ and 5 mg of Patrin-2 dissolved in acetonitrile in a 100 ml volumetric flask, thus providing a final concentration of 0.05 mg/ml for each. All the stock solutions were stored in a refrigerator at 4 °C.

2.4.2 Compatibility test of the two drugs TMZ and Patrin-2:

TMZ, Patrin-2 and a combination of TMZ and Patrin-2 stock solutions were prepared at a concentration of 0.05 mg/ml in mixture of PBS and ethanol (ratio of 8:2). The three solutions were stored at: 25, 37 and 50 °C. The samples were analysed after every 3 days. In addition to these samples a dry sample reaction was carried out, a vial consisting of 10 mg of TMZ and 10 mg of Patrin-2 were mixed and subjected to stress condition at 50 °C for 1 week. Both the compounds were then solubilised in a mixture of PBS and ethanol (ratio of 8:2) at concentrations of 0.05 mg/ml because Patrin-2 is not soluble in PBS alone ethanol was introduced to dissolve Patrin-2 in the solution maintaining the pH at 7.4.

2.5 To develop liposomal preparation of TMZ:

2.5.1 Freeze–Thaw method:

Liposomal formulation of TMZ was carried out by combining 10 mg of TMZ with phospholipids in ratios 1:1, 1:2 and 1:3 (calculated in moles) and the phospholipid providing the better entrapment was further combined with cholesterol in ratios 1:1:1, 1:2:1, 1:2:2, 1:3:1 and 1:3:2 (calculated in moles). To this mixture 2 ml of phosphate buffered saline (PBS) of pH 7.01 was added and then placed on the rotor set at 34 rpm for 30 min. The dispersion was vortexed intermittently for 30 min, this step was performed in six cycles i.e. 2 min vortexing, 3 min interval in a water bath kept at 65 °C. After vortexing the dispersion, the mixture was immersed in liquid nitrogen for 3 min and then immediately transferred to a water bath kept at 65 °C for 6 min (the freeze–thaw cycle was repeated six times) (Shazly *et al.*, 2008). The entrapment of the drug was aided by the freezing process, while the thawing process was used to rupture multilamellar vesicles in order to help the incorporation of the enclosed contents with the release medium. After completion of the freeze-thaw cycles, the liposomal

dispersion was allowed to stand for 1 hr to maximise further encapsulation of the drug (Mohammed *et al.,* 2004)**.** The determination of the encapsulation efficiency (EE %) was carried out as described in section 2.5.3.

[Phospholipid: lα-phosphatidylcholine, DMPC, DSPC, phospholipon 90H and lecithin]

2.5.2 Dehydration-Rehydration method:

Similar to thin film method: Liposomal formulation of TMZ was carried out by combining 10 mg of TMZ with phospholipids in ratios 1:1, 1:2 and 1:3 (calculated in moles) and the phospholipids providing the best entrapment was further combined with cholesterol/stearic acid in ratios 1:1:1, 1:2:1, 1:2:2, 1:3:1 and 1:3:2 (calculated in moles). To this mixture 2 ml of methanol and 10 ml of chloroform was added and then transferred to a 50 ml round bottom flask. The flask containing the mixture was placed on a rotary evaporator set at 65 °C for 4 hr to produce a thin film of the mixture. The thin film was flashed with nitrogen gas for 15 min at approx. 4 psi in order to help eliminate traces of the solvent in the film. The thin film was rehydrated with 2 ml of PBS and the suspension was vortexed for 4 min (along with hand shaking the flask and keeping it in the water bath at 40 °C) to form liposome (Glavas-Dodov *et al.,* 2005). After vortexing of the suspension, it was allowed to stand at room temperature for 1 hr for further encapsulation of the drug to form liposome.

[Phospholipid: lα-phosphatidylcholine, DMPC, DSPC, phospholipon 90H and lecithin]

2.5.3 Determination of EE %:

The EE is the actual weight of the drug entrapped in the liposome compared to the initial amount of drug weighed out. The determination of EE % of TMZ for freeze-thaw and dehydration-rehydration method was carried out as described below.

Before sample (AD_b): After 1 hr of incubation of the liposomal dispersion at 25 °C, 100 µl was taken and mixed with 2 ml of triton X-100 (10 %) and 7.90 ml HPLC grade water to make it up to a final volume of 10 ml. The solution was maintained in a water bath for 5 min at 65 °C and vortexed for 2 min and then analysed by HPLC (Maestrelli *et al.*, 2006).

After sample (AD_a): The remaining liposome suspension was washed twice with PBS (10 ml) and centrifuged for 30 min at 10000 g at a temperature of 4 °C. The pellet obtained from the centrifugation was mixed with 2 ml of PBS. From the liposome suspension 100 µl was removed and mixed with 2 ml of triton X-100 (10 %) and 7.90 ml HPLC grade water to produce a final volume of 10 ml. The solution was maintained in a water bath at 65 °C for 5 min and vortexed for 2 min and then analysed by HPLC (Kawano *et al.*, 2003).

The EE % was determined by the following equation (Shazly *et al.*, 2008):

$$EE\ \% = \frac{AD_a}{AD_b} \times 100$$

Where AD_b and AD_a are peak area obtained for TMZ in liposome before and after washing with PBS, respectively.

2.6. Methods used for preparation of PLGA microparticles were emulsifying-solvent evaporation method and spray dry method:

2.6.1. Emulsifying-solvent evaporation method:

Solution A: 200 mg of PLGA was dissolved in 10 ml of HPLC grade dichloromethane to which 5 mg of TMZ was added and sonicated for 10 min.

Solution B: Polyvinyl alcohol (PVA) (2 %) solution was prepared by dissolving 2 g of PVA in 100 ml of HPLC grade water continuously stirring the solution at 50 °C for about 45 min.

80 ml of a volume of PVA (2 %) was kept at room temperature continuously stirred at 600 rpm using magnetic stirrer to which solution A was added. The emulsion was kept continuously stirring at 600 rpm overnight in order to evaporate dichloromethane from the solution. Once the organic solvent was completely removed, the suspension was washed with HPLC grade water and the recovered microparticles were filtered using 0.7 µm glass fibre filter paper fitted in to a grade 3 glass sinter. The microparticles were stored overnight in a desiccator in order to remove any residue of water from the collected powder (Zhang and Gao, 2007). The microparticles were solubilised in 10 ml of dichloromethane using a sonicator and 30 ml of methanol was added to the solution in order to precipitate PLGA. The suspension was centrifuged for 30 min at 10000 g and the supernatant was analysed by HPLC.

2.6.2 Determination of EE % of the TMZ loaded PLGA microparticles:

The EE is the actual weight of the drug entrapped in the polymeric carrier compared to the initial amount of drug weighed out. The drug content in the supernatant was then checked using HPLC.

The EE was determined from the following equation (Seong *et al.,* 2003):

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

Where D_t is the peak area obtained from the standard solution of TMZ and D_m is the peak area obtained for TMZ in the microparticles.

Each preparation also involved the preparation of a standard solution (D_t) along with the reaction mixture in order to measure the amount of drug encapsulated. The standard solution was prepared by adding 5 mg of TMZ to a 40 ml volumetric flask dissolving it with mobile phase (0.5 % acetic acid and methanol in the ratio 9:1) and making up to the mark with the mobile phase. Analysis of the solution was carried out using HPLC.

Alternative method of calculation which can be used:

The EE was determined from the following equation (Seong *et al.*, 2003):

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

Where D_t is the amount of TMZ theoretically present in the microparticles collected (in weight) and D_m is the peak area obtained for TMZ in the microparticles.

2.6.3 Spray dry method:

2.6.3.1 The preparation of TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles:

A sample of 100 mg of PLGA and 5 mg of TMZ were dissolved in 30 ml of acetone. The solution was then spray dried using a mini spray dryer (Fig. 2.2). The aspirator was set at 100 % and inlet temperature was set at 60 °C (10 °C higher than the boiling point of acetone). The nitrogen q-flow was set at 30 mbar and the pump speed was adjusted to 20 %. The microparticles formed through spray drying were collected from the collection vessel and the cyclone by washing with acetone. The acetone was evaporated and the microparticle weighed out was approximately 32 mg. The microparticles were again washed with 30 ml of water in order to remove any unencapsulated drug. The suspension was centrifuged for 30 min at 20000 g with the temperature set at 4 °C and the supernatant was analysed using HPLC. The pellet obtained was treated with 4 ml of

acetone to dissolve the microparticles followed by 25 ml of methanol to precipitate PLGA. The suspension was centrifuged for 30 min at 10000 g with the temperature set at 4 °C and supernatant was analysed using HPLC (Seong *et al.*, 2003).

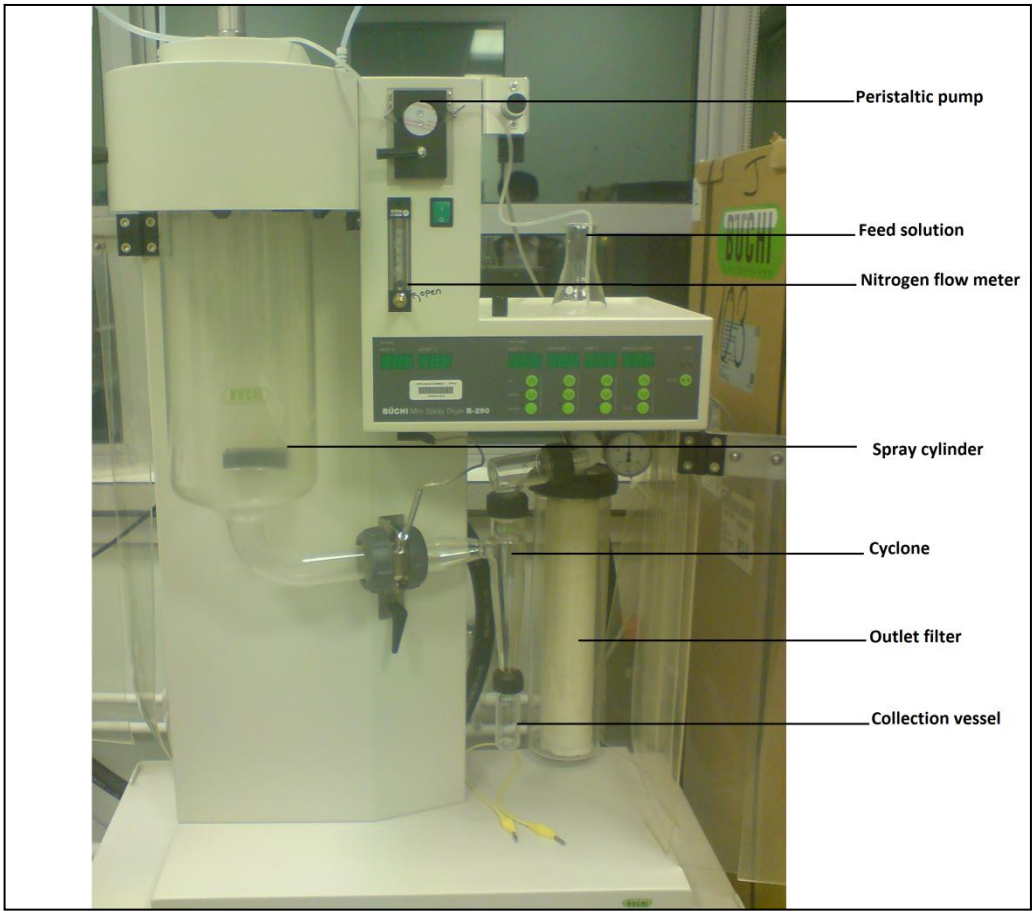


Figure 2.2 Mini Spray Dryer used for preparing the microparticles.

Patrin-2 loaded PLGA microparticles was prepared using the same protocol as described in section 2.5.3.1 except the amount of microparticle collected and weighed out was approximately 36 mg and that ethanol was used instead of water for washing the microparticles.

2.6.3.2 Determination of EE % of TMZ and Patrin-2 loaded PLGA microparticles:

The EE % of TMZ and Patrin-2 was determined from the following equation (Seong *et al.*, 2003):

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

Where D_t is the peak area obtained from the standard solution of TMZ and D_m is the peak area obtained for TMZ in the microparticles.

Each preparation also involved the preparation of a standard solution (D_t) along with the reaction mixture in order to measure the amount of drug encapsulated. The standard solution was prepared by adding 5 mg of TMZ to a 30 ml volumetric flask dissolving it with mobile phase (0.5 % acetic acid and methanol in the ratio 9:1) and making up to the mark with the mobile phase. Similarly, standard solution for Patrin-2 was prepared by adding 5 mg of Patrin-2 to a 30 ml of volumetric flask dissolving it with acetonitrile and making up to the mark with acetonitrile. Analysis of the solutions was carried out using HPLC.

Alternative method of calculation which can be used:

The EE was determined from the following equation (Seong *et al.*, 2003):

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

Where D_t is the amount of TMZ theoretically present in the microparticles collected (in weight) and D_m is the peak area obtained for TMZ in the microparticles.

2.7 Particle size analysis of liposome and microparticles:

2.7.1 Particle size analysis of liposome using the mastersizer.

Liposome prepared by using freeze-thaw and dehydration-rehydration method were analysed using the mastersizer (Fig. 2.3). Particle size distribution was plotted using a computer program Mastersizer 2000 software version 5.20 which was supplied by the manufacturer. The system was first cleaned with distilled water; the small volume dispersion unit was filled with 100 ml of distilled water to which 200 µl of the sample

was added to reach the laser obscuration up to 12-13 % in order to obtain the particle size.

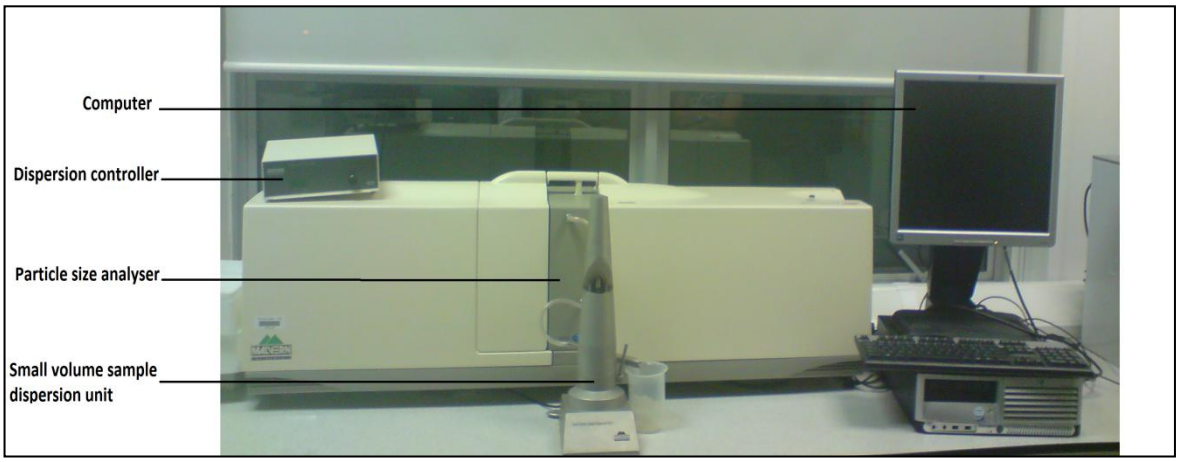


Figure 2.3 Mastersizer 2000 used for particle size analysis.

2.7.2 Particle size analysis of microparticles using the scanning electron microscopy (SEM).

The particle size and morphology was carried using a technique called scanning electron microscopy (Fig. 2.4). The technique was applied for measuring microparticles prepared by emulsifying-solvent evaporation and the spray drying method. Approximately 2 mg of the microparticles was spread evenly on an aluminium stub which was plated with gold at 50 milliamps and then placed in the chamber of the SEM for analysis. Images of the particles were obtained using a computer program Quanta-XTM 200 version 2.01 supplied by the FEI Company, UK.

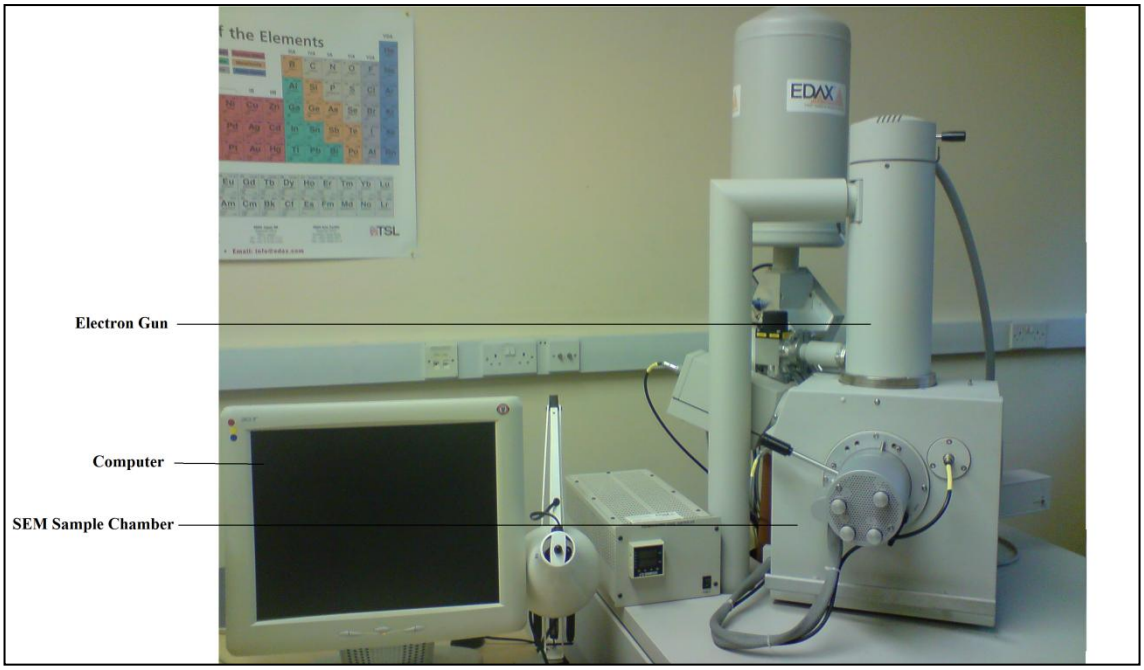


Figure 2.4 Scanning electron microscopy (SEM) used for Particle size analysis.

2.8. Dissolution techniques:

2.8.1. Dispersion method used for determination of release of TMZ and Patrin-2 from the microparticles:

In a screw-capped glass vial (30 ml) containing 25 ml of PBS at pH 7.4, 25 mg of microparticles were dispersed by shaking at 200 rpm in an incubator maintained at 37 ± 0.5 °C (Shazly *et al.,* 2008). At predetermined time intervals (0.5, 1, 2, 4, 6, 12, 24, 48,72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312 and 336 hr) an aliquot of 1 ml of the dispersion was withdrawn and replaced with 1 ml of PBS. The aliquot was centrifuged for 30 min at 13,000 *g*, and the supernatant was analysed using HPLC (Burgess *et al.,* 2002). The calculations were carried out using the amount of released compound obtained by HPLC analysis and by using the regression equation obtained from the calibration curve of both the compounds.

2.8.2. Dialysis bag method used for determination of release of TMZ and Patrin-2 from the microparticles:

During the preparation of the dialysis chamber, a 5 ml plastic tube was used with its centre of the cap removed with a heated pasteur pipette (wide end). The reaction mixture consisted of 25 mg of the microparticles to be dialyzed in a tube with 2.5 ml PBS at pH 7.4. A piece of dialysis membrane was placed loosely over top of tube. The cap of the tube was gently positioned on the tube and the tube was inverted (Fig. 2.4). The sample was placed down onto the dialysis membrane and the tube was attached to a 50 ml beaker containing 25 ml PBS. The procedure ensured that no bubbles were trapped under the membrane (Craig, 1967). The beaker was placed onto a magnetic stirrer set at 37 ± 0.5 °C and a magnetic flea was set to rotate at 200 rpm in order to encourage agitation. A control was set up along with the reaction mixture in order to make a comparison the release of the compound. At predetermined time intervals (0.5, 1, 2, 4, 6, 12, 24, 48,72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312 and 336 hr) an aliquot of 1 ml of the dispersion was withdrawn and replaced with 1 ml of PBS. The samples were analysed by HPLC (Burgess *et al.*, 2002). The calculations were carried out using the amount of released compound obtained by HPLC analysis and by using the regression equation obtained from the calibration curve of both the compounds.

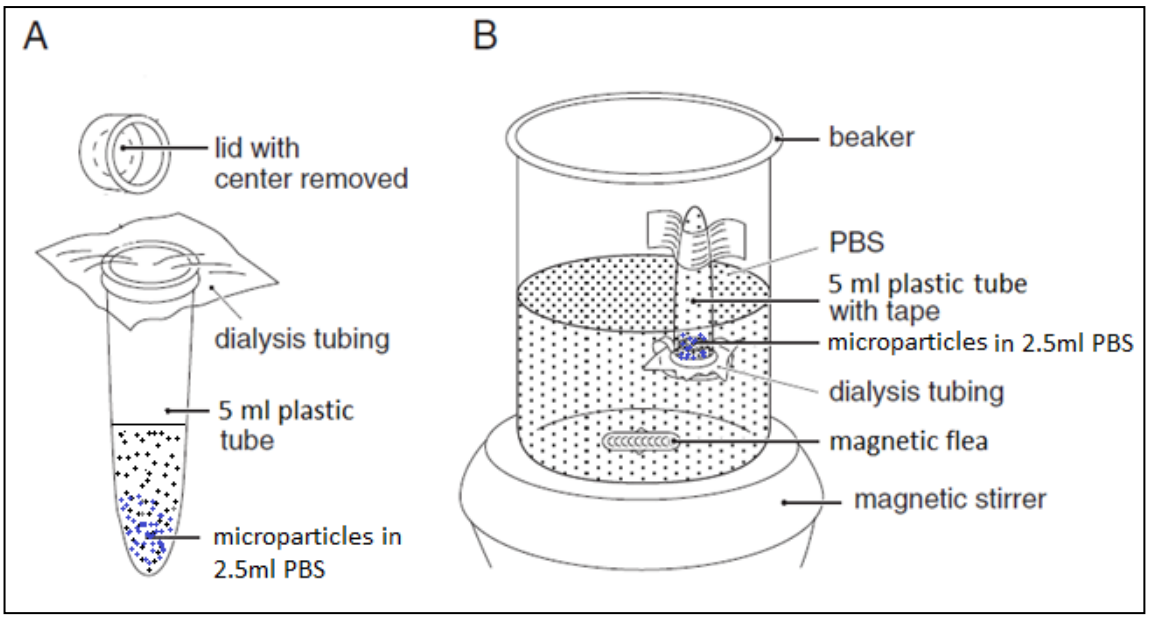


Figure 2.5 Preparation of the dialysis chamber to set the reaction (Craig, 1967).

2.9 Statistical analysis

For all the methods used for preparation of liposome and microparticles, the experiments were performed in triplicate, and the results were expressed as the mean ±Standard Deviation (SD) from the three or four independent experiments. A statistical analysis was performed using SPSS version 18.0.3 and Microsoft Excel. All data for freeze-thaw and dehydration-rehydration method were evaluated for ratio variables to compare a random sample from a normally distributed samples by one-sample "t-test", *P* values<0.005(*) and 0.001(**) are considered to be statistically significant. All the data for the Spray-dry method was evaluated for unpaired variables to compare two or more groups included pairs of the standard with the different molecular weight of polymer was tested by paired Student "t-test", *P* values<0.005(*) and 0.001(**) are considered to be statistically significant.

CHAPTER 3

RESULTS

3.1 HPLC methods for TMZ and Patrin-2 analysis.

Good peak symmetry for TMZ and Patrin-2 were achieved using the mobile phase consisting of 0.5 % acetic acid:methanol in the ratio of 9:1(v/v), (pH 2.8) and 35 % acetonitrile consisting of 10 mM TBAA, 10 mM SDS and 25 mM citric acid, (pH 3.4), respectively. TMZ was analysed at 330 nm while Patrin-2 was analysed at 287 nm. The retention time (RT) for TMZ was found to be 3.7 while for Patrin-2 was found to be 5.6 min, respectively.

Validation of the HPLC method

The parameters used for the validation included linearity, repeatability, reproducibility, limit of detection (LOD) and limit of quantification (LOQ) for the analysis of both TMZ and Patrin-2 are summarised (Table 3.6).

3.1.1 Linearity testing

Linearity for TMZ and Patrin-2 were carried out in the range of 0.01-0.1 mg/ml (Table 3.1). The calibration curve for TMZ and Patrin-2 were both linear with regression coefficients, R^2 values equal to 0.999 and 0.989, respectively (Fig. 3.1).

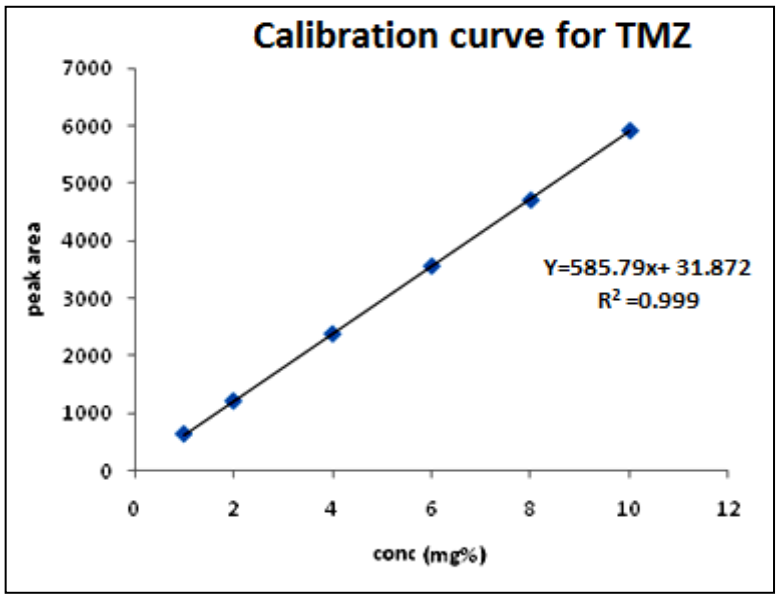
Table 3.1 Linearity testing of TMZ and Patrin-2. Data values are the average of $n = 3$.

Concentration (mg/ml)	**TMZ MPA**	**Patrin-2 MPA**
0.1	5906.59	3122.22
0.08	4697.39	2745.64
0.06	3549.57	1750.64
0.04	2369.00	1109.55
0.02	1201.12	531.87
0.01	627.07	253.42

MPA- Mean Peak Area.

The mean peak areas obtained from the solution of concentration ranging from 0.01-0.1 mg/ml for the linearity test were used to plot the calibration curve from which the regression coefficient was acquired (Fig. 3.1).

(a)



(b)

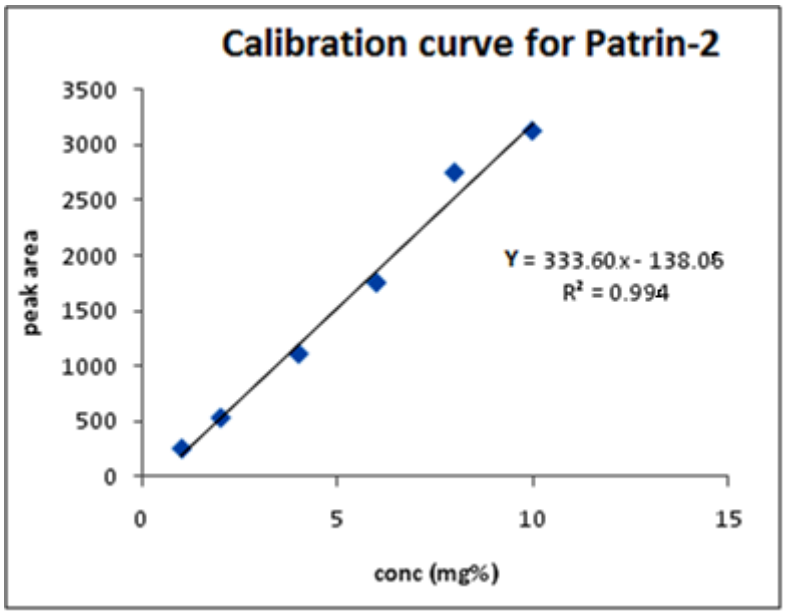


Figure 3.1 Calibration curve for TMZ and Patrin-2. (a) Calibration Curve for TMZ at concentration ranging from 0.01-0.1 mg/ml. (b) Calibration Curve for Patrin-2 at concentration ranging from 0.01-0.1 mg/ml. Y denotes dependent variable and x is the independent variable while R^2 is the regression coefficient.

3.1.2 Repeatability

Repeatability of the analytical method was carried out for TMZ and Patrin-2 using a concentration of 0.02 mg/ml (Table 3.2). The standard deviation (SD) for both TMZ and Patrin-2 were found to be 4.46 and 7.02, respectively, and the related standard deviation (RSD) for both TMZ and Patrin-2 was found to be 0.37 and 1.28, respectively. As the RSD values for both the compounds were low the repeatability was acceptable.

Table 3.2 The repeatability results for TMZ and Patrin-2 at concentration of 0.02 mg/ml. The mean of the 6 injections was calculated from which the SD and RSD values are obtained.

No. of injections of the sample	TMZ (0.02 mg/ml) Peak Area	Patrin-2 (0.02 mg/ml) Peak Area
1	1208.46	548.26
2	1214.39	540.00
3	1201.89	556.08
4	1206.48	544.25
5	1210.73	549.99
6	1212.17	558.62
Mean	1209.02	549.507
SD	4.456	7.01
RSD	0.37	1.28

SD-Standard deviation
RSD-Relative standard deviation

3.1.3 Reproducibility

The results obtained from the reproducibility determination of TMZ and Patrin-2 was carried out using six samples at a concentration of 0.02 mg/ml (Table 3.3). The SD for both TMZ and Patrin-2 were found to be 5.34 and 4.04, respectively, and RSD for both TMZ and Patrin-2 were found to be 0.44 and 0.73 respectively. The results obtained can be considered as reproducible as a low RSD value was obtained for both the compounds.

Table 3.3 The reproducibility results for TMZ and Patrin-2 at a concentration of 0.02 mg/ml. The mean of the 6 samples was calculated from which the SD and RSD values are obtained. Data values are the average of $n = 3$.

Sample No.	TMZ (0.02 mg/ml) Peak area	Patrin-2 (0.02 mg/ml) Peak area
1	1206.17	554.19
2	1210.62	558.18
3	1213.87	548.26
4	1201.23	549.13
5	1214.47	556.66
6	1204.54	555.26
Mean	1208.48	553.61
SD	5.34	4.04
RSD	0.44	0.73

SD-standard deviation
RSD- Relative standard deviation

3.1.4 Limit of detection

LOD for both TMZ and Patrin-2 were found to be 3.90 µg/ml. As the injection was not repeatable at the concentration of 3.90 µg/ml, however, a small peak could be detected for qualitative purposes but cannot be quantified.

Table 3.4 Limit of detection for TMZ and Patrin-2. Data values are the average of $n = 3$.

Concentration (µg/ml)	TMZ MPA	Patrin-2 MPA
500	300.81	117.45
250	151.23	55.73
125	60.40	25.14
62.5	29.30	12.24
31.25	14.03	5.85
15.62	7.08	3.88
7.81	3.46	1.69
3.91	1.63	0.845

MPA- Mean Peak Area.

3.1.5 Limit of quantification

The LOQ for both TMZ and Patrin-2 were 7.81 µg/ml and therefore the SD for both TMZ and Patrin-2 were found to be 0.05 and RSD for both TMZ and Patrin-2 were found to be 1.43 and 3.17, respectively.

Table 3.5 Limit of quantification of TMZ and Patrin-2 at a concentration of 7.81 µg/ml was injected 6 times. The mean of the 6 injections was calculated from which the SD and RSD values are obtained.

No. of injections	TMZ Peak Area	Patrin-2 Peak Area
1	3.58	1.64
2	3.49	1.58
3	3.61	1.55
4	3.52	1.67
5	3.62	1.57
6	3.55	1.66
Mean	3.56	1.61
SD	0.05	0.05
RSD	1.43	3.17

SD-standard deviation
RSD- Relative standard deviation

The results obtained from the HPLC analysis for validation of the system are summarised (Table 3.6). Thus from these results the HPLC method was considered to be reliable for the analysis of TMZ and Patrin-2 and further experiments were carried out using both the compounds.

Table 3.6: A summary of the parameters used to validate the analytical HPLC method for TMZ and Patrin-2.

Parameters	**TMZ**	**Patrin-2**
Analytical wavelength (nm)	330	287
Stock solution (mg/ml)	0.1	0.1
Retention time (min)	3.5	5.6
Linearity range (mg/ml)	0.01- 0.1	0.01- 0.1
Regression equation	Y=585.79x + 31.872	Y=333.60x - 138.06
Correlation coefficient (R^2)	0.999	0.994
Repeatability (RSD)	0.37	1.28
Reproducibility (RSD)	0.44	0.73
Limit of detection (µg/ml)	3.90	3.90
Limit of quantification (µg/ml)	7.81	7.81
Limit of quantification (RSD)	1.43	3.17

3.2 Stability test for TMZ and Patrin-2:

3.2.1 Compatibility test of TMZ and Patrin-2 carried out in mixture of PBS and ethanol.

In order to determine whether TMZ and Patrin-2 were compatible in PBS at a pH of 7.4 the following tests were carried out. TMZ, Patrin-2 and a combination of TMZ and Patrin-2 stock solutions were prepared at a concentration of 0.05 mg/ml in a mixture of PBS and ethanol (ratio of 8:2). The three solutions were stored at 25, 37 and 50 °C. Rapid degradation of TMZ was observed at 37 and 50 °C, whereas, Patrin-2 showed a rapid degradation at 50 °C (Table 3.7 and 3.8). A combination of both TMZ and Patrin-2 on HPLC analysis indicated rapid degradation products at 25, 37 and 50 °C (Table 3.9). Reaction between TMZ and patrin-2 has also been previously reported by Ranson and his team (Ranson *et al.*, 2007).

Degradation products of TMZ were observed at 37 and 50 °C from 72 to 288 hr while at 25 °C there was small amount of reduction in the peak area but no degradation product was detected.

Table 3.7 The results for the stability of TMZ at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for (a) 72 hr (b) 288 hr at three different temperatures. The analysis of these samples were carried out using the HPLC with mobile phase (0.5 % acetic acid:methanol in the ratio of 9:1). Data values are the average of $n = 3$.

Temperature (°C)	**Time (hr)**	**Retention time (min)**	**MPA**	**Compound**
25	72	2.08	2504.09	TMZ
	288	7.43	1160.87	UDP
37	72	2.05	594.87	TMZ
		2.22	268.32	UDP
	288	2.15	12.79	TMZ
		6.13	21.90	UDP
		17.50	1139.25	UDP
50	72	2.02	137.66	TMZ
		2.17	72.46	UDP
	288	2.17	19.33	TMZ
		6.35	18.39	UDP
		17.50	1115.70	UDP

MPA- Mean Peak Area.
UDP- Unidentified Degradation Product.

Degradation products of Patrin-2 unlike TMZ were observed at 50 °C from 72 to 288 hr while at 25 and 37 °C there was small amount of reduction in the peak area at 72 hr but no degradation product was detected.

Table 3.8 The results for the stability of Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for (a) 72 hr (b) 288 hr at three different temperatures. The analysis of these samples was carried out using HPLC with mobile phase (35 % acetonitrile consisting of 10 mM TBAA, 10 mM SDS and 25 mM citric acid). Data values are the average of $n = 3$.

Temperature (°C)	Time (hr)	Retention time (min)	MPA	Compound
25	72	6.72	1620.24	Patrin-2
	288	2.47	65.91	UDP
		3.77	10.79	UDP
		4.67	31.77	UDP
		5.93	1417.69	Patrin-2
37	72	6.97	1042.62	Patrin-2
	288	2.03	779.86	UDP
		3.73	77.85	UDP
		4.22	21.05	UDP
		4.63	36.28	UDP
		5.90	432.08	Patrin-2
50	72	2.07	753.51	UDP
		7.02	432.11	Patrin-2
	288	2.03	607.63	UDP
		3.73	87.29	UDP
		4.22	36.85	UDP
		6.18	136.64	Patrin-2

MPA- Mean Peak Area.
UDP- Unidentified Degradation Product.

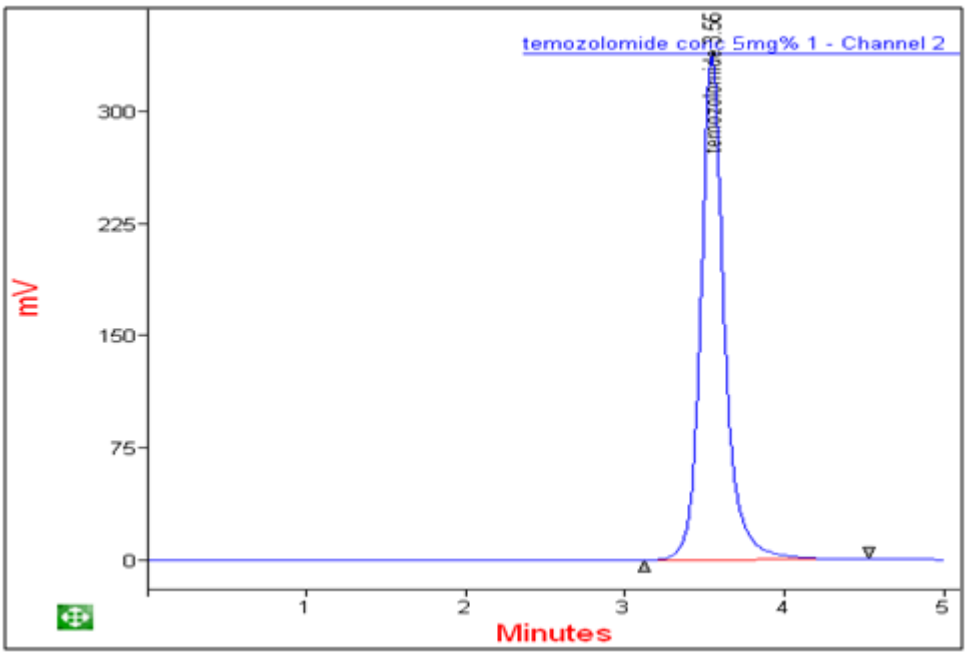
Rapid degradation products of TMZ and Patrin-2 on their combination were observed from 72 hr at all three temperatures 25, 37 and 50 °C with large amount of reduction in the peak areas of TMZ and Patrin-2. Thus the HPLC analysis of TMZ, Patrin-2 and combination of both indicated that TMZ and Patrin-2 are not stable together.

Table 3.9 The results for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for (a) 72 hr (b) 288 hr at three different temperatures. The analysis of these samples were carried out using HPLC with mobile phase (35 % acetonitrile consisting of 10 mM TBAA, 10 mM SDS and 25 mM citric acid). Data values are the average of $n = 3$.

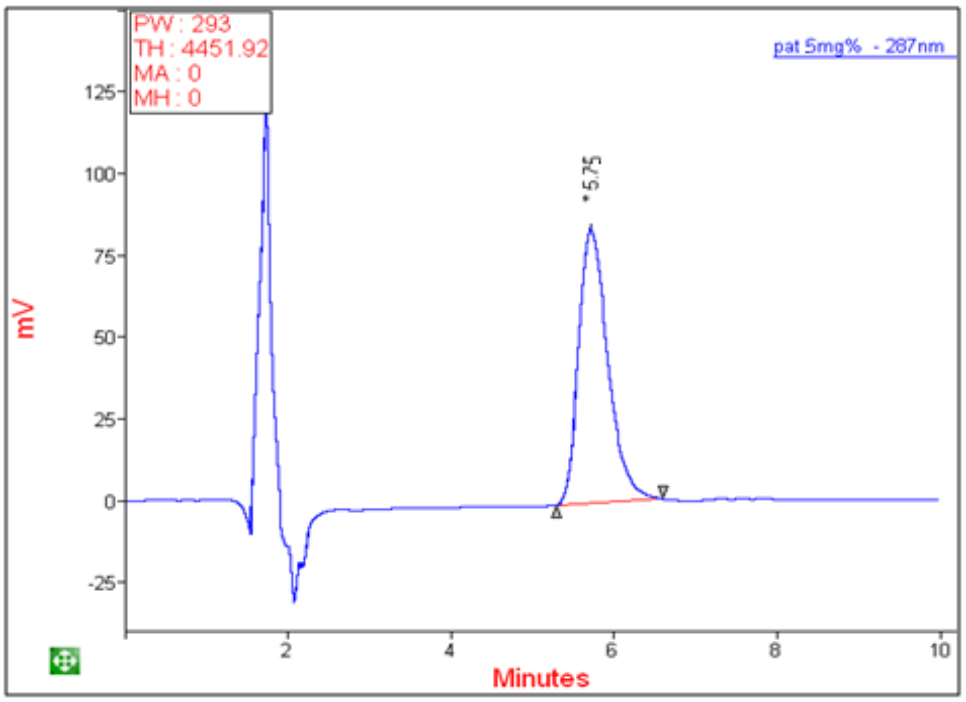
Temperature (°C)	Time (hr)	Retention time (min)	MPA	Compound
25	72	2.03	1459.65	TMZ
		6.57	1253.87	Patrin-2
		7.65	319.64	UDP
	288	2.18	231.41	TMZ
		4.12	320.23	UDP
		4.58	163.23	UDP
		10.10	533.06	UDP
37	72	2.57	499.12	TMZ
		2.93	342.71	UDP
		6.62	1208.92	Patrin-2
	288	2.37	3128.12	TMZ
		2.82	762.41	UDP
		4.35	484.07	UDP
		6.18	458.80	Patrin-2
50	72	2.60	443.66	TMZ
		2.98	356.38	UDP
		7.00	697.83	Patrin-2
	288	2.02	738.84	TMZ
		2.43	494.62	UDP
		2.77	416.74	UDP
		3.73	120.69	UDP
		4.20	48.11	UDP
		6.13	195.94	Patrin-2

MPA- Mean Peak Area.
UDP- Unidentified Degradation Product.

(a)


temozolomide conc 5mg% 1 - Channel 2
3.56
temozolomide
300
225
150
75
0
mV
1
2
3
4
5
Minutes

(b)


PW : 293
TH : 4451.92
MA : 0
MH : 0
pat 5mg% - 287nm
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125
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(c)

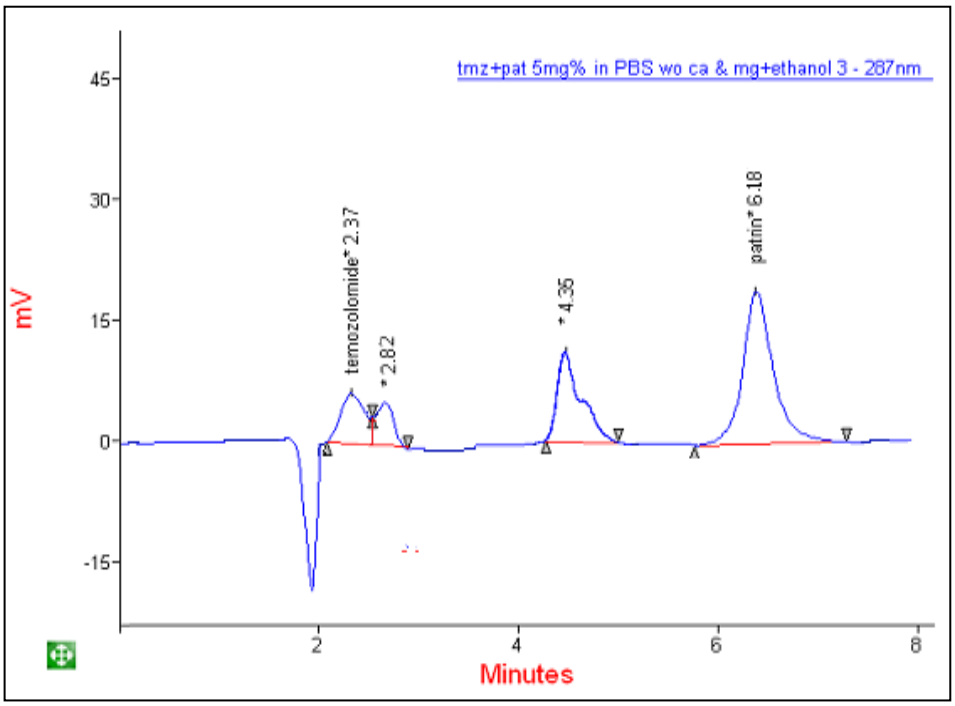


Figure 3.2 HPLC chromatogram for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml (a) TMZ Standard solution in mobile phase (*n* = 3), (b) Patrin-2 standard solution in acetonitrile (*n* = 3) and (c) TMZ and Patrin-2 mixture in PBS and ethanol (ratio 8:2) solution stored for 288 hr at 37°C (*n* = 3).

3.2.2 Dry sample reaction carried out for the determination of compatibility of TMZ and Patrin-2 at 50 °C.

When 10 mg of TMZ and 10 mg of Patrin-2 were mixed and subjected to stress conditions at 50 °C for a period of 1 week, both compounds were solubilised in a mixture of PBS and ethanol (ratio of 8:2) and a concentration made up to 0.05 mg/ml. HPLC analysis clearly indicated that degradation had occurred and thus provided further evidence that proved TMZ and Patrin-2 to be incompatible.

Table 3.10 The results testing the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol solution. The analysis of this sample was carried out using HPLC with mobile phase (35 % acetonitrile consisting of 10 mM TBAA, 10 mM SDS and 25 mM citric acid). Data values are the average of $n = 3$.

Concentration (mg/ml)	Retention time (min)	MPA	Compound
0.05	2.50	310.06	TMZ
	2.87	329.33	UDP
	4.02	4.39	UDP
	4.98	5.86	UDP
	6.43	972.39	Patrin-2

MPA- Mean Peak Area.
UDP- Unidentified Degradation Product.

3.3 Results of attempted preparations of various liposome formulations using TMZ as the active compound.

3.3.1 Freeze-Thaw method.

The liposomal formulation of TMZ was carried out by combining 10 mg of TMZ with phospholipids in different ratios and also using the combination of phospholipids and cholesterol to maximise the entrapment of TMZ. The lowest EE % obtained using the freeze-thaw method was with the use of DMPC and TMZ, use of other phospholipids such as lα-phosphatidylcholine, lecithin, phospholipon 90H and DSPC also did not proved to be suitable to give a better EE % (Table 3.11).

Table 3.11 Entrapment efficiency of TMZ loaded liposomes using different phospholipids using the freeze-thaw method. Data values ± standard deviation are average of $n = 3$.

Ratio	TMZ: Lα-phosphatidylcholine		TMZ: DSPC		TMZ:DMPC		TMZ: Phospholipon 90H		TMZ:Lecithin	
	EE %	Average Particle size (µm)	EE %	Average Particle size (µm)	EE %	Average Particle size (µm)	EE %	Average Particle size (µm)	EE %	Average Particle size (µm)
1:1	1.15 ± 0.15	3.911	1.78 ± 1.15	5.352	0.56 ± 0.55	2.821	0.60 ± 0.36	7.099	1.3 ± 0.10	4.078
1:2	1.21 ± 0.14	3.719	0.77 ± 0.40	6.979	0.17 ± 0.11	2.762	0.92 ± 0.42	7.229	2.1 ± 1.95	4.066
1:3	1.24 ± 0.21	5.368	0.63 ± 0.19	6.570	0.15 ± 0.16	3.692	3.64 ± 1.36	7.195	2.5 ± 1.47	4.248

EE %- Entrapment efficiency

The combination of phospholipids and cholesterol was experimented to improve the EE % of TMZ (Table 3.12). The combination of phospholipon 90H and cholesterol was analysed to give the maximum entrapment of 11.54 ± 0.67 % from the freeze-thaw method while the combination of lα-phosphatidylcholine and cholesterol did not indicate any difference in the EE % of TMZ when compared to the EE % of TMZ with lα-phosphatidylcholine (Table 3.11 and 3.12).

Table 3.12 Entrapment efficiency of TMZ loaded liposomes with a combination of phospholipon 90H and cholesterol and a combination of lα-phosphatidylcholine and cholesterol using the freeze-thaw method. Data values ± standard deviation are average of $n = 3$.

Ratio	**TMZ: Phospholipon 90H: Cholesterol**		**TMZ: Lα-phosphatidylcholine : Cholesterol**	
	EE %	**Average Particle size (µm)**	**EE %**	**Average Particle size (µm)**
1:1:1	7.56 ± 1.06	7.752	0.98 ± 0.61	6.821
1:2:1	6.32 ± 3.73	6.078	1.13 ± 0.16	6.799
1:2:2	11.54 ± 0.67	10.654	0.74 ± 0.37	6.910
1:3:1	11.02 ± 0.60	9.086	0.84 ± 0.44	5.912
1:3:2	10.38 ± 0.54	9.002	0.91 ± 0.75	6.871

EE %: Entrapment efficiency.

3.3.2 Dehydration-Rehydration method.

The liposomal formulation of TMZ was carried out by combining 10 mg of TMZ with phospholipids in different ratios and also using a combination of phospholipids and cholesterol/stearic acid in order to optimise the entrapment of TMZ. Use of phospholipids alone with TMZ did not proved to be suitable to give a better EE % of TMZ (Table 3.13).

Table 3.13 Entrapment efficiency achieved from TMZ loaded liposomes with the phospholipids using the dehydration-rehydration method. Data values ± standard deviation are average of $n = 3$.

Ratio	TMZ: Phospholipon 90H		TMZ: Lecithin	
	EE %	Average Particle size (µm)	EE %	Average Particle size (µm)
1:1	0.27 ± 0.16	7.116	0.57 ± 0.31	6.019
1:2	0.25 ± 0.11	7.119	0.45 ± 0.33	7.062
1:3	0.24 ± 0.20	7.231	0.53 ± 0.09	7.023

EE %: Entrapment efficiency

The combination of phospholipids and cholesterol was optimised to improve the EE % of TMZ (Table 3.14). A combination of phospholipon 90H and cholesterol was found to provide a maximum entrapment of 26.69 ± 0.34 % from the dehydration-rehydration method.

Table 3.14 Entrapment efficiency achieved from TMZ loaded liposome with the phospholipids in combination with cholesterol using the dehydration-rehydration method. Data values ± standard deviation are average of $n = 3$.

Ratio	TMZ:Phospholipon 90H: Cholesterol		TMZ:Lecithin: Cholesterol	
	EE %	Particle size (µm)	EE %	Particle size (µm)
1:1:1	2.43 ± 1.40	7.341	0.50 ± 0.54	7.189
1:2:1	2.25 ± 1.35	7.412	0.51 ± 0.60	7.061
1:2:2	26.69 ± 0.34	12.306	1.16 ± 0.11	7.621
1:3:1	13.61 ± 1.16	7.089	0.23 ± 0.08	7.168
1:3:2	19.36 ± 2.13	7.921	0.67 ± 0.46	7.211

EE %: Entrapment efficiency

The combination of phospholipids and stearic acid was experimented to for similar reasons as for cholesterol. The combination of phospholipon 90H and stearic acid did not indicate any difference in the EE % of TMZ when compared to the EE % of TMZ with phospholipon 90H while the combination of lecithin and stearic acid showed increase in the EE % as compared to the EE % of TMZ with lecithin (Table 3.13 and 3.15).

Table 3.15 Entrapment efficiency achieved from TMZ loaded liposome with phospholipids mentioned in the table below in combination with stearic acid using the dehydration-rehydration method. Data values ± standard deviation are average of $n = 3$.

Ratio	TMZ: Phospholipon 90H: Stearic acid		TMZ: Lecithin: Stearic acid	
	EE %	Average Particle size(µm)	EE %	Average Particle size(µm)
1:1:1	1.01 ± 0.35	6.834	1.98 ± 0.24	6.985
1:2:1	0.95 ± 0.33	5.126	4.94 ± 0.41	6.519
1:2:2	1.09 ± 0.24	5.981	2.73 ± 0.27	7.106
1:3:1	1.11 ± 0.19	6.286	5.09 ± 0.67	7.135
1:3:2	0.98 ± 0.30	6.121	6.65 ± 1.86	7.083

EE %: Entrapment efficiency

3.3.3 A comparison between Freeze-thaw and Dehydration-Rehydration methods used for the preparation TMZ loaded liposomes.

Different ratios of phospholipon 90H and combinations of phospholipon 90H with cholesterol were used in order to prepare TMZ loaded liposomes. In the experiment involving the freeze-thaw method there was a significant increase (*, P<0.005) in the EE % when a combination of phospholipon 90H and cholesterol was used at ratios of 1:2:2, 1:3:1 and 1:3:3 compared to the other ratios. In the dehydration-rehydration method, similar to freeze-thaw method a combination of phospholipon 90H and cholesterol showed the maximum EE %. As shown in the Fig. 3.2 there is a significant increase (**, P<0.001) in the EE % at a ratio 1:2:2, while the ratios 1:3:1 and 1:3:2 showed an increase in the EE % compared to the other ratios, however, there was a significant decrease (*, P<0.005) in the EE % compared with the ratio 1:2:2 (**, P<0.001). On comparing the freeze-thaw and dehydration-rehydration methods using a 1:2:2 ratio, the dehydration-rehydration method showed a significant increase (**, P<0.001) in the EE % as opposed to a *, P<0.005 value for the freeze-thaw method.

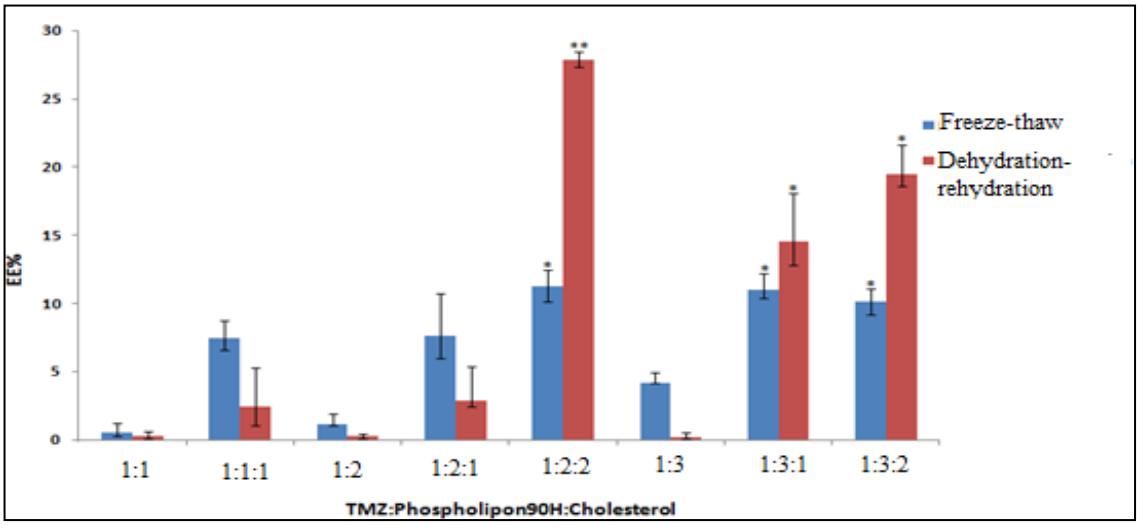


Figure 3.3 Comparison between Freeze-Thaw and Dehydration-Rehydration method. The effect of different ratios of phospholipon 90H and on combining it with cholesterol in order to encapsulate TMZ comparing two methods freeze-thaw and dehydration-rehydration using a 1:2:2 ratio, the dehydration-rehydration method showed a significant increase (**, P<0.001) in the EE % as opposed to a P<0.005 value for the freeze-thaw method. Data obtained are expressed as mean ± S.D of EE %**,** where n = 3.

3.4 Preparation of PLGA microparticles using two procedures.

Microparticles were prepared using PLGA of different molecular weights (MW). Two procedures were used for the preparation of the microparticles. However, in both procedures PLGA with an average molecular weight (MW) of $1.7x10^4$ Da showed maximum entrapment of TMZ.

3.4.1 Emulsifying-Solvent Evaporation method.

In order to prepare TMZ loaded PLGA microparticles the emulsifying solvent evaporation method was used. The maximum entrapment achieved using this method was not more than 2.76 ± 0.36 %, however modification in this technique and as from the literature it is possible to obtain the preparation of TMZ loaded PLGA microparticles with better EE% (Zhang and Gao, 2007).

Table 3.16 The entrapment efficiencies achieved when TMZ was used to prepare PLGA microparticles using the emulsifying solvent evaporation method and varying the MW of PLGA. The PLGA with a MW of 1.7×10^4 Da showed the maximum entrapment of TMZ. Data values ± standard deviation are average of $n = 3$.

PLGA MW (Da)	EE %
$1.7x10^4$	2.76 ± 0.36
$1.5x10^4$- $4x10^4$	2.56 ± 0.10
$4x10^4$- $7.5x10^4$	2.01 ± 0.36
$6.6x10^4$- $1x10^5$	1.62 ± 0.39

PLGA- Poly (DL-lactide-co-glycolide)
MW- Molecular weight
Da- Dalton
EE %- Entrapment efficiency

From the HPLC analysis the mean peak area of the standard solution used to calculate the EE % was 6456.41.

3.4.2 Spray dry method

Spray dry method used for obtaining TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles.

The spray dry method was used to prepare microparticles of both TMZ and Patrin-2 using PLGA as the vehicle. Maximum entrapment using this method was achieved when TMZ was used affording a 64.32 ± 2.58 % encapsulation efficiency while for Patrin-2 a 68.47 ± 1.47 % encapsulation efficiency was achieved. Thus this technique was found to be successful for the preparation of both TMZ and Patrin-2 loaded PLGA microparticles. However the analytical method used for calculation needs improvement in order to obtain higher encapsulation.

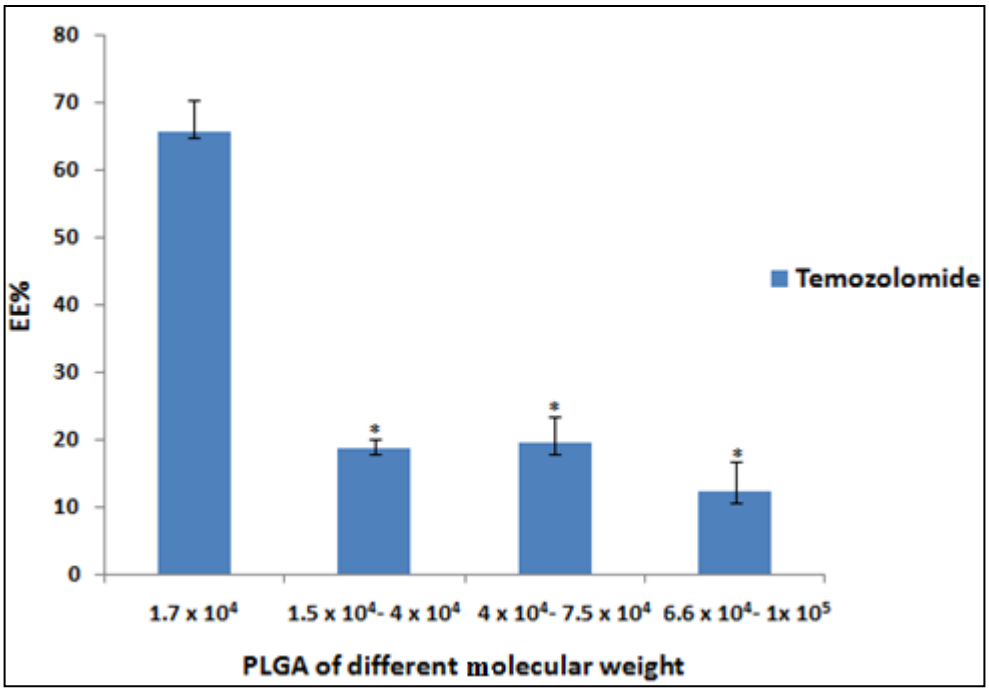
Table 3.17 The entrapment efficiency obtained from TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles by varying the MW of PLGA using the Spray Dry method with PLGA of MW 1.7 x 10^4 Da showed the maximum entrapment with TMZ. Data values ± standard deviation are average of $n = 3$.

PLGA MW (Da)	**TMZ EE %**	**Patrin-2 EE %**
$1.7x10^4$	64.32 ± 2.58	68.47 ± 1.47
$1.5x10^4$- $4x10^4$	18.77 ± 0.67	24.76 ± 1.46
$4x10^4$- $7.5x10^4$	19.35 ± 1.92	6.65 ± 0.90
$6.6x10^4$- $1x10^5$	12.15 ± 2.14	3.20 ± 0.17

PLGA- Poly (DL-lactide-co-glycolide)
MW- Molecular weight
Da-Dalton
EE %- Entrapment efficiency

From the HPLC analysis, the mean peak area of the standard solution for TMZ and Patrin-2 used to calculate the EE % was 3832.56 and 8023.08, respectively.

(a)



(b)

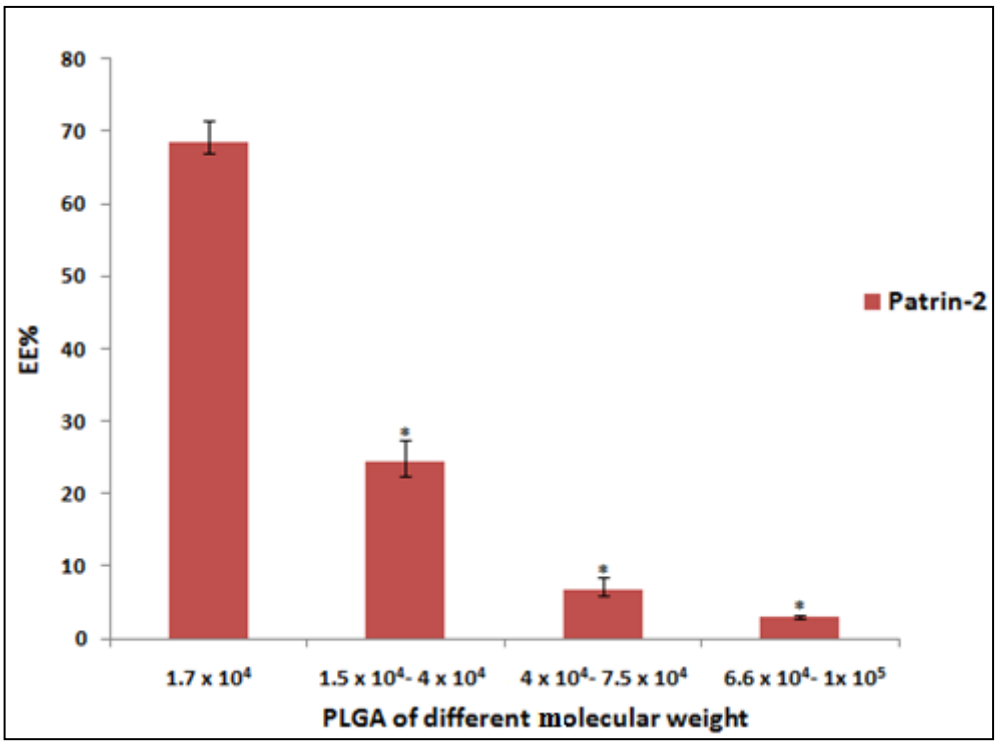


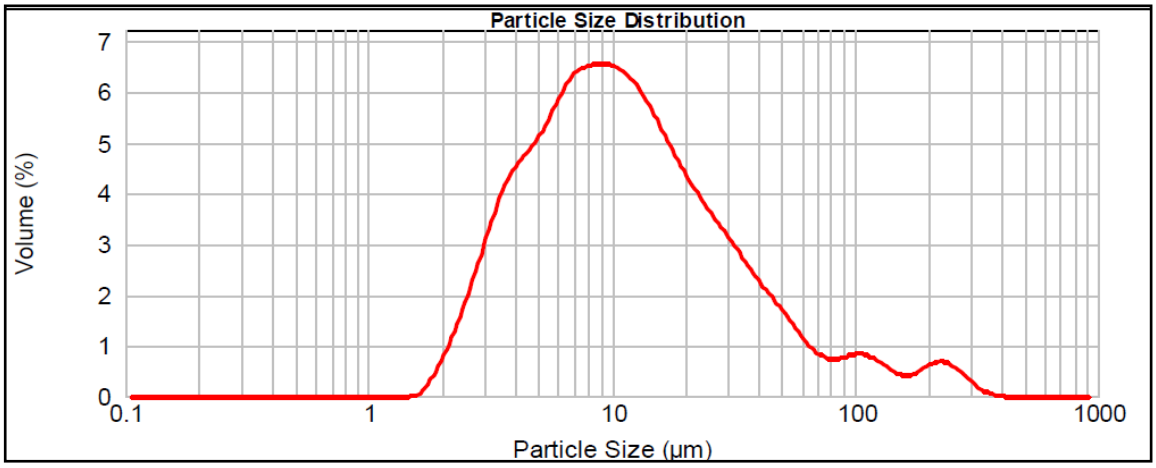
Figure 3.4 Spray dry method. The effect of varying MW of PLGA used for encapsulation of (a) TMZ and (b) Patrin-2 using Spray Dry method. Both TMZ and Patrin-2 gave maximum entrapment with the PLGA of MW of $1.7x10^4$ Da. Data obtained are expressed as mean ± S.D of EE %**,** where $n = 3$.

3.5 Particle size analysis of liposome and microparticles:

3.5.1 Particle size analysis of liposome using the mastersizer.

Liposomes prepared by the freeze-thaw and dehydration-rehydration method were analysed using the mastersizer. The maximum entrapment of TMZ in liposomes was achieved by using a combination of phospholipon 90H and cholesterol in both the methods. (Fig. 3.4).

(a)



(b)

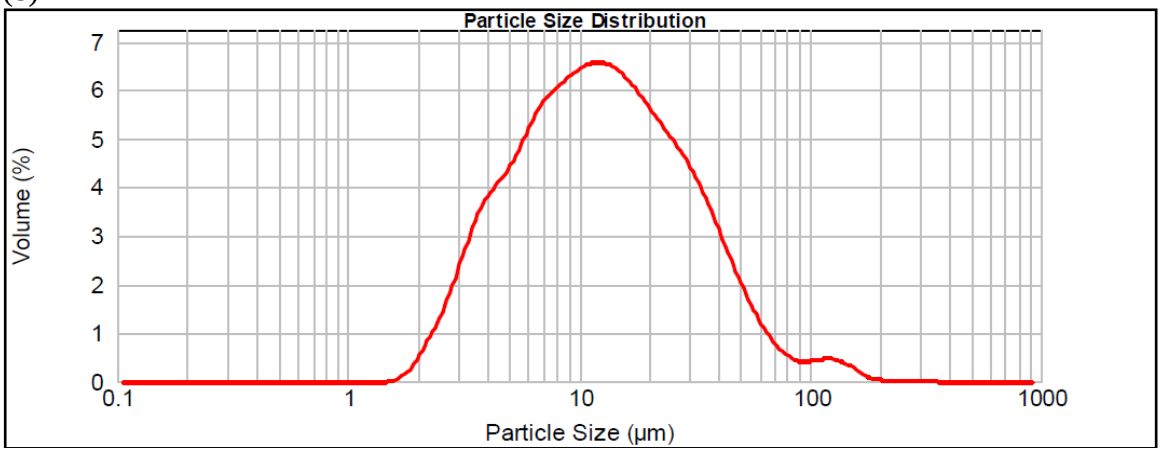


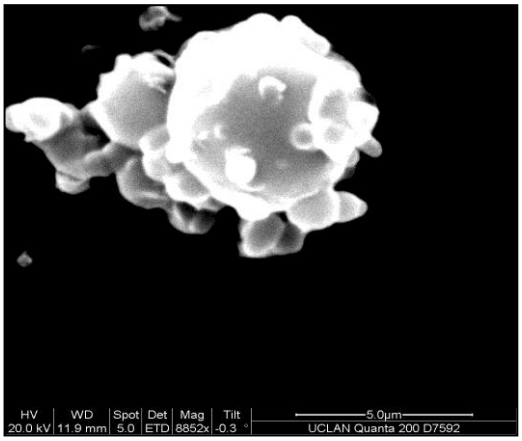
Figure 3.5 Particle size distributions. (a) Particle size of the TMZ loaded liposome consisting of phospholipon 90H and cholesterol using the freeze-thaw method is 10.654 µm. (b) Particle size of the TMZ loaded liposome consisting of phospholipon 90H and cholesterol using the dehydration-rehydration method is 12.306 µm.

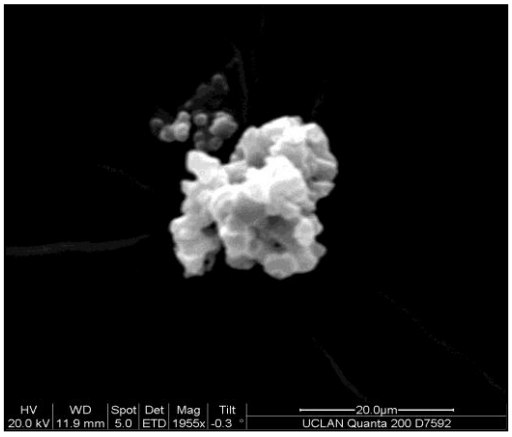
3.5.2 Particle size analysis of microparticles using the Scanning Electron Microscopy (SEM).

The technique was applied for measuring microparticles prepared by the emulsifying-solvent evaporation and the spray dry method. Maximum entrapment of TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles were achieved using the

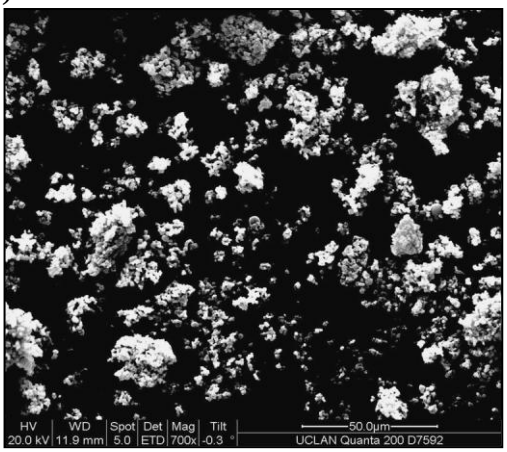
spray dry method with the PLGA of MW of $1.7x10^4$ Da. The microparticles were found to be spherical in shape (Fig. 3.5).

(a) (b)

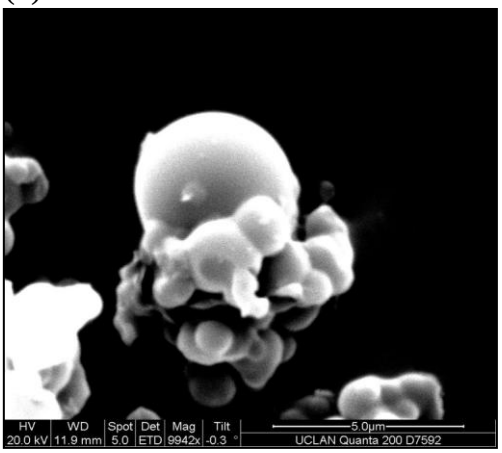




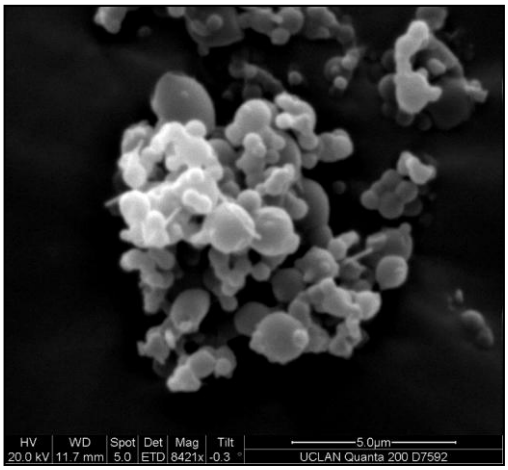
(c)



(d)



(e)



(f)

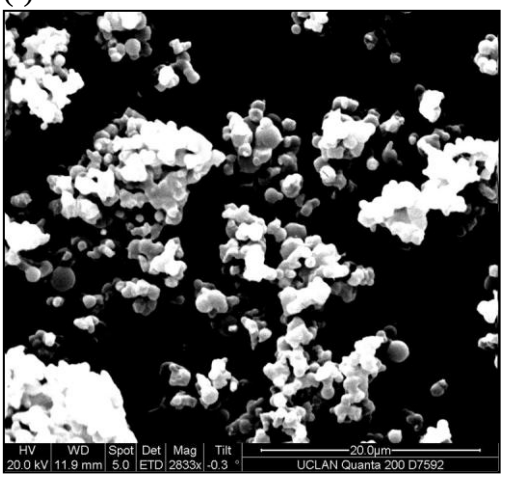


Figure 3.6 Particle size analyses. The particle size and morphology of the TMZ loaded PLGA microparticles are shown in Fig. (a), (b) and (c) while for Patrin-2 loaded PLGA microparticles is shown in Fig. (d), (e) and (f). The particles appear to be amorphous, smooth and spherical in shape. However, TMZ microparticles shown in (b) indicates small amount of pore formation on the surface of the microparticles.

3.6 Dissolution techniques

The dispersion and dialysis method were used to monitor the release of TMZ and Patrin-2 from the PLGA microparticles.

3.6.1.1 Dispersion Method used for determining the release of TMZ from the microparticles.

The release profile of TMZ from PLGA microparticles was monitored in PBS, pH 7.4 at 37 ± 0.5 °C. A plot of the percentage cumulative amount released of TMZ against time (Fig. 3.6) was obtained. From the release profiles, it was found that an initial burst of TMZ release was during the first 30 min of analysis followed by a gradual decrease in the concentration of TMZ. The cumulative mass of TMZ was calculated at 0.5 hr which is 407.06 µg in concentration as shown in Table 3.18, however, the total amount of TMZ theoretically present in TMZ loaded PLGA particles at the beginning of the in vitro release was 1200 µg. As degradation of the drug takes place are when kept at 37 °C could be the reason for not obtaining the expected amount.

Table 3.18 From HPLC analysis TMZ release from PLGA microparticles was detected and from the peak area obtained the cumulative mass release was calculated. Data value are the average of $n = 2$.

Time (hr)	MPA	Cumulative mass release (µg)
0.5	985.52	407.06
1	914.95	393.22
2	603.32	275.28
4	248.99	133.79
6	116.22	80.83
12	30.4	45.64
24	6.56	35.44
48	4.29	34.03
72	3.14	33.07
96	3.09	32.56
120	3.04	32.05
144	2.95	31.52
168	2.82	30.97
192	2.29	30.25
216	Not detected	Not detected

MPA-Mean Peak Area

The percentage of cumulative release of TMZ against time (Fig. 3.6) was plotted indicating the release pattern of TMZ. During the first 30 minutes of analysis TMZ

showed an initial burst followed by a gradual decrease in the concentration of TMZ which could be due to degradation of the drug at 37 °C.

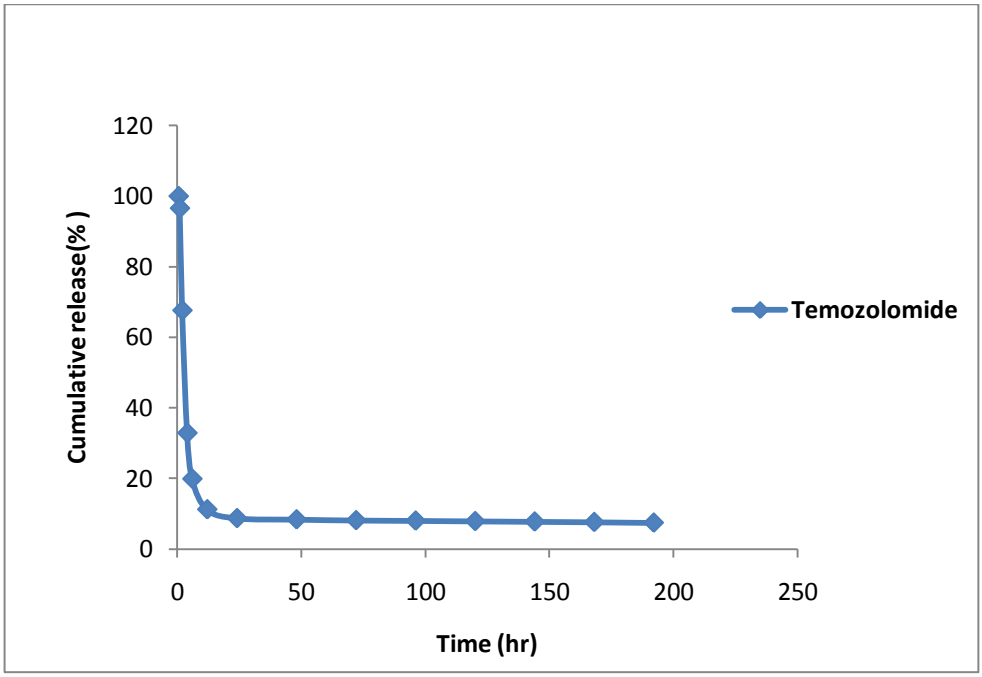


Figure 3.7 Release pattern of TMZ from PLGA microparticles. TMZ released from PLGA microparticles in PBS at pH 7.4 and at 37 ± 0.5 °C using the dispersion method.

3.6.1.2 Dispersion Method used for determining the release of Patrin-2 from microparticles.

The release profile of Patrin-2 from PLGA microparticles in PBS at pH 7.4 and at 37 ± 0.5 ℃. A plot of the percentage cumulative release of Patrin-2 against time (Fig. 3.7) was obtained. From the release profile, it was found that the release rate of Patrin-2 was slow with an initial burst of Patrin-2 released followed by an increase in peak area i.e. increase in concentration of Patrin-2. The cumulative mass of Patrin-2 was calculated the highest amount was equivalent to 196.16 µg as shown in Table 3.19, however the total amount of Patrin-2 theoretically present in TMZ loaded PLGA particles at the beginning of the in vitro release was 1500 µg. As degradation of the drug takes place are when kept at 37 ℃ could be the reason for not obtaining the expected amount.

Table 3.19 Patrin-2 released from PLGA microparticles was detected and from the peak area obtained the cumulative mass was calculated. Data value are the average of $n = 2$.

Time (hr)	MPA	Cumulative mass release (µg)
0.5	22.87	120.56
1	23.89	126.14
2	33.35	138.08
4	45.75	152.51
6	44.42	157.03
12	48.95	165.89
24	65.26	183.71
48	69.4	192.91
72	58.91	191.27
96	51.18	191.38
120	50	196.16
144	40.51	194.69
168	12.69	179.19
192	19.44	188.77
216	24.69	189.40
240	25.19	189.90
264	29.43	190.52
288	31.01	191.07
312	Not detected	Not detected

MPA-Mean Peak Area.

The percentage of cumulative release of Patrin-2 against time (Fig. 3.7) was plotted indicating the release pattern of Patrin-2. During the first 30 min of analysis Patrin-2

showed an initial burst followed by a slow increase in peak area i.e. increase in concentration of Patrin-2 which could be due to degradation of the drug at 37 °C.

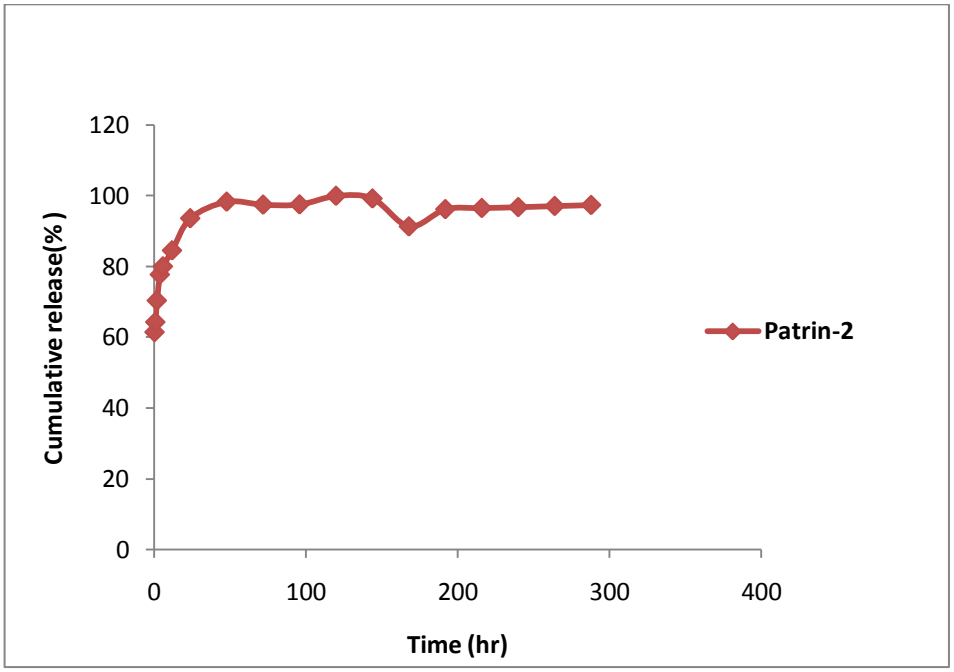


Figure 3.8 Release pattern of Patrin-2 from PLGA microparticles. Patrin-2 released from PLGA microparticles in PBS at pH 7.4 and at 37 ± 0.5 °C using dispersion method.

3.6.2 Dialysis Bag Method used for determining the release of TMZ and Patrin-2 from microparticles.

The release profiles of TMZ and Patrin-2 from PLGA microparticles in PBS at a pH 7.4 and at a temperature of 37 ± 0.5 °C. The results were analysed using HPLC. The peak area obtained for both TMZ and Patrin-2 was too low to carry out the cumulative amount released. The peak area of the control as well for both TMZ and Patrin-2 was found to be low.

CHAPTER 4

DISCUSSION, CONCLUSION

AND FUTURE PROSPECTS

4.1 Validation of HPLC system

A validation of the HPLC method was carried out following standard procedures using validation of compendial methods of USP. The mobile phase selection for TMZ was carried out by referring to a previously established method, i.e. 0.5 % acetic acid:methanol in the ratio of 9:1 (pH 2.8) while 35 % acetonitrile consisting of 10 mM TBAA, 10 mM SDS and 25 mM citric acid (pH 3.4) was found to be suitable selection of mobile phase for analysis of Patrin-2 (Moffat *et al.*, 2004; Shervington *et al.*, 2005). The retention time of TMZ was found to be 3.7 min while Patrin-2 the retention time was found to be 5.6 min. A calibration curve measuring the peak area against six concentrations for TMZ and Patrin-2 were found to be linear in the range investigated. The regression coefficient, R^2 values for TMZ and Patrin-2 were found to be 0.999 and 0.994, respectively. The SD and RSD indicated acceptable precision of the methods and the limit of detection (LOD) and limit of quantification (LOQ) were determined (Table 3.6). The LOD and LOQ concentration of TMZ and Patrin-2 was found to be the same which is possible as both the compounds are chromophores responsible for DNA cleavage (Shen *et al.*, 1995). Based on the data obtained from the validation procedures, the methods were found suitable for carrying out the experiments involving the preparations of liposome and microparticles.

4.2 Stability test of TMZ and Patrin-2:

An initial compatibility test was carried out to investigate whether TMZ and Patrin-2 could be formulated together. Thus, to ensure the stability of TMZ and Patrin-2, both the compounds were stored individually and as a mixture in PBS and ethanol (ratio of 8:2) at pH 7.4 at temperature 25, 37 and 50 °C. Rapid degradation of TMZ was observed at both 37 and 50 °C when stored for 72 hr, whereas Patrin-2 showed excessive degradation after storage at 50 °C for 72 hr. When in combination both TMZ and Patrin-2 showed early and rapid degradation at all three incubating temperatures at

a period of 72 hr. Powdered samples of TMZ and Patrin-2 were also stored at 50 °C for 1 week and then analysed after solubilising in PBS/ ethanol (8:2) at pH 7.4. Although there was a considerable quantity of both TMZ and Patrin-2 intact, there were several peaks observed that corresponded to degradation products (Table 3.10). Based on these observations, a personal communication and a previous report by Ranson and his team it was decided not to combine TMZ and Patrin-2 for preparing of either liposomes or PLGA microparticles (Ranson *et al.,* 2007). Ranson *et al* also reported that TMZ and Patrin-2 when given in combination to the patient results in increased incidence of haematological adverse events like thrombocytopenia and neutropenia (Ranson *et al.,* 2007).

4.3 Attempted preparation of various liposome formulation of TMZ using freeze-thaw and dehydration-rehydration method.

Different ratios of phospholipid individually as well as in combination with cholesterol were used to prepare TMZ loaded liposomes. It was observed that the EE % varied from one phospholipid to another, Ostro, 1983 also reported similar observations. The highest value of EE % calculated for this method was found when phospholipon 90H and cholesterol were used, while the lowest EE % was estabilished when liposomes were prepared with DMPC. The differences may be due to the variation in the phase transition temperatures and in the acyl chain length (Anderson and Omri, 2004). As shown in Fig. 1.13 phospholipon 90H has longer acyl chain and higher phase transition temperature (54 °C) compared to DMPC (Fig. 1.11b). Phospholipon 90H was comparatively better than DMPC, however, when phospholipon 90H was used alone there was no improvement in the EE %, and hence was combined with cholesterol (shown in Fig. 3.2). The maximum EE % achieved using this method was still rather low, with a maximum value of 11.54 ± 0.67 %. This may have been attributed to the

low affinity and solubility of TMZ with the lipid which constitutes the liposomal membrane increasing the chances of TMZ being entrapped in the aqueous compartment, thus, less amount of medium entrapped gives lower EE % (Vadiei *et al.,* 1989; Gulati *et al.,* 1998). Hence, the use of cholesterol increases the EE % as it makes the membrane more rigid and reduces the drug from escaping (Liu et al., 2002).

Similar to the freeze-thaw method different ratios of phospholipid were used in addition to combinations with cholesterol/stearic acid to prepare TMZ loaded liposomes using dehydration-rehydration method. The highest of EE % obtained was when TMZ: phospholipion 90H: cholesterol in a ratio 1:2:2 was used achieving 26.69 ± 0.34 %. When TMZ and phospholipon 90H was used, the EE % was very low. Cholesterol was included in the formulation in order to increase the strength of the membrane. With the inclusion of stearic acid there was no significant improvement in the EE % (Gomez-hens and Fernandez- Romero, 2005). Thus showing cholesterol to be more suitable for preparing TMZ loaded liposomes. Although the EE % achieved with phospholipon 90H and cholesterol was not high, this may have been attributed to the low affinity and solubility of TMZ with the lipid (Vadiei *et al.,* 1989; Gulati *et al.,* 1998).

On comparing the EE % between freeze-thaw and dehydration-rehydration methods using the same concentration of phospholipids and TMZ, the EE % achieved by the dehydration-rehydration method was up to twice that of the freeze-thaw method (Refer to result section: 3.3.3). This could have been attributed to the dehydration-rehydration method involving the simultaneous trapping of most of the components. The membrane boundaries of the vesicles are reported to protect the encapsulated material under extreme conditions (Monnard *et al.,* 1997). Important features of the two methods in addition to significant entrapment of the active compound is the broad range of

biologically active agents that can be used and liposomes prepared present stable solute retention characteristics. These two methods are relatively simple and do not require use of organic solvents or detergents (Ostro, 1983l; Mayer *et al.*, 1986). In this study different ratios of phospholipid were used individually as well as in combination with cholesterol/stearic acid were used in both the methods. However, a combination of phospholipon 90H together with cholesterol showed maximum entrapment in both methods. In the freeze-thaw method there was a significant increase (*, $P<0.005$) in the EE % when a combination of TMZ, phospholipon 90H and cholesterol were used at ratios of 1:2:2, 1:3:1 and 1:3:3 (Refer to result section: 3.3.3). When only TMZ and phospholipon 90H were used at ratios of 1:1, 1:2 and 1:3 low entrapment of TMZ was achieved as shown in Fig 3.2 which reinforced the importance of the use of cholesterol. In dehydration-rehydration method using similar combinations to the freeze-thaw method the combination of phospholipon 90H and cholesterol showed maximum EE %. As shown in the Fig. 3.2 there is a significant increase (**, $P<0.001$) in the EE % at 1:2:2 ratio, while the 1:3:1 and 1:3:2 ratios showed less significant increase (*, $P< 0.005$) in the EE % when compared to other ratios such as 1:1:1 and 1:2:1 as well as with ratios of phospholipon 90H alone with TMZ i.e. 1:1, 1:2 and 1:3. In comparing the EE % shown in table 3.13 and 3.14, a combination of the ratios of phospholipon 90H alone with TMZ is considerably low compared to the combination of phospholipon90H and cholesterol with TMZ (Ostro, 1983; Gomez-hens and Fernandez- Romero, 2005). Similar to the freeze-thaw method, it justifies that the use of cholesterol supports the increase of EE % of TMZ and also according to the results obtained as mentioned in table 3.14 and 3.15, cholesterol is useful in increasing the rigidity of the membrane (Bangham *et al.*, 1965; Ostro, 1983). It was found that the dehydration-rehydration method was found to be more suitable for encapsulating TMZ when compared to freeze-thaw method. From the results obtained it was found that the

phospholipids and the methods used were not useful for the encapsulation of TMZ into liposomes which might be due to TMZ's high solubility in aqueous medium and low affinity with the lipids. Therefore, the investigation was carried out using polymer PLGA for preparation of microparticles which are also reported previously to carry more advantages over liposomes (Kreuter, 1991). The body distribution of the carriers can be altered by modification of their surface properties, especially the surface properties of the microparticles are easily changed by simple coating of the particles with certain biocompatible surfactants (Bertling *et al.*, 1991).

4.4 Preparation of PLGA microparticles using emulsifying-solvent evaporation and spray dry methods.

Two methods were used for preparing TMZ loaded PLGA microparticles, one i.e. emulsifying solvent evaporation method, while the other, the spray dry method. The main aim of this study was to find a suitable method to encapsulate TMZ into microparticles and then to combine TMZ with Patrin-2. Because of the mode of action of a pseudo substrate i.e. Patrin-2 combining with TMZ would be suitable way of enhancing the effect of TMZ, however due to the incompatibility of these two compounds described in section 4.2 the strategy was abandoned, microparticles of TMZ and Patrin-2 were prepared separately using the spray dry method which gave higher EE % than the emulsifying solvent evaporation method. The spray dry method, compared with the emulsifying solvent evaporation method, produced more regular shape microparticles with a relatively small size distribution, similar observations were also reported by Pavanetto *et al.*, 1992.

TMZ loaded PLGA microparticles were prepared using different MW of PLGA which included $1.7x10^4$, $1.5x10^4$ - $4x10^4$, $4x10^4$ - $7.5x10^4$ and $6.6x10^4$ - $1x10^5$ using emulsifying solvent evaporation method. Though the EE % obtained was very low to be

considered, PLGA with MW of $1.7x10^4$ showed EE % of only 2.76 ± 0.36 % which was found to be comparatively higher than other PLGA of different MW as shown in table 3.16. As reported in previous studies, preparation variables can be responsible for giving poor EE % such as the selection of the organic solvents or stirring rate of the emulsion or sometimes the organic phase acts as a barrier between the internal aqueous phase and the continuous aqueous phase (McGinity and O'Donnell, 1997). Due to high solubility of some drug in the aqueous medium prevents the preparation of the saturated solution in the organic solvents (Herrmann and Bodmeier, 1995). As TMZ is highly soluble in aqueous medium these might be the reason responsible for obtaining the low entrapment efficiency.

TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles were prepared by varying the MW of PLGA using the spray dry method, to determine their effect on EE % of TMZ. Different molecular weights of PLGA used in the study included $1.7x10^4$, $1.5x10^4$ – $4x10^4$, $4x10^4$ – $7.5x10^4$ and $6.6x10^4$ – $1x10^5$. The maximum entrapment achieved using this method for TMZ was 64.32 ± 2.58 % while for Patrin-2 the EE % was 68.47 ± 1.47 % thus, this procedure was found to be suitable for the preparation of both TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles. On increasing the MW of PLGA i.e. MW in range of $1.5x10^4$ - $4x10^4$, $4x10^4$ - $7.5x10^4$ and $6.6x10^4$ - $1x10^5$, a significant decrease (*, $P< 0.001$) in the EE % of both TMZ and Patrin-2 is obtained. Previous studies have found that a decrease in the EE % could be attributed either to thermal degradation of the compound or the accumulation of the active compound on the surface of PLGA microparticles (Sacchetti and Van-Oort, 1996; Seong *et al.,* 2003). The main purpose of using the spray dry method was to achieve a molecular dispersion of the compound in the final product (Takashima *et al.,* 2007; Lee *et al.,* 2008). This method was found to be more effective

in producing microspheres with high EE % (Sacchetti and Van-Oort, 1996). As reported by previous studies high entrapment efficiency is typical using the spray dry method because the drug cannot partition into an external phase as compared to the emulsifying-solvent evaporation method (O'Hara and Hickey, 2000).

4.5 Particle size analysis of liposomes and microparticles.

Phospholipids when dispersed in an aqueous medium instantaneously form spherical structures called as vesicles or liposomes, having sizes ranging from that of bacterial cells first established by Bangham and co-workers (Bangham *et al.,* 1965). The average particle size obtained in this study varied depending on the type of lipids, the concentration of the lipids and the EE % or the type of liposome formed. Depending upon the liposome formed, as the EE % increases the average particle size increases (Table 3.12 and 3.14 and Chou *et al.,* 2003). In the freeze-thaw and dehydration-rehydration the particle sizes tends to increase slightly on addition of cholesterol.

The SEM technique was applied for measuring microparticles prepared by emulsifying-solvent evaporation and the spray dry method. The maximum entrapment of TMZ loaded PLGA microparticles and Patrin-2 loaded PLGA microparticles were achieved using the spray dry method with PLGA of MW equal to $1.7x10^4$. The morphology of the microparticles from the spray dry method was found to be spherical and smooth, however small pores were observed on the surface of TMZ loaded microparticles as shown in Fig. 3.5 (b). The microparticles prepared with PLGA of higher MW were aggregated and lost the spherical shape, which is in agreement with the literature that states that microparticle produced with high molecular weight PLGA tend to lose the spherical shape and form fibrous particles (Seong *et al.,* 2003; Lee *et al.,* 2008). No morphological difference was observed between the microparticles of TMZ and Patrin-2. There were a few large particles, which lost their spherical shape. It may have been

that as indicated in a recent study that during spraying of the solution some droplets may have slightly different sizes and the particles formed from such droplets could shrink due to evaporation of the solvent during the drying process (Pamujula *et al.*, 2004). However, the particle size distribution of the microparticles of both compounds was reproducible (Wang and Wang, 2003).

4.6 Dissolution techniques

The dispersion and dialysis methods were used to monitor the release of TMZ and Patrin-2 from the PLGA microparticles and to compare the *in vitro* release of TMZ and Patrin-2 (Shazly *et al.*, 2008). An *in vitro* release profile can reveal basic information on the structural properties of the formulation as well as on possible interactions between drug and polymer and their effectiveness on the rate and mechanism of drug release (Washington, 1990). Such information is important in facilitating a scientific approach to the design and development of sustained drug delivery systems with specific properties (D'Souza and DeLuca, 2006).

4.6.1. Dispersion Method used for determination of release of TMZ and Patrin-2 from the PLGA microparticles.

The release profiles of both TMZ and Patrin-2 from PLGA microparticles were monitored in to PBS, pH 7.4 at 37 ± 0.5 °C was carried out. From the release profiles it was observed that for TMZ loaded PLGA microparticles, there was an initial burst release of TMZ during the first six hours, followed by a decrease in the release of TMZ (Fig. 3.6). As observed from % cumulative release, 100 % release of TMZ was observed at 0.5 hr which is 407.06 µg (Refer table 3.18). However, the total amount of TMZ theoretically present in TMZ loaded PLGA microparticles at the beginning of the *in vitro* release was 1200 µg (Refer to EE % in table 3.17). Similarly from the release profile of Patrin-2 loaded PLGA microparticles, an initial burst release of Patrin-2 was

observed followed by a small amount of release of Patrin-2 (Fig. 3.7).The % cumulative release of Patrin-2 was calculated to be 100 % at 120 hr which is 196.16 µg in concentration (Table 3.19) however, the total amount of Patrin-2 release theoretically should have been 1500 µg (Refer to EE % in table 3.17). The initial burst release of TMZ and Patrin-2 might be due to diffusion of drug particles present on the surface of microparticles as well as the release of the encapsulated (Bodmeier and Chen, 1988; Shazly *et al.,* 2008). Also the possible reason for the results obtained could be the degradation products formed of both the compounds at 37 °C due to which the expected amount of the drugs was not possible to calculate.

As mentioned in section 1.5.4 the release of the drug from the polymer occurs in different phases, rapid release of the drug sometimes occurs due to pore formation, if the drug is bonded weakly to the polymer surface it is released with an initial burst. Initial burst release phase also occurs sometimes due to bulk degradation of the polymer (Allemann et al., 1993; Polakovic *et al.,* 1999; Panyam *et al.,* 2004; Liu *et al.,* 2005; Tamber *et al.,* 2005). If the bulk degradation of the polymer is made to exhibit surface degradation or layer-by-layer degradation, the polymer could present a promising control release of the drug also termed as sustained release (Conte *et al.,* 1993; Abdul and Poddar, 2004; Chen *et al.,* 2005; Loo *et al.,* 2005; Frank *et al.,* 2005; Loo *et al.,* 2010). The fast release rate of TMZ could be due to its high affinity for water, using a more hydrophobic polymer PLGA 85:15 might support a much slower release rate than PLGA 75:25 used in this study (Lee *et al.,* 2008). In previous studies it has been proved that the use of different monomer ratios of copolymer have helped to alter the *in vitro* release of the drug (Zhao *et al.,* 2010). Other factors such as pH of the medium and porous surface might be responsible for the initial rapid release of TMZ and Patrin-2. The pH of the dissolution medium affects the drug significantly and may

be responsible for the rapid burst effect (O'Hara and Hickey, 2000). The differences in particle shape can also be a contributing factor to the high initial release of the spray dried particles (Pavanetto *et al.,* 1992; Lalla and Sapna, 1993). In the previous studies, it has been demonstrated that particle porosity was found to be a greater determinant of total surface area than gross morphology (Pavanetto *et al.,* 1992). One of the reasons for a decrease in concentration i.e. when TMZ was not been detected could be due to the rapid degradation of TMZ in PBS at pH 7.4. From our stability studies in section 4.2 and data from others, it might be difficult to monitor the total amount of drug being released when degradation takes place (Wagenaar and Muller, 1994; Zhang and Gao, 2007). This theory could apply to both TMZ and Patrin-2. However, sometimes the release pattern of the drug is also dependent not only on the diffusion of the drug through the matrix of the polymer but also on the degradation rate of the polymer (Schwendeman *et al.,* 1996). For Patrin-2 an initial burst release with a continuous slow release was observed, again this could be either due to degradation of the compound in PBS at pH 7.4 or could be that the compound was in the lag phase and may be the monitoring should have been conducted for a longer period of time (Loo *et al.,* 2010). A difference in the release pattern could have occurred due to early matrix erosion of the polymer, however, human error has not been ruled out (Spenlehauer *et al.,* 1989; O'Hara and Hickey, 2000). The stage when there is no drug released is termed the lag phase where the drug is not diffusing out of the polymer and can be overcome by treating the sample with radiation (e-beam) which modifies the physical properties of the polymer and thereby reduces the lag phase (Miao *et al.,* 2009; Loo *et al.,* 2010).

4.6.2 Dialysis Bag Method used for determination of release of TMZ and Patrin-2 from the PLGA microparticles.

The release profiles of TMZ and Patrin-2 from PLGA microparticles in to PBS, pH 7.4 at 37 ± 0.5 °C was carried out. At predetermined time intervals (0.5, 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312 and 336 hr) an aliquot of 1 ml of the dispersion was withdrawn and replaced with 1 ml of PBS. The results were analysed using HPLC, however, the peak area obtained was too low to carry out the cumulative amount release calculation. The result obtained from the control was also too low. In the dialysis method the loaded microparticles were separated from the dissolution media by the dialysis membrane. The passage of the compound occurs through the membrane into the dissolution media and the sample is withdrawn for analysis (Berthold *et al.,* 1996). The low concentration of the compound measured could have been due to the type of dialysis bag used was not suitably matched for the compounds for this reason sometimes compound takes longer time to diffuse out of the membrane (Larsen *et al.,* 2000). This draw back could possibly be overcome by using varying MW exclusion cutoffs dialysis bags in the future studies to determine a suitable MW cut off range of the dialysis bag (Park *et al.,* 1995). Some studies have reported that low concentration of drugs released could be attributed to equilibration with the outer dissolution media being slow because of the small membrane surface area available for the drug to transport (D'Souza and DeLuca, 2005). Slow equilibration causes limitations in accurate analysis of initial drug levels being released (Diaz *et al.,* 1999). These disadvantages could be overcome by using a commercial dialysis setup which has a large surface area for facilitation of drug transport (D'Souza and DeLuca, 2005). *In vitro* analysis revealed that slower rates and incomplete drug release is sometimes observed when using a membrane system for dissolution tests (Gido *et al.,* 1993). Previously studies have been undertaken to reduce the time span of the *in vitro* release

experiments, certain factors were taken into consideration for this purpose like addition of preservatives and impose certain limitations on the method such as compatibility of the constituents of the release device like membrane and its stability (Burgess *et al.,* 2002). Thus short term release experiments might be more reliable for quality analysis purposes. Some alterations in the conditions might help in accelerating the release of the compound from the membrane such conditions include elevated temperature, altering pH and use of surfactants (D'Souza and DeLuca, 2006).

In comparison of the dissolution techniques based on the results obtained, the dispersion method proved to be better than the dialysis method, similarly in previous literature dispersion method was found to be better than dialysis method, however as both the methods were not completely explored and due to degradation of the drugs taking place it is not concluded as which method would have been better (Shazly *et al.,* 2008). The reasons for obtaining such lower values in the dialysis technique consists of certain limitations such as the outer media is stirred, however, the media inside the dialysis bag is not stirred accurately and sometimes it is subjected to accumulation of the polymer or the drug on the surface of the dialysis bag making the release process slower (Park *et al.,* 1995). An additional limitation in this procedure of drug release is when the drug strongly binds to the polymer or membrane, however, this can be all evicted to some degree by sampling and buffer replacement (Kinget *et al.,* 1979).

CONCLUSION

Validated HPLC methods were used for the analysis of both TMZ and Patrin-2. TMZ is known to be effective in patients that lack MGMT activity since MGMT counteracts the effect of TMZ by removing the methyl group (originally added by temozolomide). Patrin-2 is used to counteract the effect of MGMT and thus enhances the activity of TMZ. Stability tests were carried out to determine whether or not TMZ and Patrin-2 could be combined, however, degradation was observed on analysis of the combined mixture and therefore the work involved formulating these two actives in drug delivery systems separately and not in combination.

TMZ loaded liposomes were prepared using different ratios of phospholipid individually and in combination with cholesterol/stearic acid. A combination of phospholipon 90H and cholesterol at a ratio of 1:2:2 was found to be more effective in encapsulating TMZ as compared to other phospholipids such as lα-phosphatidylcholine, DSPC, DMPC and lecithin using freeze-thaw and dehydration-rehydration methods. It was found that the EE % varied depending on the type of lipid used. From the particle size analysis, it was noted that with an increase in concentration of certain lipids with the addition cholesterol, a slight increase in the average particle size was observed in both methods. Of the two methods used in the study involving the encapsulation of TMZ into liposomes the dehydration-rehydration method was found to be favoured since a significant increase in encapsulated ratio of 1:2:2 occurred when was used with TMZ, phospholipon 90H and cholesterol.

Two methods were used for preparing TMZ loaded PLGA microparticles namely emulsifying solvent evaporation and spray dry methods. The EE % obtained for the emulsifying solvent evaporation method was found to be too low to be considered, however, the spray dry method gave a significantly high EE %. The spray dry method used for the preparation of Patrin-2 loaded PLGA microparticles also resulted in a

significantly high EE %. The SEM of the microparticles showed that the microparticles were smooth and spherical, however some TMZ loaded microparticles showed pore formation. From the findings it can be concluded that the lower the MW of PLGA used the higher the EE % in both methods. From the results one can conclude that, TMZ and Partin-2 were successfully encapsulated into PLGA microparticles using the spray dry method.

To study the release profile of TMZ and Patrin-2 from the PLGA microparticles two methods were carried out namely the dispersion and dialysis bag diffusion method. The dispersion method was found to be more efficient and reliable, however as both the methods were not completely explored it is not concluded as which method would have been better. TMZ and Patrin-2 showed an initial burst release, however TMZ later showed a decrease in concentration over a period of time while Patrin-2 showed a slow release with a steady increase in concentration. Both compounds did not show complete release from the microparticle in expected concentration which could be due to degradation of the drug taking place at the set temperature and pH, however future work could focus on improving the release of these compounds by altering the hydrophobic nature of the PLGA by varying the copolymer monomer ratio.

FUTURE PROSPECTS

Based on the results obtained from the current research, the spray dry method was found to give the highest EE % for TMZ and Partin-2, however in order to achieve a higher EE % different solvent systems such as dichloromethane or chloroform could be investigated or the calculations of determining the EE% should be altered which would give higher encapsulation i.e. the amount of TMZ obtained from the microparticles using the HPLC should be compared with the amount of TMZ that could be theoretically present in the same amount of microparticles weighed out. Different monomer ratios of copolymer of PLGA consisting of greater hydrophobic polymers such as PLGA 85:15 of different molecular weights as well as PLGA polymers of MW less than $1.7x10^4$ Da could be investigated. The use of hydrophobic PLGA polymers may also enhance release profiles of TMZ and Patrin-2. Lower MW PLGA could be used to accelerate the release pattern of Patrin-2. The pH of the dissolution medium affects the drug significantly and is responsible for the burst effect. Altering the pH and the use of surfactants could also be included in a study. For the dialysis bag diffusion technique, different dialysis bags with varying MW's could be investigated to find which would be most suitable in assisting the release of TMZ and Patrin-2. The microparticles could also be treated with radiation (e-beam) for faster release and modification in the technique by increasing the surface area of the dialysis bag available for the drug to diffuse out of the membrane might also enhance the release pattern of the drug.

The viabilities of treated and untreated glioma cells could be determined using the standard MTT and ATP assays. To evaluate the cytotoxicity of TMZ loaded liposomes, TMZ and Patrin-2 loaded microparticles should be tested on glioma cell lines and their effect should be compared with the cytotoxicity of TMZ and Patrin-2 applied

separately. The effect of PLGA microparticles should also be tested on the glioma cell lines.

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APPENDIX

Appendix 1

Validation of HPLC system for Temozolomide (TMZ)

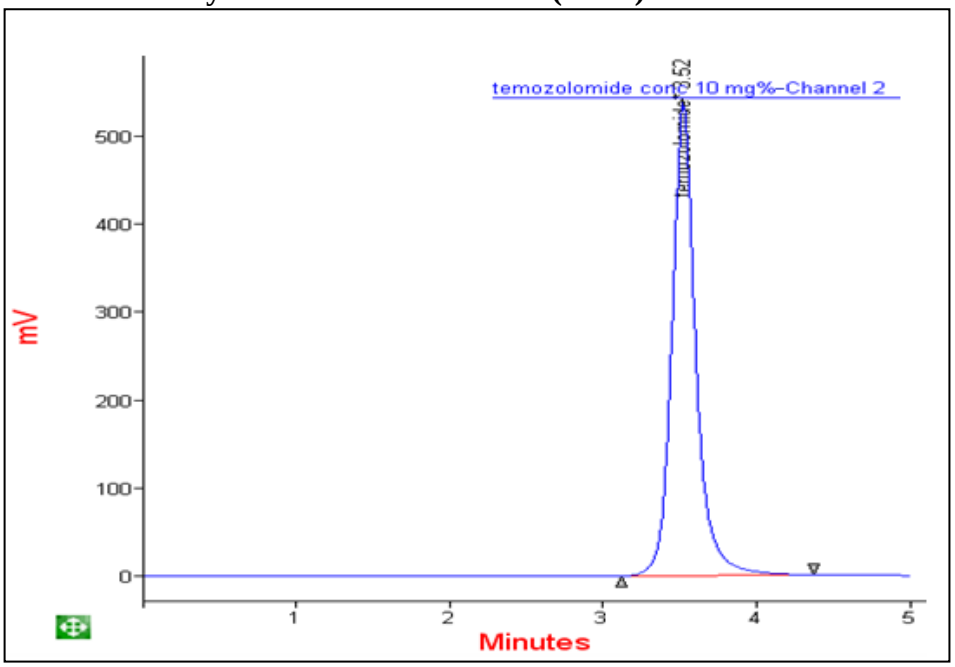

Figure 6.1 HPLC chromatogram for TMZ (0.1 mg/ml) (*n* = 3).

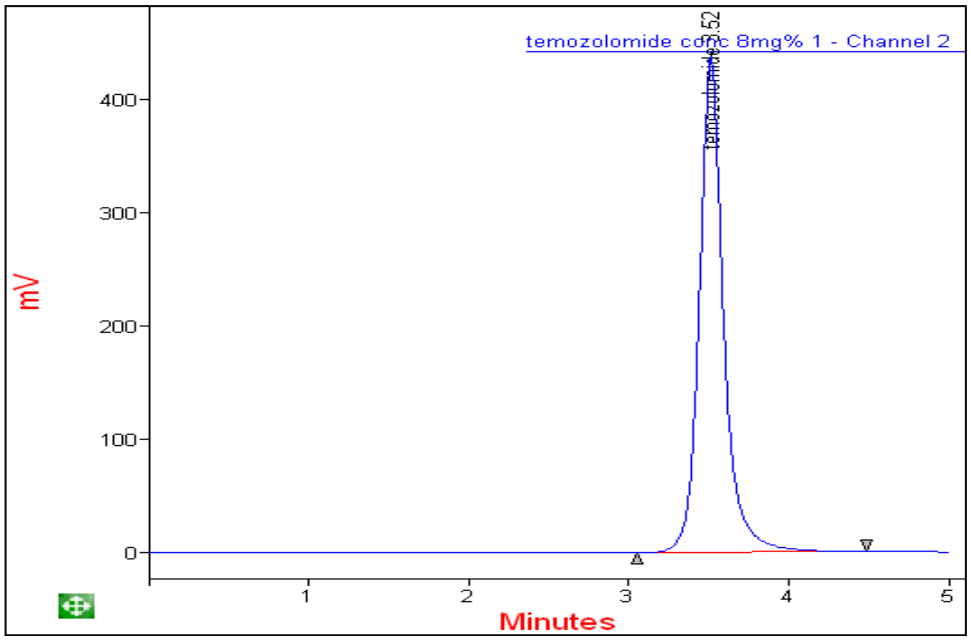

Figure 6.2 HPLC chromatogram for TMZ (0.08 mg/ml) (*n* = 3).

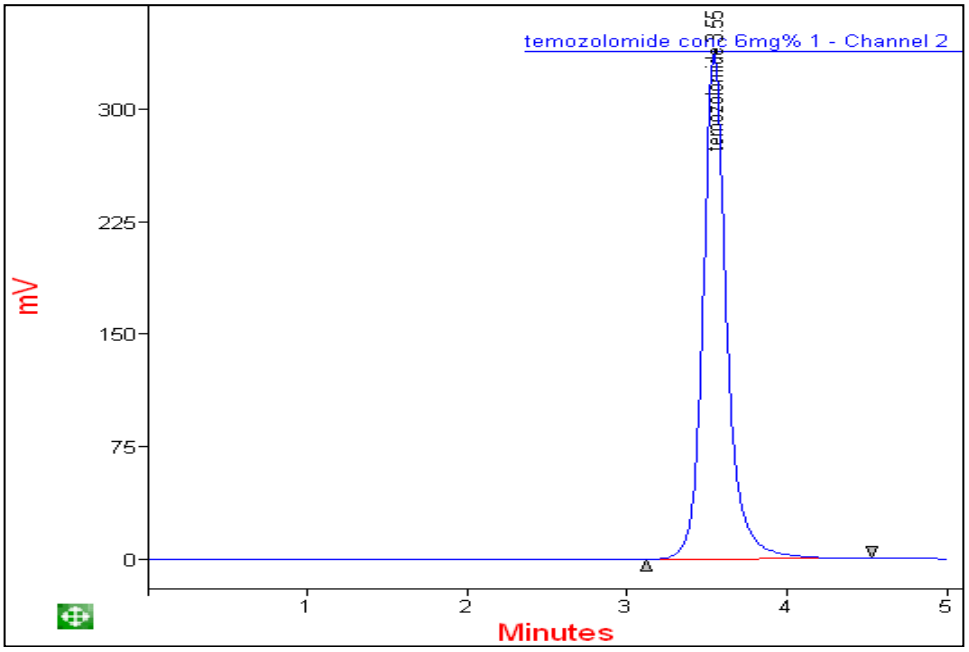

Figure 6.3 HPLC chromatogram for TMZ (0.06 mg/ml) (*n* = 3).

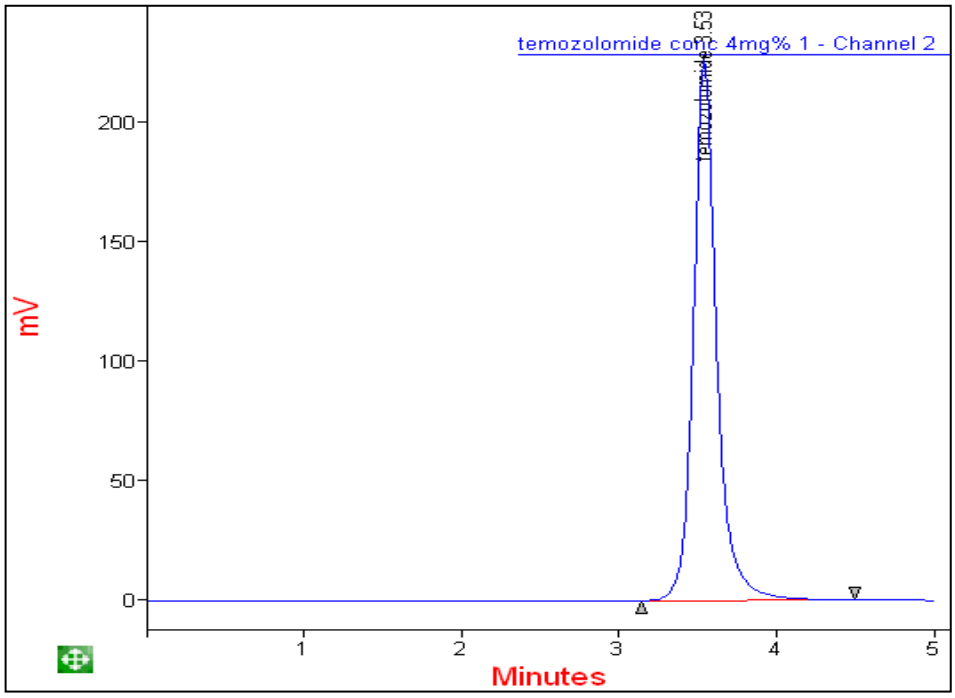

Figure 6.4 HPLC chromatogram for TMZ (0.04 mg/ml) (*n* = 3).

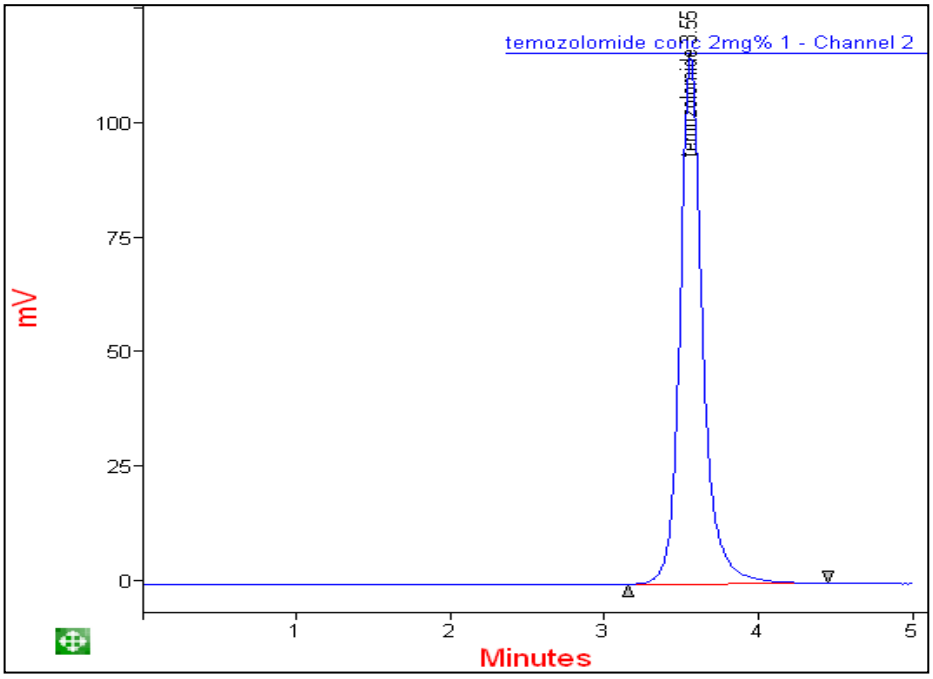

Figure 6.5 HPLC chromatogram for TMZ (0.02 mg/ml) (*n* = 3).

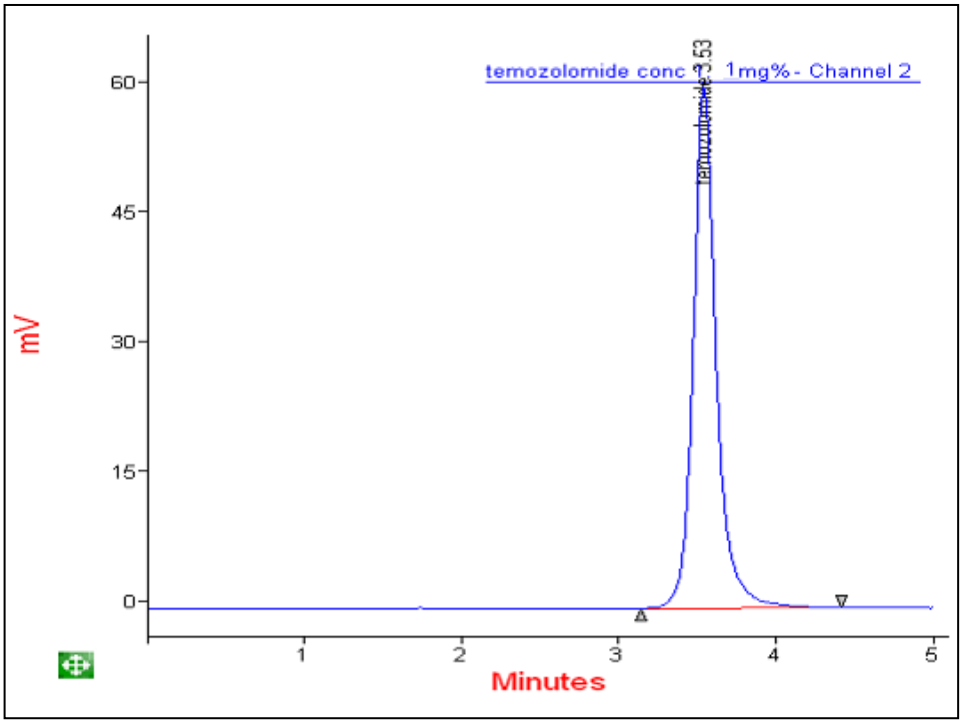

Figure 6.6 HPLC chromatogram for TMZ (0.01 mg/ml) (*n* = 3).

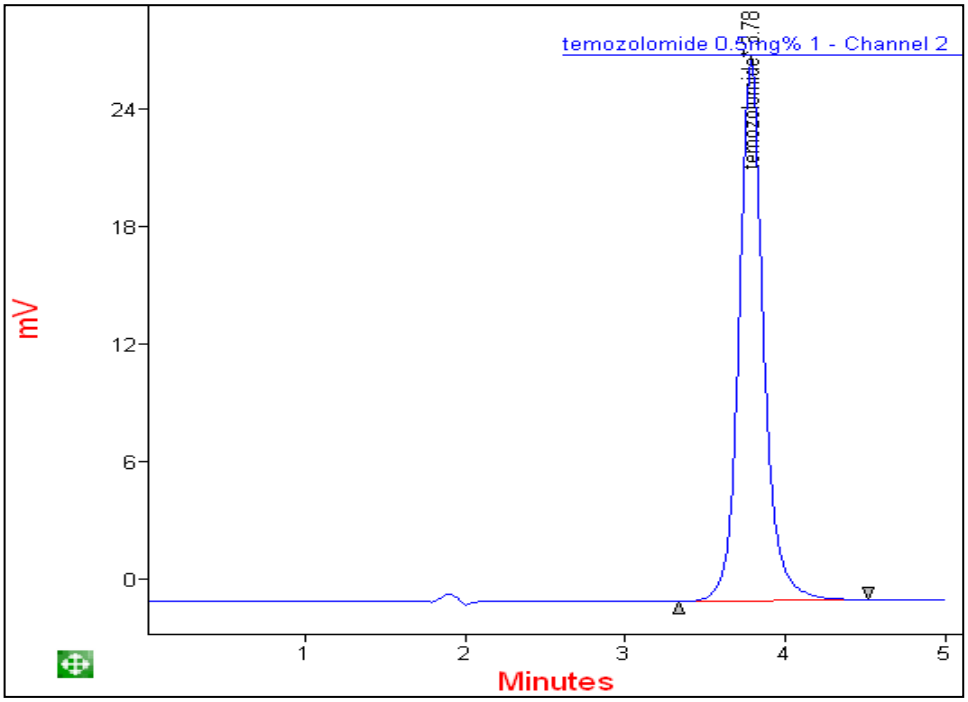

Figure 6.7 HPLC chromatogram for TMZ (0.005 mg/ml) (*n* = 3).

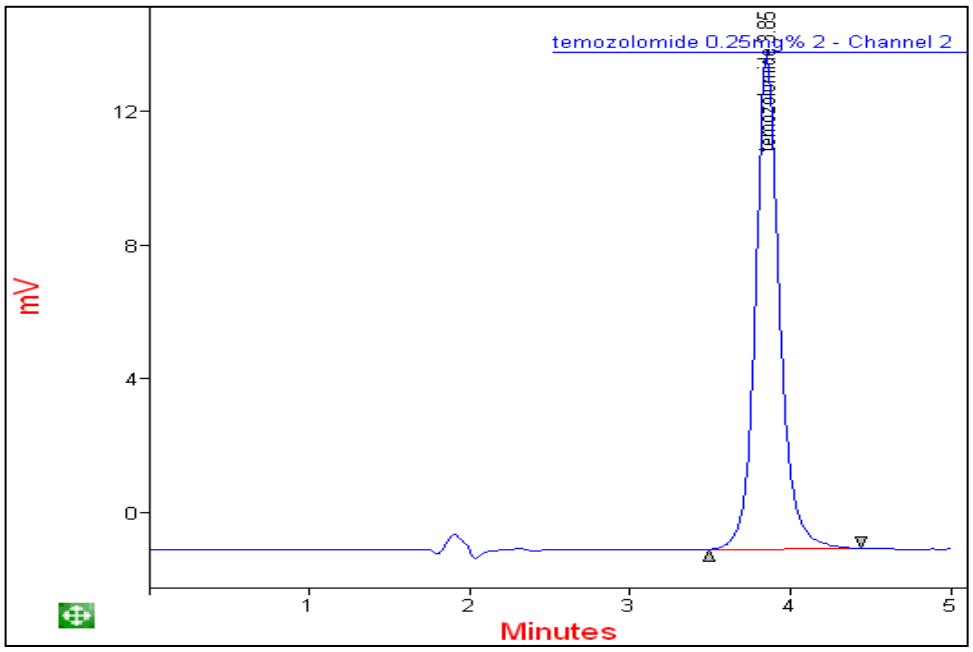

Figure 6.8 HPLC chromatogram for TMZ (0.0025 mg/ml) (*n* = 3).

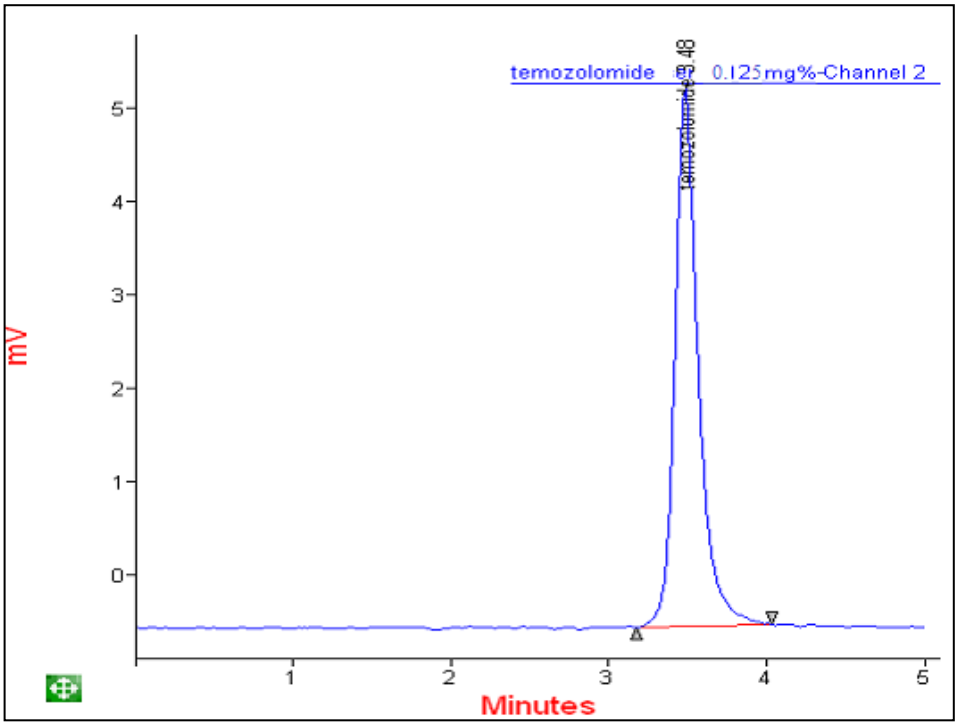

Figure 6.9 HPLC chromatogram for TMZ (0.00125 mg/ml) (*n* = 3).

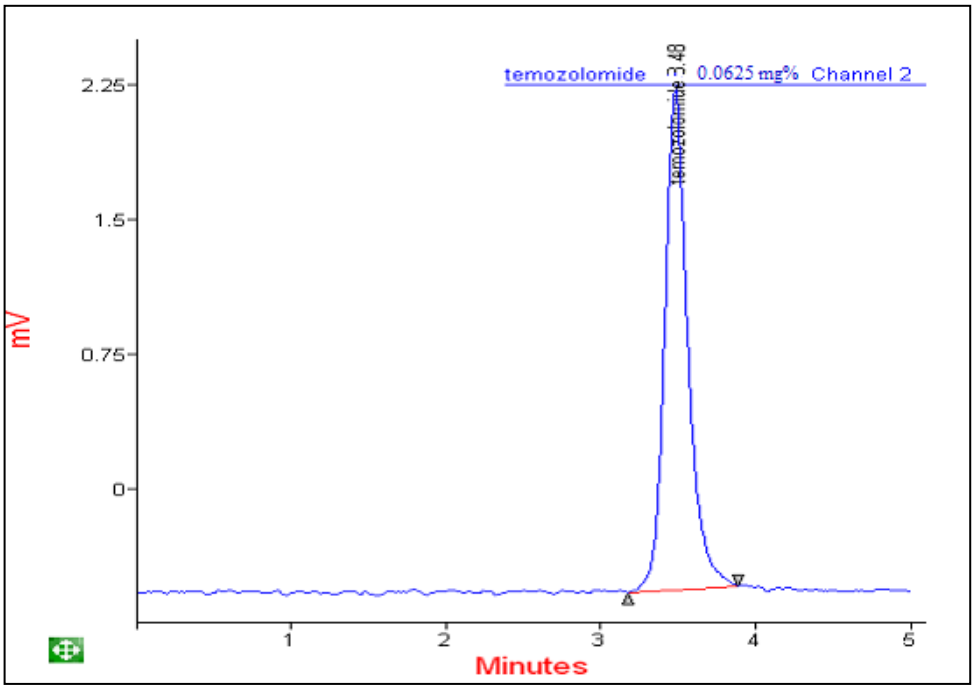

Figure 6.10 HPLC chromatogram for TMZ (0.000625 mg/ml) (*n* = 3).

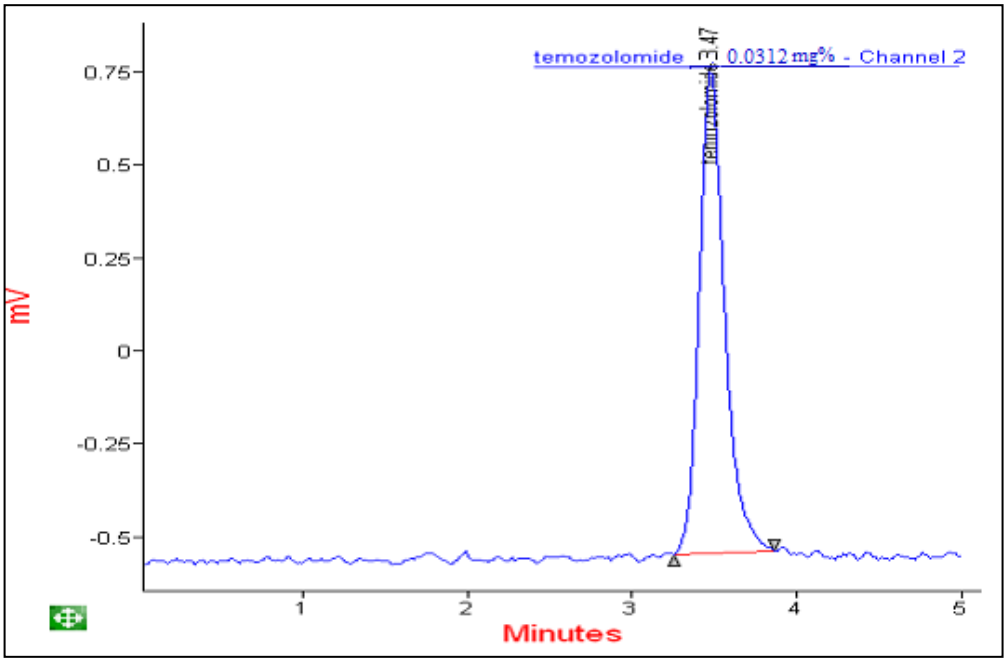

Figure 6.11 HPLC chromatogram for TMZ (0.000312 mg/ml) (*n* = 3).

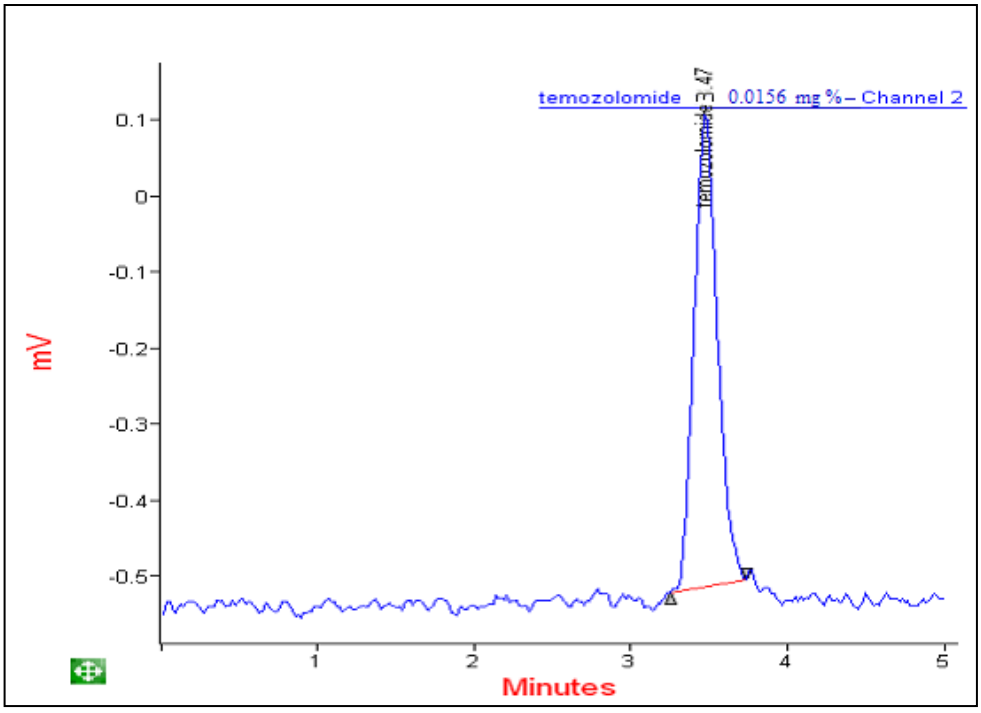

Figure 6.12 HPLC chromatogram for TMZ (0.000156 mg/ml) (*n* = 3).

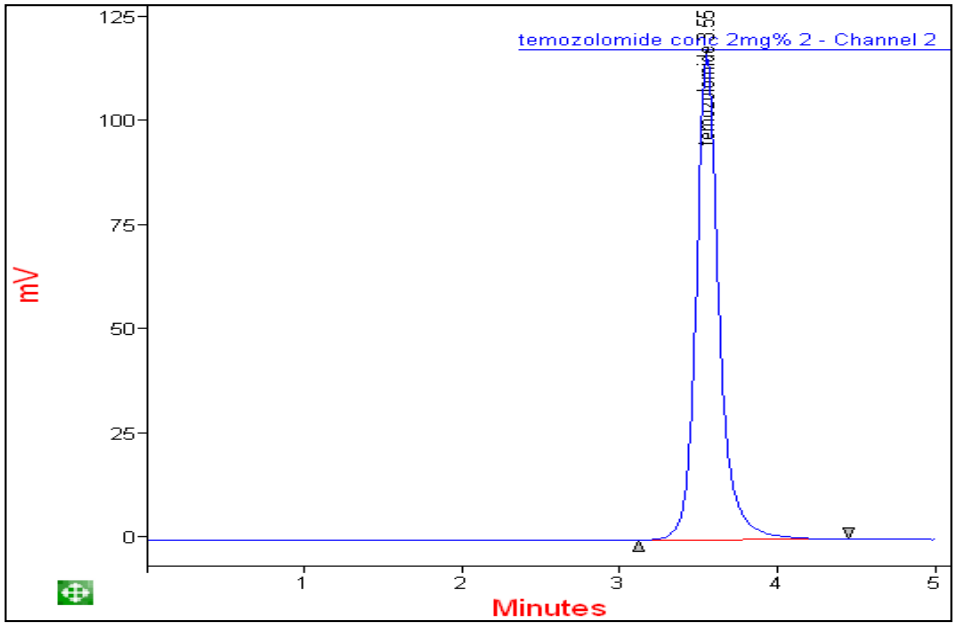

Figure 6.13 HPLC chromatogram for reproducibility of TMZ (0.02 mg/ml) (*n*=6).

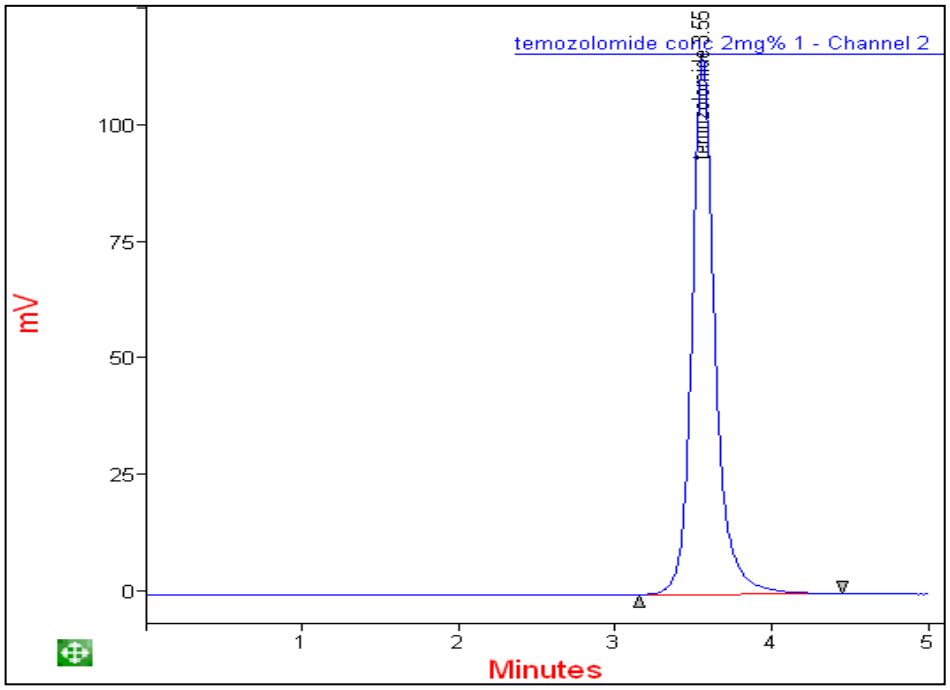

Figure 6.14 HPLC chromatogram for repeatability of TMZ (0.02 mg/ml) (*n*=6).

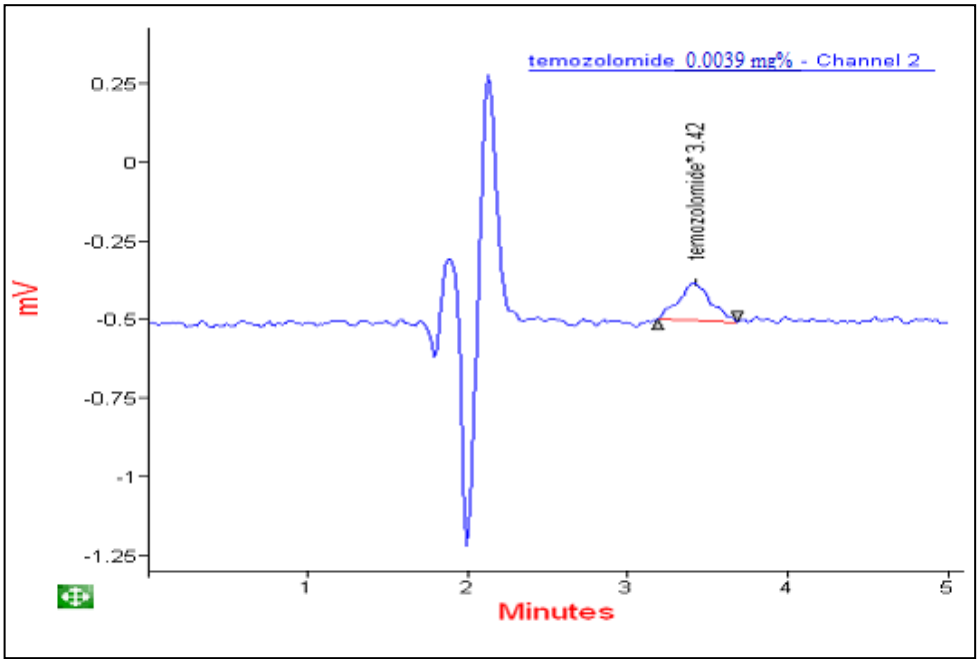

Figure 6.16 HPLC chromatogram showing LOD for TMZ (0.000039 mg/ml) (*n* = 3).

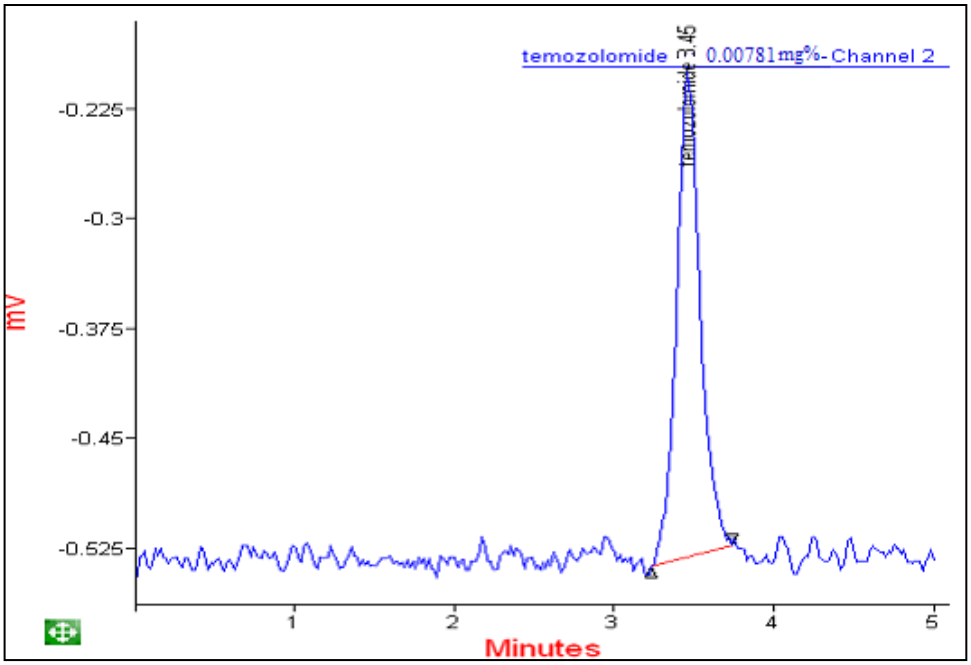

Figure 6.15 HPLC chromatogram showing LOQ for TMZ (0.0000781 mg/ml) (*n*=6).

Appendix 2

Validation of HPLC system for Patrin-2

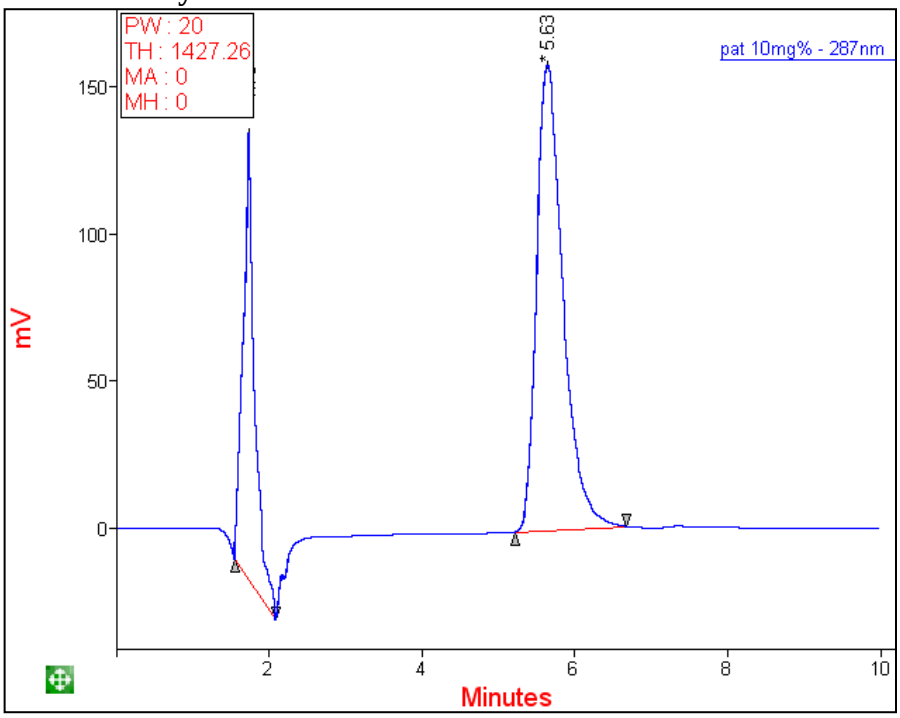

Figure 6.17 HPLC chromatogram for Patrin-2 (0.1 mg/ml) (*n* = 3).

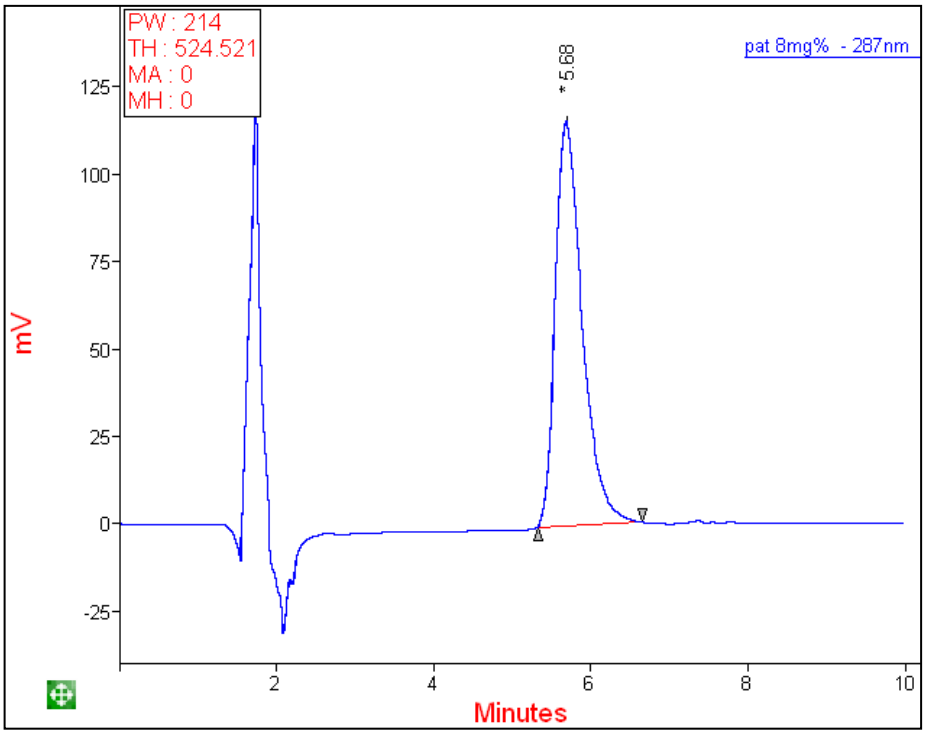

Figure 6.18 HPLC chromatogram for Patrin-2 (0.08 mg/ml) (*n* = 3).

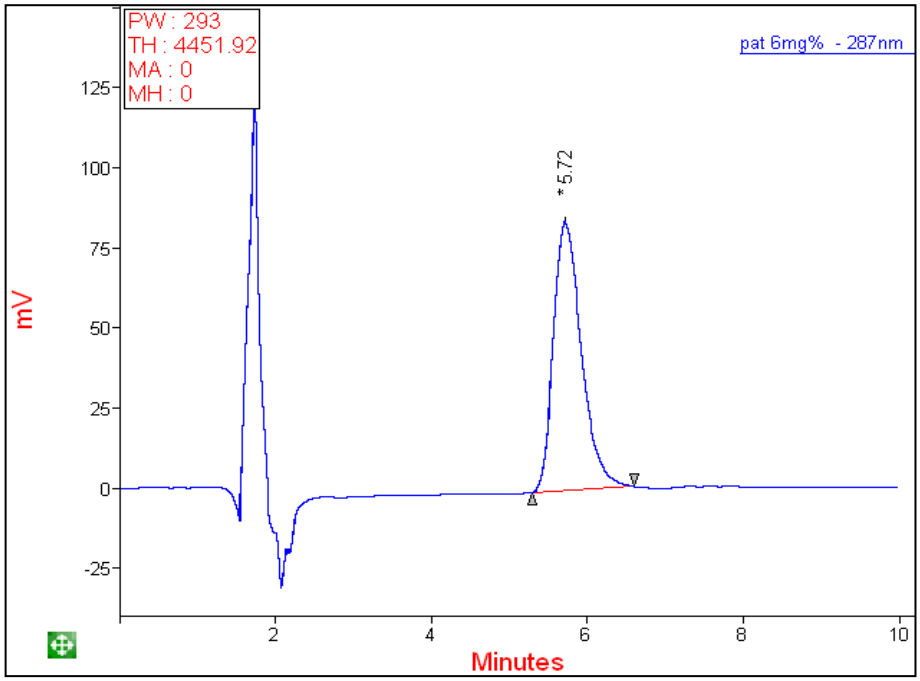

Figure 6.19 HPLC chromatogram for Patrin-2 (0.06 mg/ml) (*n* = 3).

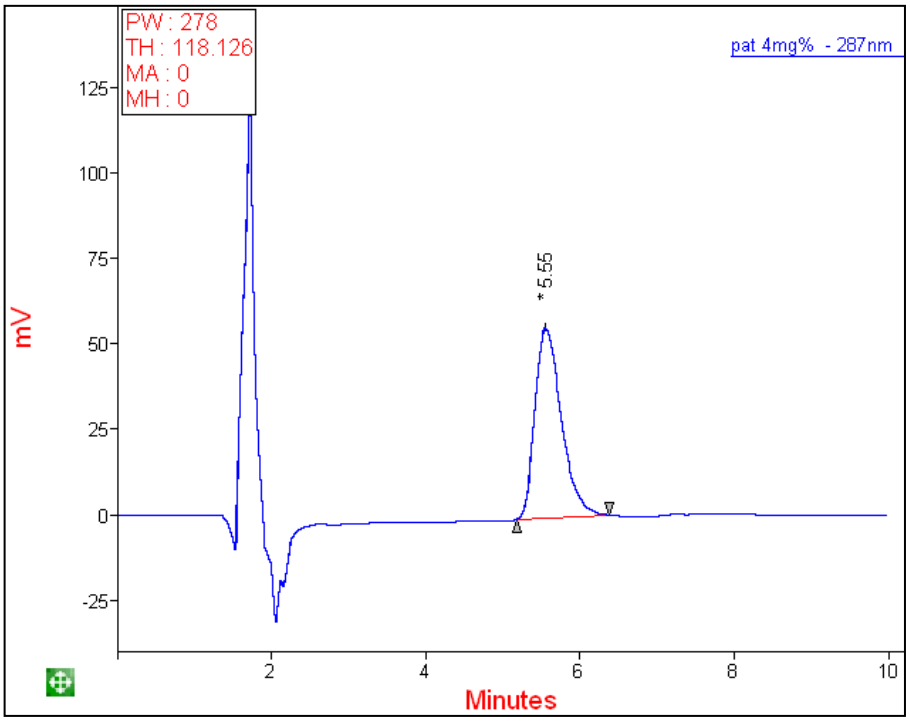

Figure 6.20 HPLC chromatogram for Patrin-2 (0.04 mg/ml) (*n* = 3).

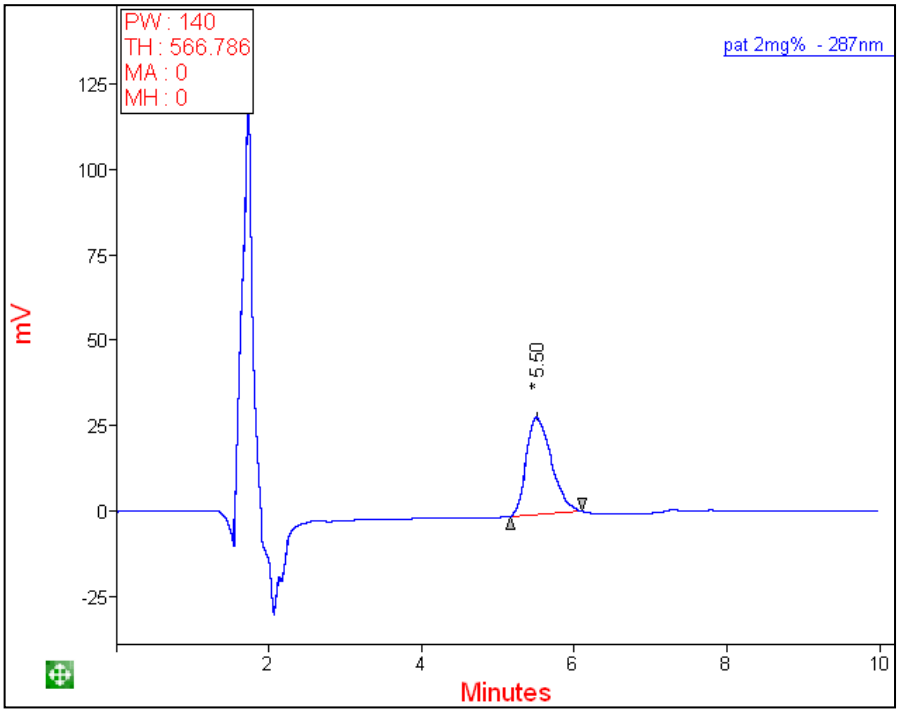

Figure 6.21 HPLC chromatogram for Patrin-2 (0.02 mg/ml) (*n* = 3).

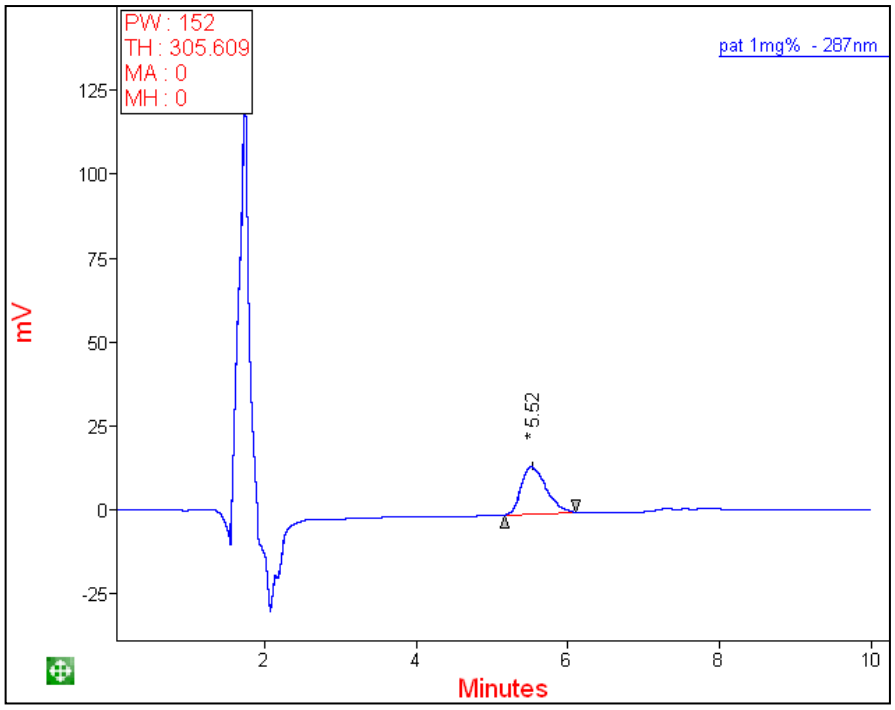

Figure 6.22 HPLC chromatogram for Patrin-2 (0.01 mg/ml) (*n* = 3).

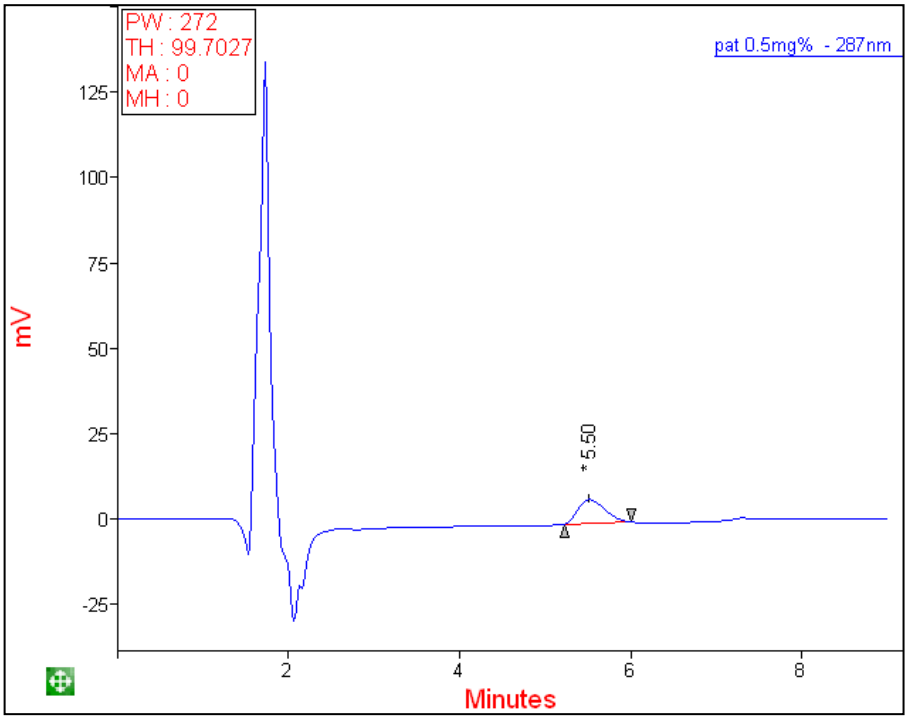

Figure 6.22 HPLC chromatogram for Patrin-2 (0.005 mg/ml) (*n* = 3).

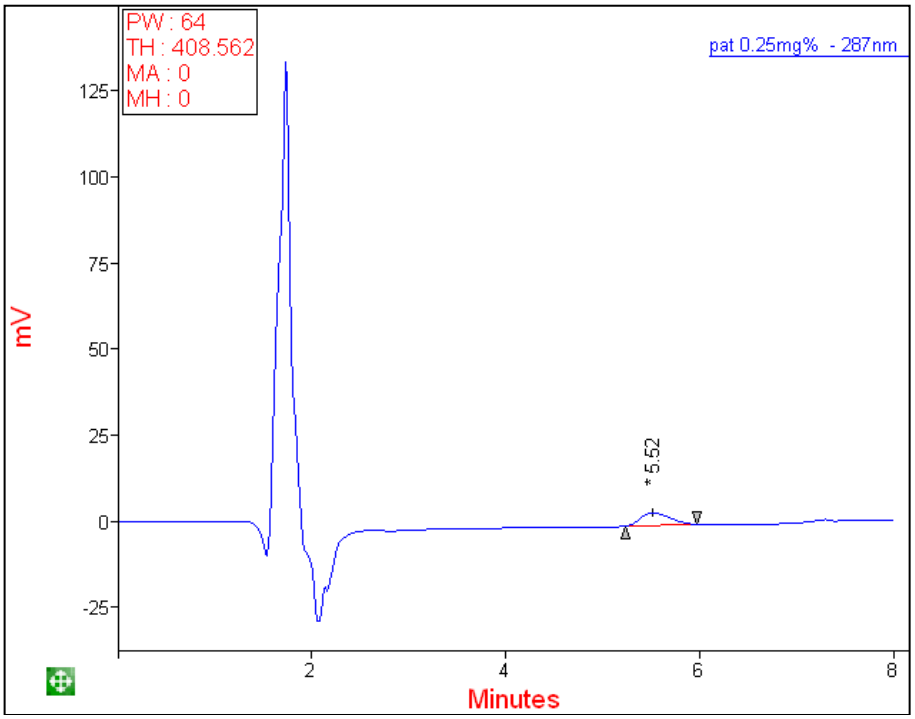

Figure 6.23 HPLC chromatogram for Patrin-2 (0.0025 mg/ml) (*n* = 3).

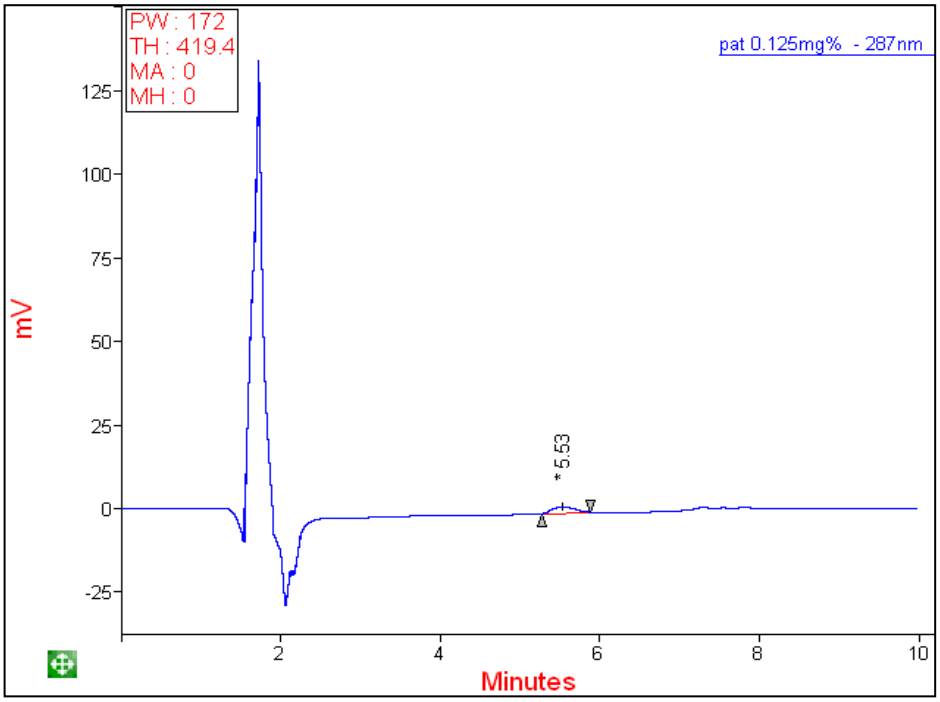

Figure 6.24 HPLC chromatogram for Patrin-2 (0.00125 mg/ml) (*n* = 3).

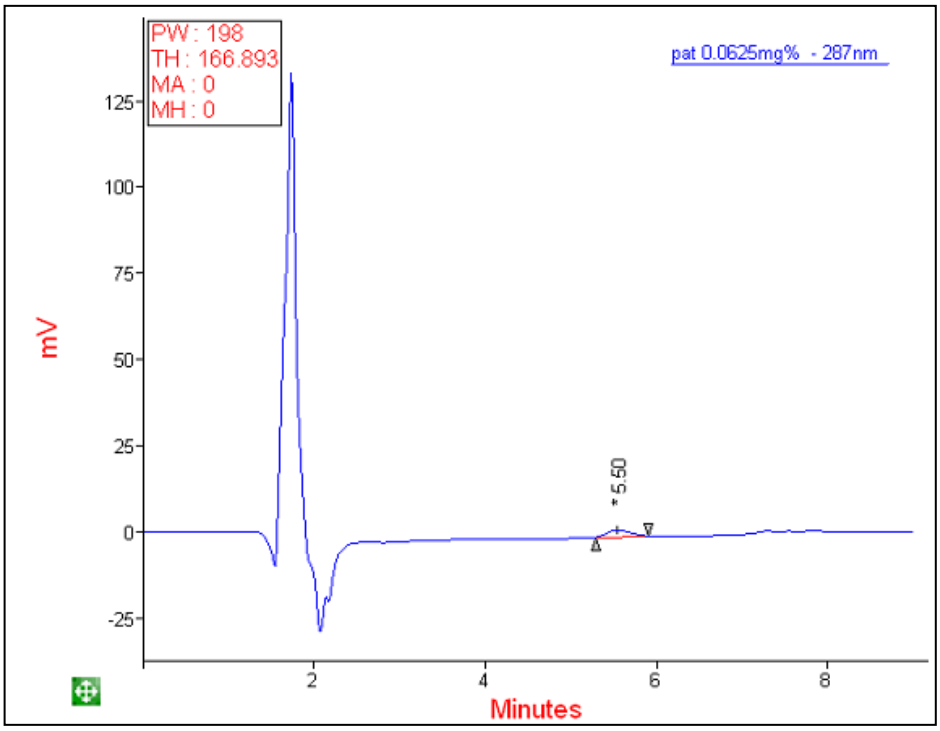

Figure 6.25 HPLC chromatogram for Patrin-2 (0.000625 mg/ml) (*n* = 3).

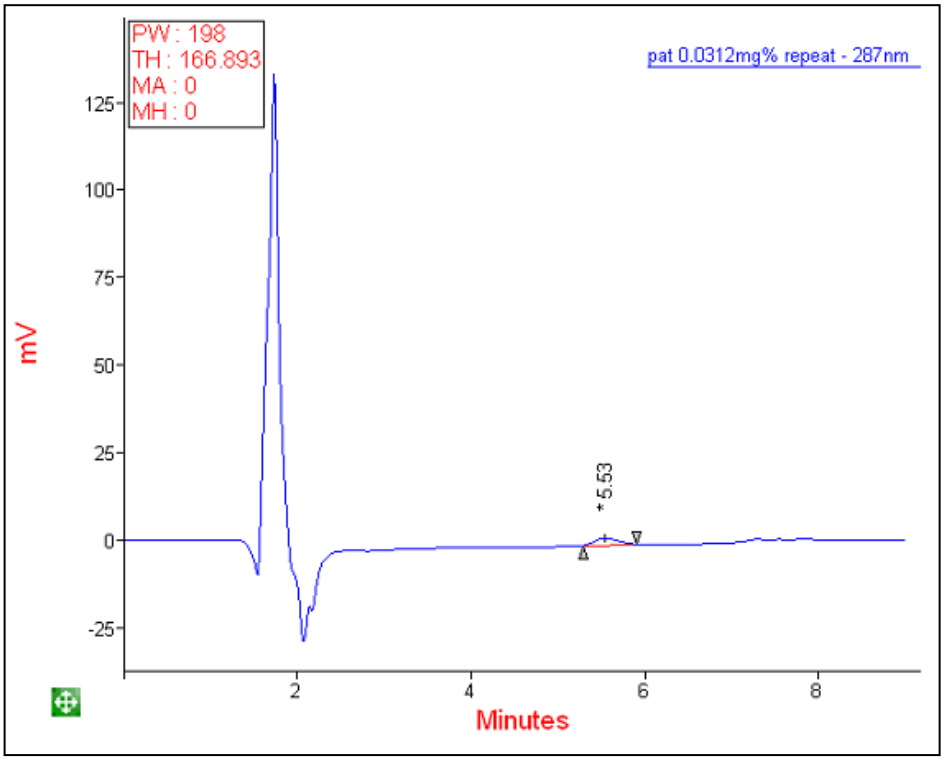

Figure 6.26 HPLC chromatogram for Patrin-2 (0.000312 mg/ml) (*n* = 3).

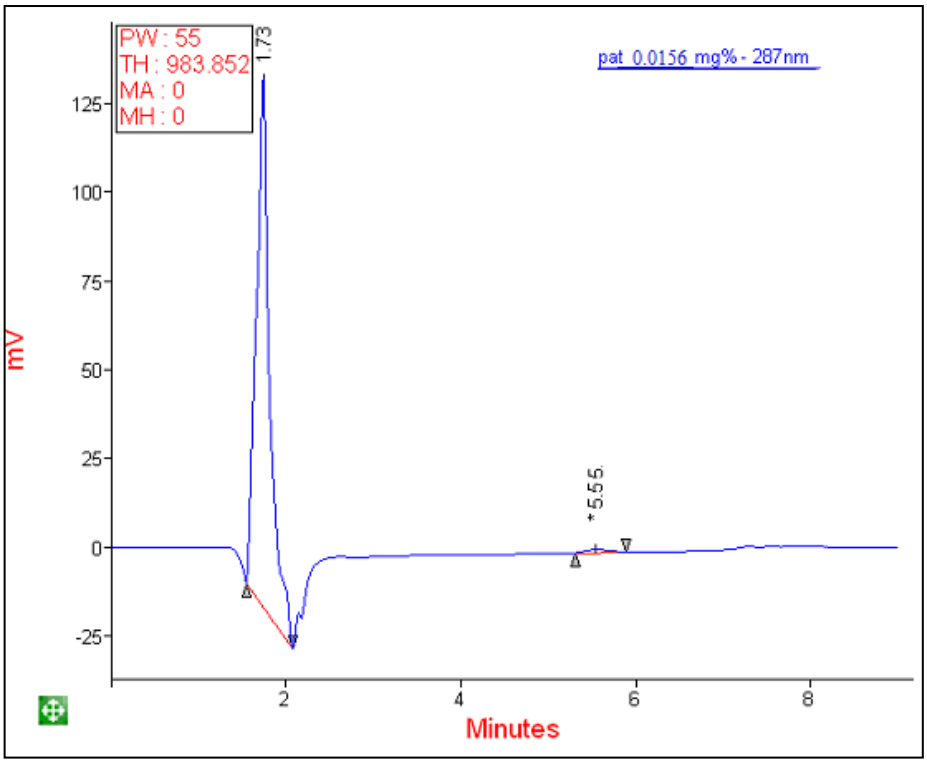

Figure 6.27 HPLC chromatogram for Patrin-2 (0.000156 mg/ml) (*n* = 3).

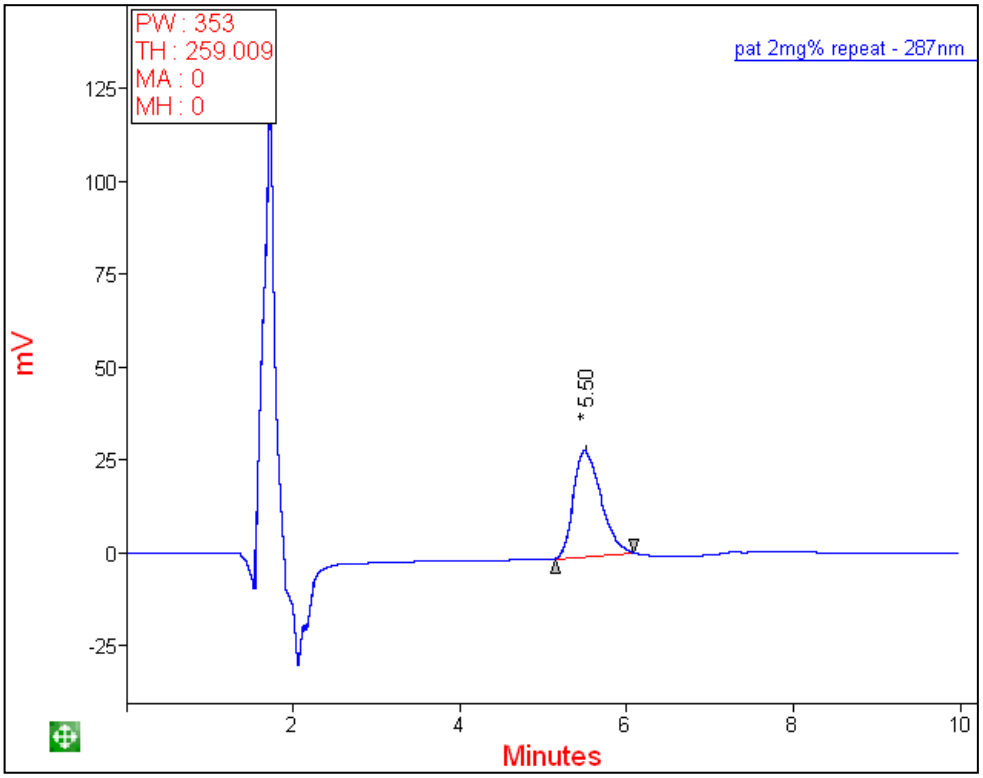

Figure 6.28 HPLC chromatogram for repeatability of Patrin-2 (0.02 mg/ml) (*n*=6).

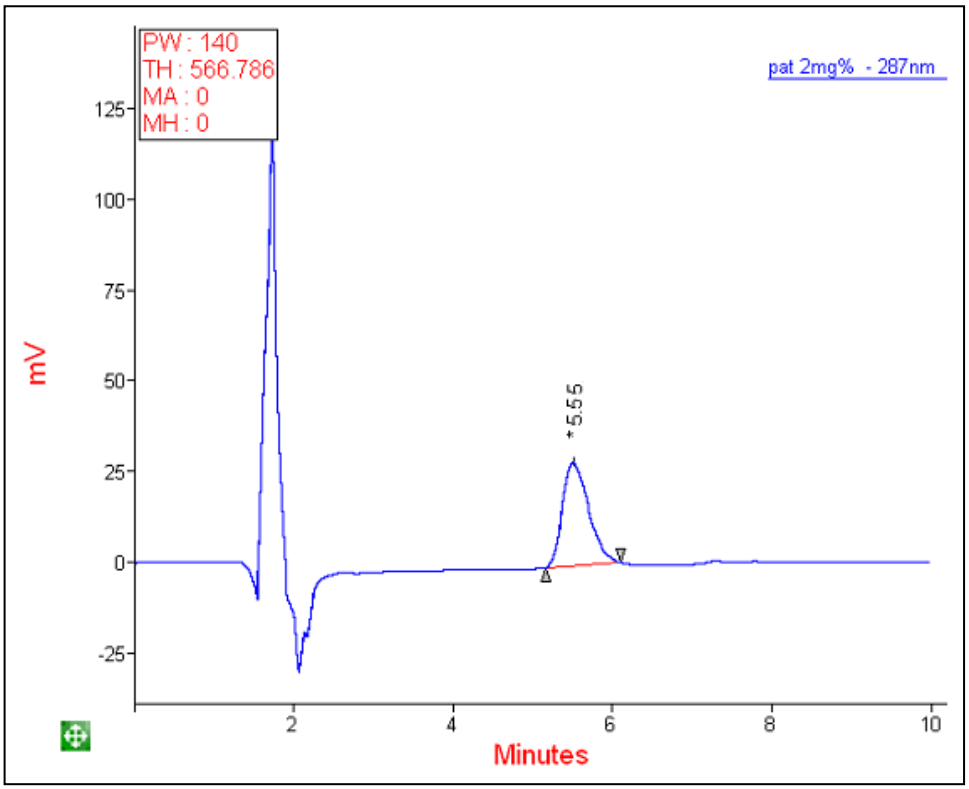

Figure 6.29 HPLC chromatogram for reproducibility of Patrin-2 (0.02 mg/ml) (*n*=6).

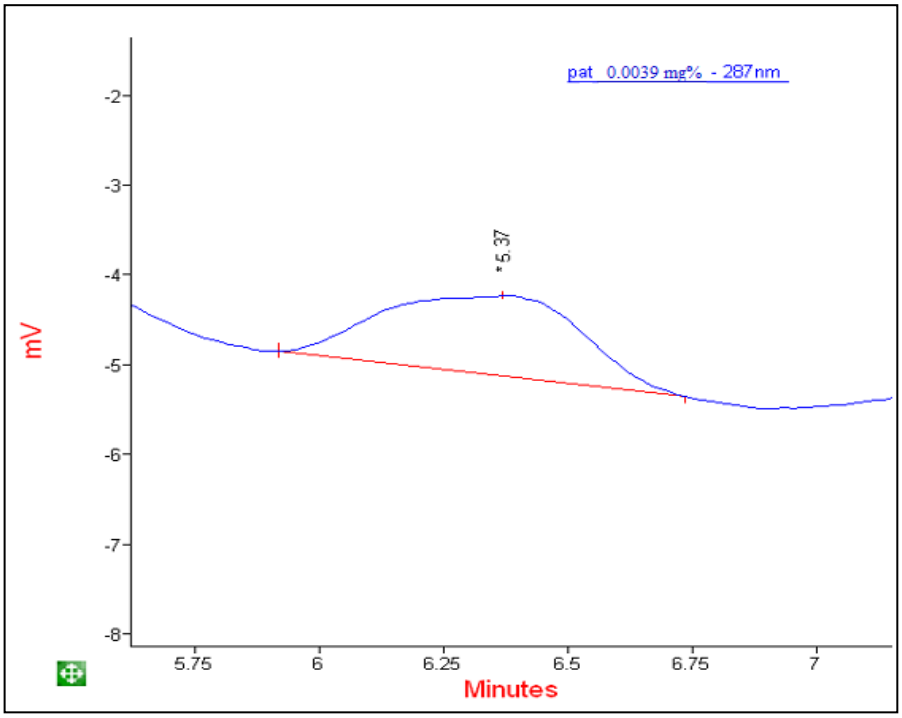

Figure 6.31 HPLC chromatogram for LOD of Patrin-2 (0.000039 mg/ml) (*n* = 3).

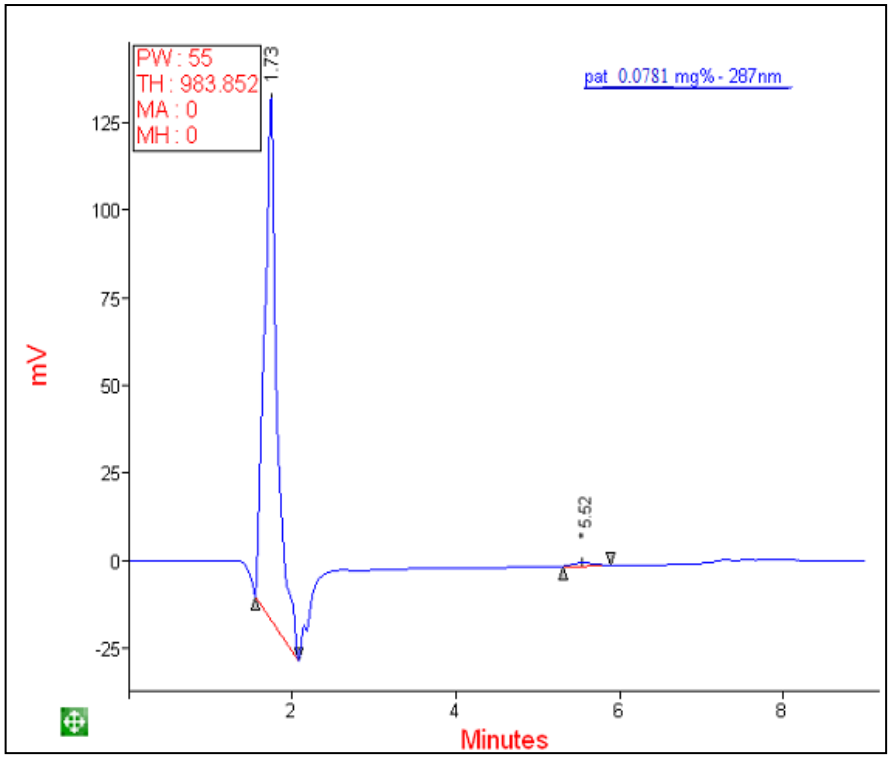

Figure 6.30 HPLC chromatogram for LOQ of Patrin-2 (0.0000781 mg/ml) (*n*=6).

Appendix 3

Stability test for TMZ and Patrin-2

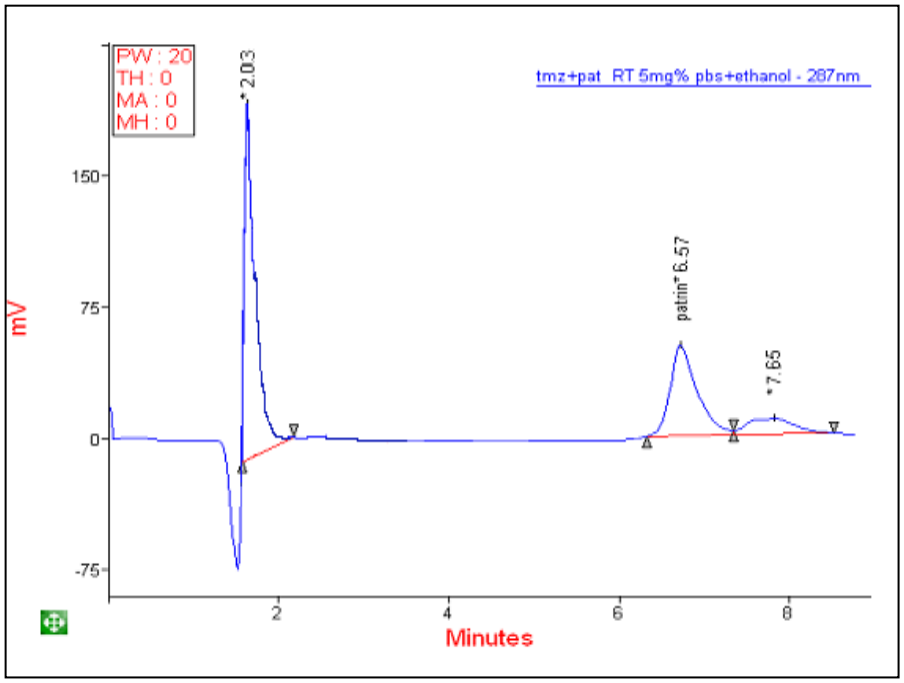

Figure 6.32 HPLC chromatogram for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for 72 hr at 25°C (*n* = 3).

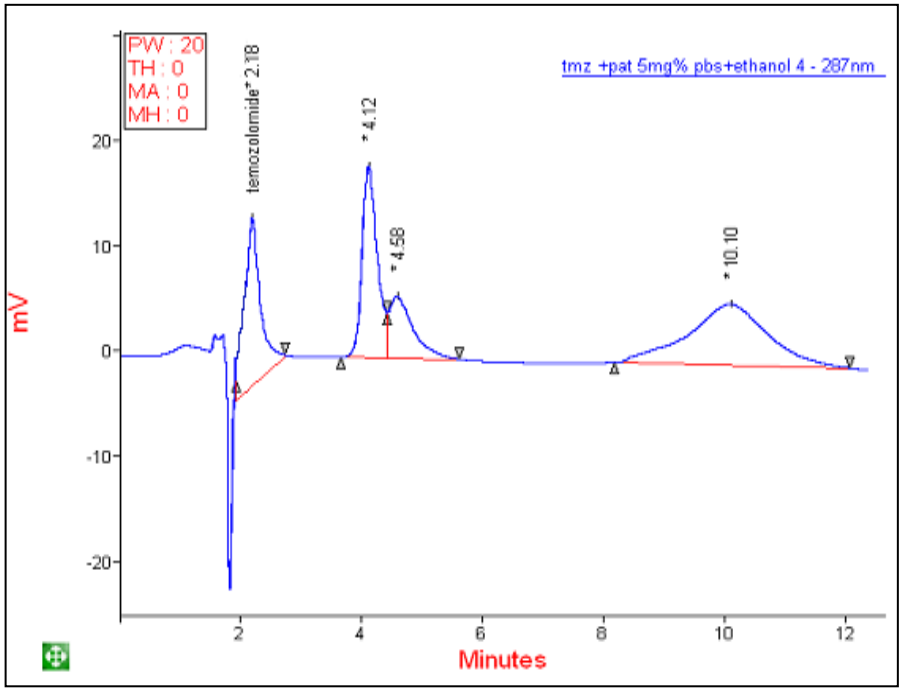

Figure 6.33 HPLC chromatogram for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for 288 hr at 25°C (*n* = 3).

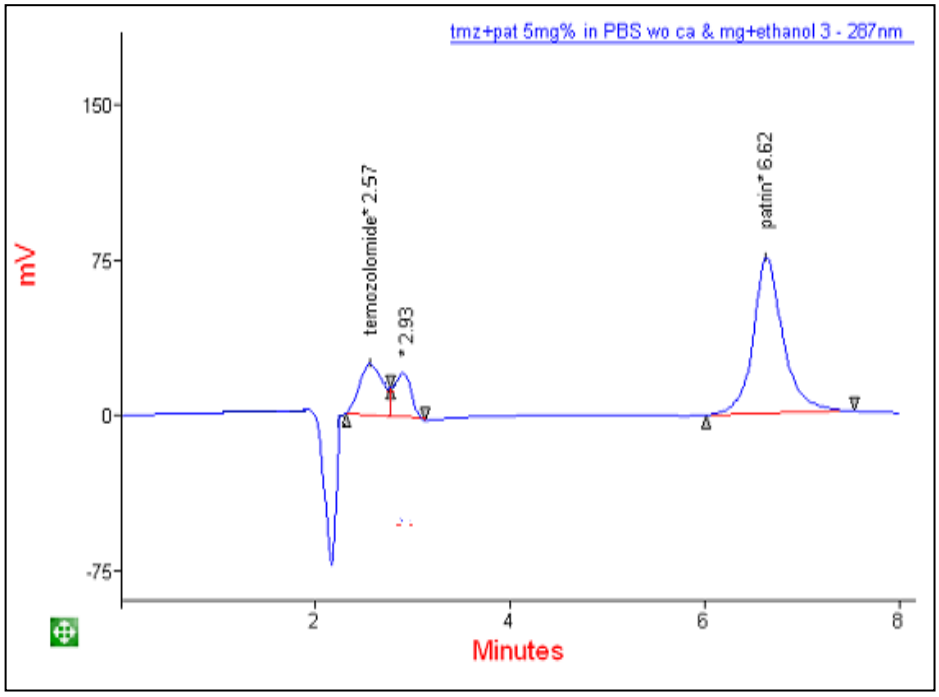

Figure 6.34 HPLC chromatogram for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for 72 hr at 37°C (*n* = 3).

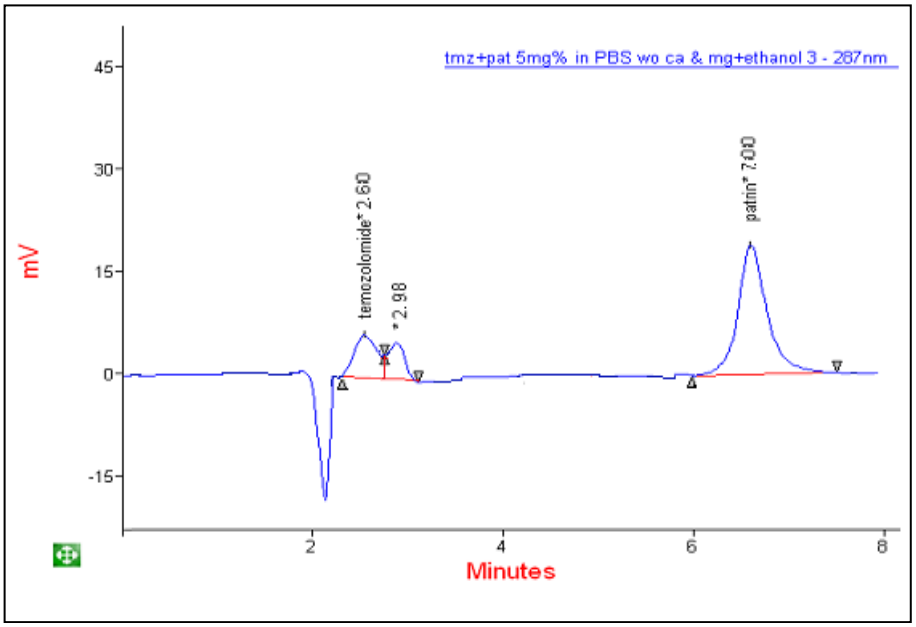

Figure 6.35 HPLC chromatogram for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for 72 hr at 50°C (*n* = 3).

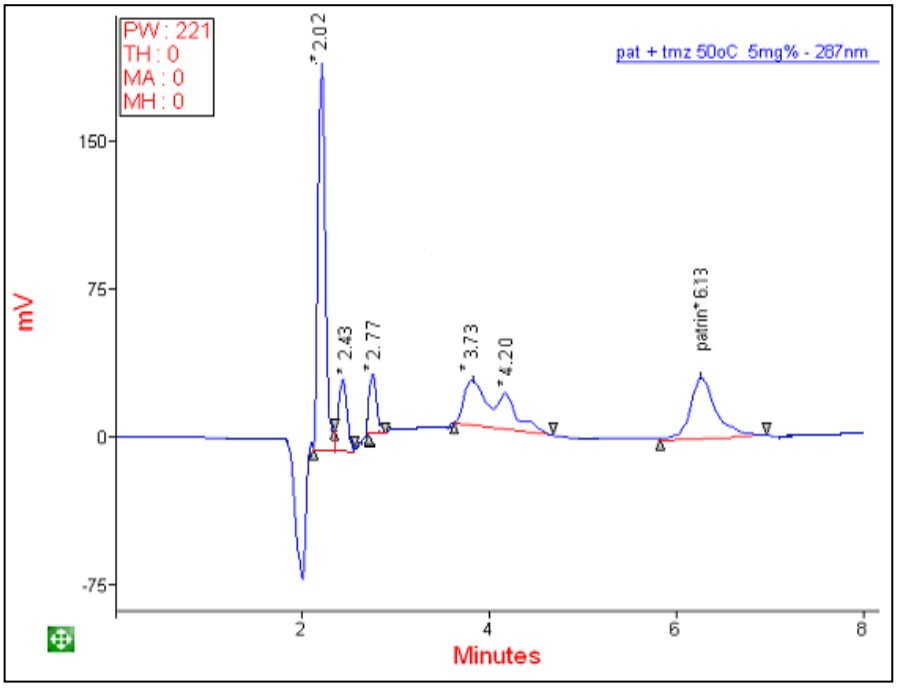

Figure 6.36 HPLC chromatogram for the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) solution stored for 288 hr at 50°C (*n* = 3).

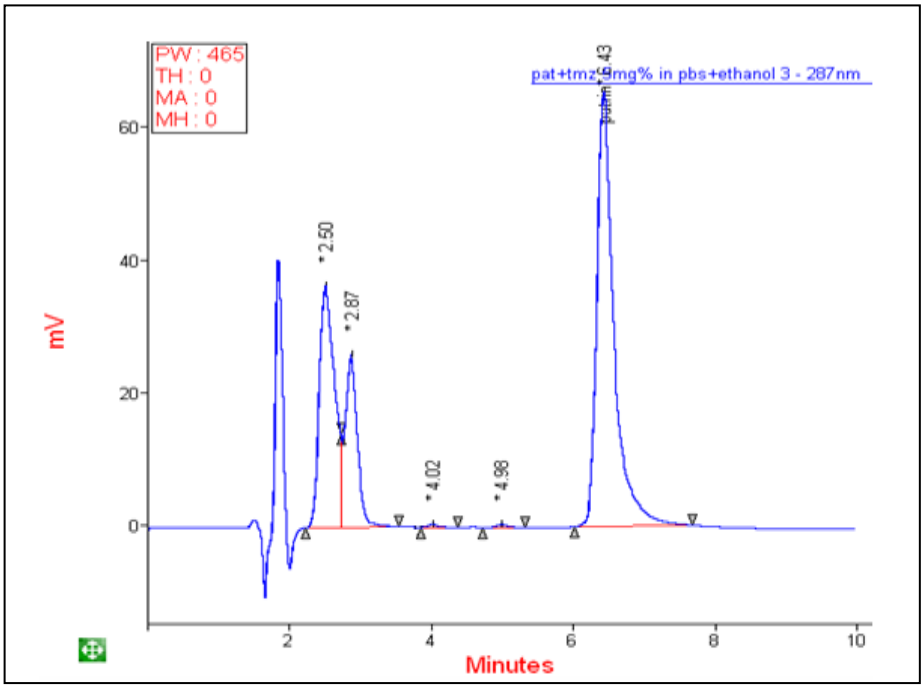

Figure 6.37 HPLC chromatogram for the dry sample reaction carried out to determine the compatibility of TMZ and Patrin-2 at a concentration of 0.05 mg/ml in PBS and ethanol (ratio 8:2) (*n* = 3).

Appendix 4

Calculation of EE % for TMZ loaded liposomes:

1) Freeze-Thaw method

$$EE\ \% = \frac{AD_a}{AD_b} \times 100$$

$AD_b \rightarrow$ mean peak area obtained for TMZ in liposome before washing with PBS

$AD_a \rightarrow$ mean peak area obtained for TMZ in liposome after washing with PBS

$AD_a = 23.025$

$AD_b = 199.43$

$$EE\ \% = \frac{23.025}{199.43} \times 100$$

$$= 11.54\%$$

2) Dehydration-Rehydration method

$$EE\ \% = \frac{AD_a}{AD_b} \times 100$$

$AD_b \rightarrow$ mean peak area obtained for TMZ in liposome before washing with PBS

$AD_a \rightarrow$ mean peak area obtained for TMZ in liposome after washing with PBS

$AD_a = 163.12$

$AD_b = 611.01$

$$EE\ \% = \frac{163.12}{611.01} \times 100$$

$$= 26.69\%$$

Calculation of EE % for TMZ and Patrin-2 loaded PLGA microparticles:

1) Emulsifying-solvent evaporation method for preparation of TMZ loaded microparticles.

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

$D_m \rightarrow$ mean peak area obtained for TMZ in microparticle

$D_t \rightarrow$ mean peak area obtained for standard solution of TMZ

$D_m = 178.83$

$D_t = 6456.41$

$$EE\ \% = \frac{178.83}{6456.41} \times 100$$

$= 2.76\ \%$

2) Spray dry method for preparation of TMZ loaded microparticles.

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

$D_m \rightarrow$ mean peak area obtained for TMZ in microparticle

$D_t \rightarrow$ mean peak area obtained for standard solution of TMZ

$D_m = 5161.22$

$D_t = 8023.08$

$$EE\ \% = \frac{5161.22}{8023.08} \times 100$$

$= 64.32\ \%$

3) Spray dry method for preparation of Patrin-2 loaded microparticles.

$$EE\ \% = \frac{D_m}{D_t} \times 100$$

$D_m \rightarrow$ mean peak area obtained for Patrin-2 in microparticle

$D_t \rightarrow$ mean peak area obtained for standard solution of Patrin-2

$D_m = 2619.75$

$D_t = 3825.92$

$$EE\ \% = \frac{2619.75}{3825.92} \times 100$$

$$= 68.47\ \%$$

Calculation of cumulative mass release (µg) for TMZ loaded PLGA microparticle:

$T_1 = T_1$ conc (µg/ml) * X

T_2 = (T_2 conc (µg ml) * X) + T_1 cumulative mass release-(T_1 conc (µg /ml)* Y)

T_3 = (T_3 conc (µg /ml) * X) + T_2 cumulative mass release-(T_2 conc (µg /ml)* Y)

T4 = and so on…

T= Sample collection timepoint

X= Tolal volume of release medium (i.e. 25ml in this study)

Y= Tolal volume of release medium-Sampling volume (i.e. 25ml-1ml=24ml)

Calculations:

T_1 = 16.28 × 25 = 407.06

T_2 = (15.07 × 25) + 407.06 - (16.28 × 24) = 393.22

Similarly the calculations were carried out for the remaining samples.

Calculation of cumulative mass release (µg) for Patrin-2 loaded PLGA microparticle:

T_1 = T_1 conc (µg /ml) * X

T_2 = (T_2 conc (µg /ml) * X) + T_1 cumulative mass release-(T_1 conc (µg /ml)* Y)

T_3 = (T_3 conc (µg /ml) * X) + T_2 cumulative mass release-(T_2 conc (µg /ml)* Y)

T4 = and so on…

T= Sample collection timepoint

X= Tolal volume of release medium (i.e. 25ml in this study)

Y= Tolal volume of release medium-Sampling volume (i.e. 25ml-1ml=24ml)

Calculations:

T_1 = 4.82 × 25 = 120.56

T_2 = (4.85 × 25) + 120.56 - (4.82 × 24) = 126.14

Similarly the calculations were carried out for the remaining samples.