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Solid Lipid Nanoparticulate Formulation for ifosfamide:
Development and Characterization

By

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A THESIS

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ABSTRACT

The present research focuses on the development and characterization of a delivery system for ifosfamide to enhance its stability and sustaining *in vitro* release. Solid lipid nanoparticles (SLNs) of ifosfamide were developed by double emulsion solvent evaporation technique using glycercyl monooleate and chitosan. The chitosan present in the delivery system was cross-linked using sodium tripolyphosphate. The effect of chamber pressure during lyophilization of emulsions was studied by lyophilizing the emulsion at low pressure (high vacuum) and high pressure (low vacuum). Two products having distinct morphologies were obtained as a result of lyophilizing emulsion under different vacuum conditions. They were named as matrix system and nanoparticles and both these systems were characterized. The particle size and surface charge of the delivery systems were determined using a zetasizer. The surface morphology was analyzed using scanning electron microscopy (SEM). Moisture content and weight loss on heating were assessed using Karl Fischer titrimetry and thermogravimetric analysis, respectively. The physical state of the drug in the particles was analyzed using differential scanning calorimeter (DSC) and X-ray diffractometer (XRD). Fourier Transform Infrared Spectroscopy (FTIR) was used to evaluate the cross-linking of chitosan in the delivery system. *In vitro* drug release at pH 7.4 and pH 6.8 was carried out and samples were analyzed using High Performance Liquid Chromatography (HPLC). The degradation of ifosfamide in solution and in the delivery systems at pH 2.5 was evaluated using and Liquid Chromatography/Mass Spectroscopy (LC/MS). Cellular permeability studies of ifosfamide solution and the delivery systems were performed using Caco-2 cells. Subcellular localization studies using rhodamine loaded nanoparticles were performed using Caco-2 cells. Different chamber pressures during lyophilization produced products having different morphology and moisture content. The drug in
the delivery systems was found to exist in a non-crystalline state. Both the delivery systems showed high drug loading efficiency and were able to sustain the release of the drug in pH 7.4 as well as pH 6.8. The stability of ifosfamide in the delivery system was found to be better than free drug in acidic medium. Drug in solution and nanoparticles showed a significantly higher permeability from the apical to the basolateral side while no significant difference was observed in the case of matrix system. The nanoparticles were found to be localized in the lysosomes of the Caco-2 cells.
PREFACE

MANUSCRIPT:


ABSTRACTS:


2010 world congress in association with the AAPS annual meeting and exposition, New Orleans, Louisiana.

Dedicated to my parents
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I would like to express my earnest gratitude to my advisor, mentor and chair of my supervisory committee, Dr. Alekha K. Dash for his tremendous support, able guidance, visionary expertise and encouragement throughout my stay at Creighton University. He has been instrumental in developing in me a scientific outlook, problem-solving skills, and sincerity towards my research work. He has always laid emphasis on honesty and integrity in scientific research being conducted, which keeps on improving the quality of research.

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CHAPTER 1

Introduction
1.1. Cancer

Cancer is defined as a condition in which there is an abnormal growth and division of cells without any inhibitory control which can eventually invade nearby cells and tissues. These cells are called cancerous or tumorous cells. Tumors can be further classified as- benign or malignant. Cells grow in an uncontrolled manner in both kinds of tumors but malignant tumors are characterized by their ability to revert back into cells having capacity to differentiate, their invasiveness and capability to metastasize. Tumor cells may metastasize via blood or lymphatics or through both the routes. When the cancer spreads to other tissues and organs there are increased risk of patient fatality. Various factors- both environment and genetic, are responsible for causing cancer which includes:

i. **Chemicals** – These are called carcinogens (chemicals causing cancer). They include mutagens (chemicals causing gene mutations), tobacco, alcohol, asbestos etc [1-3].

ii. **Ionizing radiation** – Ionizing radiations like gamma rays and ultraviolet rays [4,5].

iii. **Infections** – Viruses are responsible for many cases of cancers in human beings. These include hepatitis B and hepatitis C viruses, human papillomavirus, human polyomaviruses, Kaposi’s sarcoma herpes virus, Human T-cell leukemia virus-1, Epstein-Barr virus and Helicobacter pylori [6].

iv. **Heredity** – Cancer is not generally passed from one generation to the other unless it occurs in germ cells. However, sometimes defects in genes that protect cells from tumors may predispose next generation to cancer. Mutations in genes like BRCA1, BRCA2 and p53 have been shown to increase risk for cancer [7,8].
A variety of treatments are available to treat cancer. Surgery, chemotherapy, radiotherapy or a combination of these options are currently used for treating cancer. Chemotherapy includes drugs from many classes such as DNA alkylating agents, cytotoxic agents affecting purine and pyrimidine synthesis, cytotoxic antibiotics obtained from microbial origin, plant derivatives affecting microtubule synthesis, hormones, and other miscellaneous agents. DNA alkylating agents are widely used in the treatment of tumors [9]. These include the agents from following categories:

i. **Nitrogen mustards** – They are derived from the ‘mustard gas’ used in World War I. They have a basic formula (R-N-bis-(2-chloroethyl)). Examples are cyclophosphamide, ifosfamide, mechloethamine, uracil mustard, melphalan and chloambucil.

ii. **Nitrosoureas** – They are highly lipid soluble and can easily cross the blood brain barrier and hence used for treating tumors of the brain and meninges. Examples are lomustine, carmustine and streptozotocin.

iii. **Alkyl sulfonates** – Busulfan is a prototypical drug of this class which is used for treating chronic granulocytic leukemia.

Ifosfamide is relatively new to the class of deoxy ribonucleic acid (DNA) alkylating agents. It is a structural analogue of cyclophosphamide. It was approved as new molecular entity by FDA in 1988 for use in germ cell testicular cancer. It is also used for the treatment of pancreatic cancer, soft tissue sarcoma and cervical carcinoma as a single agent or in combination with other chemotherapeutic agents. It is available either as a powder which is reconstituted with diluents for injection, or as injectable solution. No sustained-release formulations have been developed so far for ifosfamide. Various methods are utilized to provide sustained-release of
drugs. Some of the methods include formulation of nanoparticles, in situ gels, microparticles, and liposomes [10-13]. Preparation of nanoparticles is one of the most common methods to sustain and control the release of drugs [14].

1.2. Nanoparticles

Nanoparticles can be defined as solid colloidal particles ranging from 10 nm to 1000 nm in size. They consist of macromolecular material in which the active principle (drug or biologically active material) is dissolved, entrapped or encapsulated and/or to which the active principle is adsorbed or attached [15]. Nanoparticles have various bio-applications like drug delivery, gene delivery, magnetic resonance imaging (MRI) and in vitro diagnostics [16]. Nanoparticles offer several advantages over other drug delivery systems. To mention a few, nanoparticulate systems can be used to acts as carriers for drugs due to their capacity to release drugs [17], they can be easily taken up by cells due to their small size thus effecting in higher concentration of drug within cells [18], they can be used to improve stability of drugs [19] and can be biocompatible if formulated from biocompatible and biodegradable polymers [20]. A wide variety of polymers have been used for drug and gene delivery applications which are obtained from natural as well as synthetic sources. Given below is a table showing the most widely used polymers to formulate nanoparticulate formulations [21].
Table 1.1 Most Widely Used Polymers Constituting Nanoparticles Designed as Drug Carriers and Their Abbreviations [21].

<table>
<thead>
<tr>
<th>Material</th>
<th>Full name</th>
<th>Abbreviation or Commercial names</th>
<th>Reference</th>
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<tr>
<td>Synthetic homopolymers</td>
<td>Poly(lactide)</td>
<td>PLA</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>Poly(lactide-co-glycolide)</td>
<td>PLGA</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>Poly(epsilon-caprolactone)</td>
<td>PCL</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>Poly(isobutylcyanoacrylate)</td>
<td>PICBA</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Poly(acrylate), poly(methacrylate)</td>
<td>Eudragit*</td>
<td>Evonik®</td>
</tr>
<tr>
<td>Natural polymers</td>
<td>Chitosan</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alginate</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Gelatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copolymers</td>
<td>Poly(lactide)-poly(ethylene glycol)</td>
<td>PLA-PEG</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>Poly(lactide-co-glycolide)-poly(ethylene glycol)</td>
<td>PLGA-PEG</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>Poly(epsilon-caprolactone)-poly(ethylene glycol)</td>
<td>PCL-PEG</td>
<td>[27]</td>
</tr>
<tr>
<td>Colloid stabilizers</td>
<td>Dextran</td>
<td>F68</td>
<td>BASF</td>
</tr>
<tr>
<td></td>
<td>Pluronic F68</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poly(vinyl alcohol)</td>
<td>PVA</td>
<td></td>
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<tr>
<td></td>
<td>Tween® 20 or Tween® 80</td>
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1.3. Methods of preparation of nanoparticles

Many methods have been tested for the preparation of nanoparticles. These include nanoprecipitation, emulsification-diffusion, emulsification-coacervation, double emulsification and, polymer-coating. These methods are described in brief in the figure below [28].

5
The choice of method for preparation of nanoparticles depends on the properties of drug and the nature of polymer to be used. Multiple emulsion solvent evaporation methods are commonly used for encapsulating both hydrophilic and hydrophobic drugs [10,29]. These systems use lipids as the internal phase which is emulsified with a surfactant. This emulsion is further emulsified and coated with a polymer and the solvent is then evaporated giving nanosized particles. This technique is a modification of solid lipid nanoparticles where the drug is dispersed in the melted lipid which is then emulsified with a surfactant followed by solvent
evaporation. Many techniques have been utilized for production of solid lipid nanoparticles (SLNs). These techniques have been briefly described below [30].

1.4. Preparation of SLN by high pressure homogenization

This involves production of SLN by either the hot homogenization technique or the cold homogenization technique. In both the techniques, the lipid is first melted and the drug is either dissolved or solubilized in the melted lipid. This mixture of melted lipid containing the drug is called lipid melt. For hot homogenization technique, the drug-containing melt is dispersed under stirring in a hot aqueous surfactant solution at identical temperature and homogenized. This (o/w) emulsion is then cooled to allow the lipid to solidify to form the SLNs. For lipids like glycerides having a low melting point, lower temperatures may be needed to initiate solidification. Lyophilization is carried out to remove the solvent from the system.

The process of cold homogenization is used for compounds which are highly sensitive to elevated temperatures. In this process, the drug containing lipid melt is cooled in order to solidify it. Size reduction of this lipid melt is performed to yield micron sized particles, which are then dispersed in a cold surfactant solution. A pre-suspension is obtained by this process. Homogenization of this pre-suspension is done to break the microparticles directly to SLNs.

1.4.1. SLN produced by microemulsion technique

In this process, the lipid is heated at a temperature slightly above its melting point and the surfactant is also heated at the same temperature. The hot surfactant solution is then added in appropriate ratio to the lipid melt under mechanical stirring to form microemulsion. This
microemulsion is further dispersed in a cold aqueous medium under mild mechanical mixing so that the small sized SLNs are formed due to precipitation.

1.4.2. Precipitated lipid particles

This process of production of SLNs is similar to that of the production of polymeric nanoparticles by solvent evaporation. In this method, the lipid is dissolved in an organic solvent and this solution is emulsified with an aqueous phase consisting of surfactant. The organic solvent is then evaporated, which leads to the formation of SLNs. The use of organic solvents and problems with scaling up are the major disadvantages of this technique.

Solid lipid nanoparticles have several advantages over other colloidal carriers include [31]:

i. Drug release can be controlled.

ii. Stability of pharmaceuticals can be improved.

iii. Can be used to incorporate higher drug load of hydrophilic as well as hydrophobic drugs.

iv. Targeted release of active constituents can be obtained.

v. Use of biodegradable lipids for preparing SLNs makes them biocompatible.

vi. Preparation of SLNs avoids use of organic solvents.

vii. Easier to prepare as compared to polymeric nanoparticles.

viii. Easy to scale up and sterilize.

SLNs can be coated with hydrophilic polymers to enhance their stability in plasma, augment biodistribution, and increase their bioavailability [32]. The nature of coating can also
affect the release characteristics of the drug from SLNs. Hydrophilic coating has been shown to retard the release of hydrophobic drugs from SLNs [32]. Hydrophilic substances like poloxamers, chitosan, polyvinyl alcohol, and PEG are used to coat SLNs to reduce their phagocytic uptake [32]. Coating of SLNs with chitosan has been shown to increase their permeability across Caco 2 cells [33]. Due to the aforementioned advantages, hydrophilic polymers like chitosan can be used to coat SLNs.

1.5. Chitosan

![Molecular structure of chitosan consisting of glucopyranose rings](image)

**Figure 1.2** Molecular structure of chitosan consisting of glucopyranose rings

Chitosan is derived by deacetylation of chitin, a homopolymer of 2-acetamido-2-deoxy-β-D-glucopyranose [34]. Chitin is obtained from the exoskeletons of crustaceans, cell wall of fungi, cuticles of insects, and shells of mollusks [35]. The molecular structure of chitosan is similar to that of other polysaccharides such as cellulose but its properties are modified by the presence of primary aliphatic amines. Presence of free amino groups makes chitosan basic and hence, it can accept protons in an acidic environment thus rendering it an overall positive charge. Chitosan mostly occurs as high molecular weight heteropolysaccharide with some N-acetyl groups. The degree of deacetylation and molecular weight of chitosan are important factors affecting solubility, viscosity, anti-microbial activity, and drug loading [36,37]. The degree of deacetylation is defined as the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-
deoxy-D-glucopyranose in the structure of chitosan. The N-acetyl groups in chitosan can be arranged randomly or as blocks and this arrangement may affect the solution properties, binding and enzymatic hydrolysis of chitosan [38]. The pKa value for chitosan is 6.5 indicating that at a pH less than its pKa, chitosan is a polycation since the amino groups in chitosan are protonated. This positive charge leads to development of repulsive forces causing chitosan to have an extended structure and thus have a high viscosity [39].

1.5.1. Applications of chitosan in drug delivery

Chitosan could be potentially used for drug and vaccine delivery in veterinary species. It has many applications in the field of drug delivery for systemic as well as local delivery of drugs, genes and vaccines [40]. There are no Food and Drug Administration (FDA) approved formulations containing chitosan available as yet. Due to their biocompatibility and ability to form small sized nanoparticles, natural polymers like chitosan are being increasingly used to prepare nanoparticles [41]. The physicochemical properties of these polymers can be used to form ionic or covalent bonds with drug molecules thus improving their solubility, stability and permeability [42]. The labile bonds, which chitosan forms with drugs can be broken at physiological pH due to a change in the degree of ionization of chitosan. Changes in the pH can influence the ionization of Chitosan is widely used to formulate microspheres which are used as drug delivery systems for the controlled release of drugs, such as antibiotics, anticancer agents, proteins, peptide drugs and vaccines. It is used to coat surface of liposomes for improving bioavailability of poorly absorbed oral drugs thus increasing their mucoadhesiveness and enhancing their contact time with intestinal mucosa [43]. Thiolated derivatives of chitosan have been used for oral peptide delivery thus providing more conformational stability to these
peptides [44]. Chitosan has been used to formulate microparticles for pulmonary delivery of active medicaments. On interaction with mucus, they swell giving a two-fold advantage of prolonging the release of drugs as well as eluding uptake by alveolar macrophages, thus, acting as sustained-release delivery agent [45]. Chitosan has been widely used to formulate nanoparticles which are used for diagnostic as well as drug delivery purposes. Chitosan nanoparticles have been demonstrated to control the release of active medicaments. One of the major advantages of chitosan to prepare nanoparticles over other polymers is that they avoid use of hazardous organic solvents e.g. dichloromethane, chloroform, etc. for their preparation. Chitosan nanoparticles have been prepared for delivering hydrophobic as well as hydrophilic anti-cancer drugs like paclitaxel and doxorubicin respectively [46,47]. Insulin has been successfully incorporated in chitosan nanoparticles prepared by polyelectrolyte complexation [48]. Nanoparticles prepared by cross-linking chitosan with sodium tripolyphosphate have been extensively studied.

1.5.2. Cross-linking of Chitosan with TPP

Chitosan bears positive charge in acidic media and hence reacts with sodium tripolyphosphate (TPP), which is negatively charged throughout the physiologically acceptable pH range [37]. This is an electrostatic interaction between the NH\textsuperscript{3+} groups present on chitosan and OH\textsuperscript{−} and P\textsubscript{3}O\textsubscript{10}\textsuperscript{5−} ions provided by TPP. The presence of these negatively charged ions depends on the pH of TPP solution and hence, the pH of TPP solution has a significant effect on the cross-linking of chitosan [36]. Two proposed schemes for reaction of chitosan with TPP solutions of different pH are given below.
At higher pH of TPP solution, the cross-linking is effected by deprotonation (A) while at a lower pH of TPP when phosphoric ions are abundant, chitosan molecules are cross-linked ionically. The ratio of chitosan to TPP plays an important role in the degree of cross-linking and hence controlling the particle size during preparation of nanoparticles [49]. The formation of nanoparticles is possible only at certain concentrations of chitosan and TPP and that the size of the particles is small when lower concentrations of chitosan and TPP (ratio 3:1 to 5:1) are used [50]. When TPP solution is added to chitosan solution, three kinds of phenomena are observed: solution, aggregates and opalescent suspension [51]. Aggregates give particles having size range in microns while solution fails to yield any nanoparticles. The zone of opalescent suspension gives nanoparticles. A larger chitosan to TPP ratio (20:1) leads to formation of particles with larger size due to inadequate cross-linking of chitosan. The pH of the TPP and chitosan solutions dictates the degree of ionization and hence, plays a significant role in the degree of cross-linking of chitosan [36,37].
Chitosan particles cross-linked with TPP have been prepared for delivery of several drugs. Particles containing ciprofloxacin hydrochloride, a hydrophilic drug, showed a sustained-release up to 10 hours in PBS [49]. Streptomycin, gentamicin and tobramycin loaded chitosan-TPP nanoparticles have been evaluated for oral use against Mycobacterium tuberculosis [52]. Paclitaxel has been prepared and delivered to HeLa cells by folate-conjugated chitosan which were ionically cross-linked with TPP. These particles showed a higher uptake and significant toxicity to HeLa cells [53]. Gene delivery is one of the most modern techniques being investigated to overcome diseased conditions by targeting nanoparticles containing specific genes or proteins. Chitosan-TPP nanoparticles containing plasmid DNA have been studied for transfection efficiency, cellular uptake, in vivo gene expression and toxicity following intratracheal administration to mice [54]. Ionically cross-linked chitosan nanoparticles have been demonstrated to successfully deliver shRNA (short hairpin RNA) to inhibit expression of TGF-beta1 which is involved in metastasis. These nanoparticles were found to be effective in TGF-beta1 gene silencing [55]. The suitability of these nanoparticles to deliver genes and peptides indicates that chitosan-TPP nanoparticles could be used for delivery of hydrophilic drugs.

1.6. Lyophilization

The origin of the term lyophilization is from the porous nature of the product formed and it has a ‘lyophil’ characteristic meaning an affinity to reabsorb the solvent and revert itself back to original state [56]. Lyophilization is often used synonymous to freeze drying, which refers to removal of both aqueous and organic solvents. The modern definition of lyophilization defines it as a stabilizing process whereby a substance is first frozen, then subjected to primary drying meaning reducing the quantity of solvent by sublimation and finally most of the remaining
solvent is removed by desorption (secondary drying) so that the product no longer supportsiological growth or chemical reactions [57]. A brief description of the processes involved in
lyophilization is provided below.

Freezing- It separates solutes from the solvent. For an aqueous system, the transformation
of water to ice crystals will take place and the solutes will be confined to interstitial regions
between the ice crystals. Primary Drying- After freezing the formulation completely, the pressure
in the chamber is reduced; heat is supplied to the formulation to effect sublimation of ice
crystals. Removal of all the ice crystals marks the completion of this process. The resultant cake
has a volume equivalent to the frozen formulation.

Secondary Drying- After completion of primary drying, some residual moisture remains
adsorbed to the cake which could be lethal for the stability of the product. This is removed by
increasing the temperature of the product and decreasing the partial pressure of water vapor in
the chamber. This process should lead to a cake without decreasing the volume of the interstitial
cake to give a stable product.

1.6.1. Cosmetic properties of product

The appearance or cosmetic properties of the cake depend on the primary and secondary
drying steps of lyophilization. A uniform cake structure is formed when there is proper
formation of ice crystals during the freezing process (Figure 1.3 (a)). Ideally, the lyophilized
cake should have volume equivalent to that of frozen matrix (Figure 1.3 (b)). When the ice-
product matrix is not completely frozen, there is collapse of the cake during primary drying
(Figure 1.3 (c)). If liquid phase is present in the ice-product matrix, there is meltback during the
process of primary drying (Figure 1.3 (d)).

![Figure 1.3](image.png)

**Figure 1.4** (a) Cake matrix as a result of formation of uniform matrix during freezing process, (b) Non-uniform matrix as a result of heterogeneous ice formation, (c) Collapse of cake structure as a result of primary drying process, and (d) Meltback of the product during lyophilization process.

Thermal analysis of the product of lyophilization is an important tool to assess the effect of process of lyophilization on the product and this is critical for formulation development. Thermal characterization helps to study about the solid-state form, whether the material is in crystalline or amorphous state or a mixture of both. It also tells about the compatibility of different components of the formulation and their physical stability. This information is quite useful in selecting product having desired morphology and physical state and thus indirectly helping to optimize the process of lyophilization [58]. Thus, study of lyophilization parameters and its effect on the product morphology is an interesting area of research.

1.7. Hypotheses and specific aims

SLNs prepared from Glyceryl monooleate (GMO) and surface modified with chitosan ionically cross-linked with TPP were prepared and characterized in the present study. The
underlying hypothesis for this investigation were:

**Hypothesis I:** “Solid lipid nanoparticles will provide a sustained-release of hydrophilic drug ifosfamide *in vitro*”.

**Hypothesis II:** “Acid stability of ifosfamide will be enhanced in SLNs”.

In order to test these two hypotheses, following specific aims were achieved:

**Specific Aim 1:** Preparation of the SLNs for sustained-release of ifosfamide.

**Specific Aim 2:** Characterization of the SLNs.

**Specific Aim 3:** Evaluation of the SLNs.
CHAPTER 2

Ifosfamide (Physicochemical properties and Pharmacology)
2.1. Drug Type

As discussed earlier, ifosfamide is a structural isomer of cyclophosphamide and is highly hydrophilic molecule. It is a small molecule with a molecular weight of 260 amu (Atomic Mass Unit). Due to the isotopic distribution of chlorine, the molecular weight is sometimes calculated as 261 amu. It was first approved by the USFDA in 1988 as a third-line treatment for testicular cancer. It is most commonly used in combination with mesna which is a prophylactic agent for hemorrhagic cystitis. Since its first entry in the market, many formulations of ifosfamide have received FDA approval.

1988 - December 30 - New molecular entity (NME)

Ifex<sup>®</sup> injectable; injection (1gm/vial, 3gm/vial)

Active Ingredient(s): ifosfamide

Applicant: Baxter Healthcare

Chemical Type: New molecular entity (NME)

Marketing status: Prescription

2002 - May 28

Ifosfamide injectable; injection (1gm/vial, 3gm/vial)

Active Ingredient(s): ifosfamide

Applicant: APP Pharms

Marketing status: Prescription

2007 - April 4
Ifosfamide injectable; injection (1gm/20mL (50mg/mL), 3gm/60mL (50mg/mL))

Active Ingredient(s): ifosfamide

Applicant: Teva Parenteral

Marketing status: Prescription

2009 - September 22

Ifosfamide injectable; injection (1gm/20mL (50mg/mL), 3gm/60mL (50mg/mL))

Active Ingredient(s): ifosfamide

Applicant: APP Pharms

Marketing status: Prescription

2.2. Nomenclature

2.2.1. Chemical IUPAC name

The IUPAC name of ifosfamide is N-3-bis(2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine-2-oxide.

2.2.2. Synonyms

Asta Z 4942, I-Phosphamide, Ifosfamid, ifosfamide Sterile, Ifsofamide, Iphosphamid, Iphosphamide, Isofosfamide, Isophosphamide

2.2.3. Brand names

Cyfos®, Holoxan 1000®, IFEX®, Ifex/Mesnex Kit®, Ifosfamide/Mesna Kit®, Mitoxana®, Naxamide®
2.3. Formulae

2.3.1. Empirical

The empirical molecular formula for ifosfamide is \( \text{C}_7\text{H}_{15}\text{Cl}_2\text{N}_2\text{O}_2\text{P} \).

2.3.2. Structural

![Structural formula for ifosfamide](image)

**Figure 2.1** Structural formula for ifosfamide

2.4. Molecular weight

The average molecular weight is 261.1 amu.

2.5. CAS number

3778-73-2
2.6. Physicochemical properties of ifosfamide

Table 2.1 Physicochemical properties of ifosfamide

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical state and appearance</td>
<td>White crystalline powder</td>
<td>Baxter MSDS</td>
</tr>
<tr>
<td>Melting Point</td>
<td>48-51°C</td>
<td>Baxter MSDS</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td>0.57</td>
<td>[59]</td>
</tr>
<tr>
<td>Solubility</td>
<td>Water – 100mg/ mL</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>Methylene Chloride – 1g/ mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Readily soluble in methanol</td>
<td></td>
</tr>
<tr>
<td>pKₐ</td>
<td>1.45</td>
<td>[59]</td>
</tr>
</tbody>
</table>

2.7. Pharmacology

2.7.1. Drug category

On the basis of its mechanism of action, ifosfamide has been categorized as a DNA alkylating agent which is cell cycle non-specific. It may also have some immunosuppressant effect. It is included in category D for pregnancy by FDA meaning that it may causes birth defects in an unborn baby.

2.7.2. Mechanism of action

Ifosfamide is a prodrug, which is activated on metabolism by hepatic enzymes to yield cytotoxic nitrogen mustards. These nitrogen mustards react with the DNA in the cells and lead to
cell death [61]. At physiological pH, conversion of the generated nitrogen mustard to carbonium ion takes place. The carbonium ion, thus formed, reacts with the N-7 of the guanine residue in DNA thus forming a covalent bond. The cross-linking of DNA strands takes place when the second side-chain of the phosphoramidate mustard reacts with guanine moiety in the same DNA strand or in the opposite DNA strand [62]. Thus, it destroys the cancer cells by initiating programmed cell death (apoptosis) which is effected by damage to DNA and modulation of cell cycle to bring about anti-proliferative changes [63][64]. Ifosfamide has also been found to inhibit protein synthesis [65] and its metabolite chloroacetic acid (CAA) has been shown to interfere with the cell’s glycolytic pathway thus decreasing glucose metabolism and hence causing cell death [66]. Some studies have even reported decrease of adenosine triphosphate (ATP) turnover in the cells but this effect has been mostly associated with unwanted nephrotoxicity [67].
2.7.3. Indications

**Table 2.2 Marketed formulations for ifosfamide and their indications**

<table>
<thead>
<tr>
<th>Company</th>
<th>Formulation</th>
<th>Indication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baxter Healthcare</td>
<td>1g and 3g vials</td>
<td>Third line of chemotherapy of germ cell testicular cancer. Used in combination with a prophylactic agent for hemorrhagic cystitis, such as mesna.</td>
<td>FDA</td>
</tr>
<tr>
<td>Teva Pharmaceuticals</td>
<td>1g/20 mL and 3g/60 mL solutions in sterile water for injection</td>
<td>Same</td>
<td>FDA</td>
</tr>
<tr>
<td>Pharmaceutical Partners of Canada (PPC)</td>
<td>1g vials</td>
<td>Soft tissue sarcoma (first-line single agent therapy and second-line single agent therapy for relapsed patients), pancreatic carcinoma (second-line single agent therapy for relapsed patients) and cervical carcinoma (single agent or in combination with cisplatin and bleomycin).</td>
<td>PPC package insert</td>
</tr>
</tbody>
</table>

2.7.4. Contraindications

Continued use of ifosfamide is contraindicated in patients with severely depressed bone marrow function. Ifosfamide is also contraindicated in patients who have demonstrated a previous hypersensitivity to it.

2.7.5. Adverse effects and toxicity
Myelosuppression and urotoxicity are the major dose limiting toxicities in patients receiving ifosfamide as a single therapy. Gross hematuria associated with hemorrhagic cystitis is a major adverse effect, to overcome which requires administration of mesna, vigorous hydration and dose fractionation. Given below is a table describing the major side effects of ifosfamide and their % incidence.

**Table 2.3** Adverse effects of ifosfamide and incidence of their occurrence. *

* - Baxter Package insert

<table>
<thead>
<tr>
<th>Adverse Reaction</th>
<th>*Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alopecia</td>
<td>83</td>
</tr>
<tr>
<td>Nausea-Vomiting</td>
<td>58</td>
</tr>
<tr>
<td>Hematuria</td>
<td>46</td>
</tr>
<tr>
<td>Gross Hematuria</td>
<td>12</td>
</tr>
<tr>
<td>CNS Toxicity</td>
<td>12</td>
</tr>
<tr>
<td>Infection</td>
<td>8</td>
</tr>
<tr>
<td>Renal Impairment</td>
<td>6</td>
</tr>
<tr>
<td>Liver Dysfunction</td>
<td>3</td>
</tr>
<tr>
<td>Phlebitis</td>
<td>2</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
</tr>
</tbody>
</table>

*Based on 2070 patients

Hematologic toxicity occurs as a result of myelosuppression which is dose dependent. Thrombocytopenia is common in patients receiving escalated doses of ifosfamide [68] while the WBC count drops in the patients receiving ifosfamide in combination with cisplatin or docetaxel [69,70].

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Urotoxicity is one of the most severe adverse effects observed with ifosfamide therapy. It consists of hemorrhagic cystitis, dysuria, urinary incontinence or retention and bladder irritation. This urotoxicity is caused by CAA produced by the metabolism of ifosfamide. CAA depletes ATP levels, glutathione content, and cell growth [71]. Cell culture, animal and clinical studies have reported that CAA reacts with the sulfhydryl (SH) groups in the cells, which in turn deplete the anti-oxidant reserves of the cell thus causing cell death [72].

Neurotoxicity occurs in about 10-30% patients following intravenous infusion therapy of ifosfamide. This neurotoxicity could result in clinical manifestations ranging from mild somnolence and confusion to severe encephalopathy [73]. It increases incidences of CNS toxicity in 50% patients after oral administration because after oral administration, ifosfamide is preferentially metabolized to toxic metabolites following oral administration [74]. The major toxic metabolite is CAA which can cross the blood-brain barrier. There are three mechanisms reported for causing CNS toxicity are (i) a direct neurotoxic effect, (ii) depletion of CNS glutathione, (iii) inhibition of mitochondrial oxidative phosphorylation resulting in impaired fatty acid metabolism [73].

Nausea and vomiting occurs in about 60% of the patients who receive ifosfamide. These adverse effects can be controlled by antiemetics. Other gastrointestinal side effects include anorexia, diarrhea, and in some cases, constipation.

2.7.6. Metabolism

Being a structural analogue of cyclophosphamide, ifosfamide undergoes metabolism in a manner similar to cyclophosphamide. Being a prodrug, it could undergo either activation or deactivation depending upon a critical balance of enzymes. The activation pathway involves
activation through an intermediate 4-hydroxylation to finally form 4-hydroxyifosfamide. The inactivation involves conversion of 4-hydroxyifosfamide to 4-ketoifosfamide by alcohol dehydrogenase (ADH) or, alternatively, to 4-thioifosfamide upon conjugation with reduced glutathione. 4-hydroxyifosfamide being unstable undergoes interconversion into its tautomer aldofosfamide. This aldofosfamide can be converted to either carboxyifosfamide by aldehyde dehydrogenase (ALDH1A1) or to aldoifosfamide by aldo-keto reductase (AKR1) [75]. In a different route of metabolism, aldofosfamide is decomposed to give Ifosforamide mustard [75]. This is the final metabolite, which forms covalent linkage with DNA molecule and cause cross-links between DNA strands [76]. A major difference between the metabolism of cyclophosphamide and ifosfamide is that 4-hydroxy-metabolite is the major product of cyclophosphamide metabolism, whereas one-fourth to about one-half of ifosfamide is metabolized to chloroacetaldehyde through 2- and 3-dechloroethylation. The dechloroethyl metabolites are formed on removal of the chloroethyl group either from the nitrogen in the side-chain in which case it forms 2-dechloroethyl ifosfamide, or from the nitrogen in the ring, in which case it forms 3-dechloroethyl ifosfamide. Both these products are inactive [77]. Liver microsomal enzymes especially cytochromes are actively involved in metabolism of ifosfamide leading to either activation or deactivation. CYP3A4 plays a role in activation by formation of 4-hydroxylation as well as deactivation by dechloroethylation [78,79]. CYP3A5 is another isoenzyme that has also been demonstrated to be involved in the dechloroethylation of ifosfamide [80]. CYP2B6 contributes significantly towards the activation and dechloroethylation of ifosfamide [77].
CHAPTER 3

Analytical Method Development and Validation
3.1. Introduction

Various analytical methods have been developed for quantitative determination of ifosfamide. A quantitative determination method for ifosfamide detection in human serum was developed by Margison, et al., 1986. This method involved solvent extraction, reverse phase HPLC and UV detection at 190 nm. The above mentioned HPLC method was modified and used for reverse-phase HPLC determination of ifosfamide using C-18 column by Mufioz et al., 1996. This modified method utilized water: acetonitrile (70: 30) as the mobile phase and detection was carried out at 210 nm. The HPLC method used in this study is a further modification of the two aforementioned methods and uses water-acetonitrile as mobile phase, C-18 column and detection is carried out at 195 nm. Ifosfamide does not have any conjugate double bonds. It does contain nitrogen which has electrons in non-bonding orbitals which can be excited to pi anti-bonding orbitals using UV light in the range of 190-210 nm. Hence, most methods use wavelength in the range of 190-210 nm capable of exciting phosphoramid group of the molecule.

3.2. Materials

Ifosfamide (≥ 98%) was obtained from Sigma Aldrich (St. Louis, MO). Phenomenex Luna C 18 column (250 x 4.6 mm, 5 µ) was obtained from Phenomenex, Torrance, CA. HPLC grade acetonitrile was sourced from Fischer Scientific (Fair Lawn, NJ).

3.3. Methods

3.3.1. Chromatography

The HPLC chromatograph obtained from Shimadzu Corporation (Tokyo, Japan) was used for the HPLC analysis of ifosfamide. The HPLC system consisted of a binary pump (Model
LC-10AT), an auto injector (Model SIL-9A), a UV-Visible Spectrophotometric detector (Model SPD-6AV), a column oven and a recorder (Model CR-501). The separation of ifosfamide consisted of reverse phase chromatography which was carried out on C-18 Luna column (250 x 4.6 mm, 5 µ; Phenomenex, Torrance, CA). The column was maintained at 40°C for separation throughout the analytical procedure. The mobile phase consisted of water: acetonitrile (70:30 v/v). The apparent pH of the mobile phase was recorded to be 5.8 measured using an UltraBASIC pH meter (Denver Instruments). The separation was carried out using isocratic mode of flow with a flow rate of 1.5 mL/min. The effluents were analyzed at 195 nm. The total run time for each run was set to 7 minutes.

3.3.2. Preparation of solutions

3.3.2.1. Mobile phase

Acetonitrile (300 mL) was mixed with 700 mL water. The solution was agitated to ensure uniform mixing. The solution was then filtered through a 0.45 µ nylon filter (Millipore, Inc) prior to be used in HPLC. The apparent pH of the mobile phase was measured using a pH meter and reported to be 5.8.

3.3.2.2. Standard solutions

The standard solutions of ifosfamide were prepared in the mobile phase. Briefly, ifosfamide powder was weighed (12.5 mg) and added to the mobile phase in a 25 mL volumetric flask to give a stock solution of concentration 500 µg/mL. Various standard solutions were prepared from this stock solution after appropriate dilution with mobile phase.

3.4. Calculations
The standard solutions of ifosfamide were injected on the HPLC. The injection volume was 20μL. A standard curve was constructed by plotting the peak height of the standard corresponding to their respective concentration. The unknown ifosfamide concentration was determined by interpolating from regression equation relating to the peak height, obtained from the standard curve. The peak height gave a better correlation with the concentration as compared to the peak area. When the samples from the release study were injected, tailing was observed in some cases. Hence peak height gave a better with the concentration as compared to the peak area in such cases.

3.5. Applications of the HPLC method

3.5.1. Determination of Drug Loading and Drug loading efficiency

The theoretical drug load of the formulation was calculated by dividing the weight of the drug added to the formulation to the total weight of the formulation ingredients. The drug loading efficiency of the formulation was then determined experimentally. Briefly, 10 mg of the formulation was dispersed in 10 mL Phosphate Buffer (PB) in triplicate and this suspension was centrifuged at 4000 rpm for 30 minutes. The supernatant was removed, filtered, diluted with HPLC mobile phase and analyzed for the drug content. This was used to calculate the percentage drug loading efficiency of the formulation. This is defined as:

\[
\text{Drug Loading Efficiency} = \frac{\text{Amount of drug extracted from formulation}}{\text{Amount of drug initially incorporated in the formulation}} \times 100
\]

3.5.2. In vitro release of ifosfamide from the formulation

The in vitro release of the entrapped drug was determined under sink conditions. The in vitro drug release profile of the formulation was determined by measuring the cumulative
amount of drug released. The *in vitro* release studies were carried out in water-jacketed side-by-side glass diffusion chambers comprising of donor and receiver compartments (3 mL capacity each). Three mL of Phosphate buffer (PB) (pH 7.4) was placed in both donor and receiver compartments. About 15 mg of formulation was dispersed in the donor compartment. The release media in both chambers were stirred using magnetic stirrers. The donor and receiver compartments were separated by a dialysis membrane having a molecular weight cut-off of 1000 Daltons (MWCO 1000 Daltons). The chambers were maintained at 37°C by circulating water through the jacketed system. For sample collection, the whole contents of the receiver compartment were removed at specific time intervals and were replaced with PB. In order to maintain the sink conditions, the amount of solution in the release medium should be < 10 % of its saturated solubility in the release medium. In this case, the amount of solute in the release medium was 0.375 % of the saturated solubility and hence, sink condition was maintained throughout the experiment. Samples were then analyzed using the HPLC method. The *in vitro* release of drug from the delivery systems was also carried out at pH 6.8 to simulate intestinal pH.

3.6. Results and discussion for Assay Validation

3.6.1. Specificity

The International Conference on Harmonization (ICH) documents define specificity as the ability to assess unequivocally the analyte in the presence of the components that may be expected to be present such as impurities, degradation products and matrix components.[81] The term ‘selectivity’ is sometimes used interchangeably with specificity with agencies like IUPAC, AOAC etc which use specificity for procedures that are completely selective. The specificity of
this reversed phase HPLC method was determined by comparing the chromatograms obtained following the injection of mobile phase without drug and the chromatogram of the mobile phase containing the drug. The representative chromatogram of the mobile phase without any drug is shown in Figure 3.1 and the representative chromatogram of the mobile phase with the drug (250 µg/mL) is shown in Figure 3.2. The retention time of the drug was found to be 5.4 minutes. The height of the peak increased proportionately to the concentration of ifosfamide. No peak from the mobile phase was found to interfere with the drug peak. Thus the specificity of this HPLC method was confirmed.

3.6.2. Linearity

The United States Pharmacopeia (USP) defines linearity of an analytical method as its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [81]. In other words, the linearity of an analytical method is defined as the concentration range within which the response of the analyte produced by the detector is linearly proportional to the concentration. The results could be directly proportional to the detector response or they have to be transformed by a well defined mathematical equation. The standard curve for ifosfamide was found to be linear over the concentration range of 5-500 µg/mL. A linear equation of the standard curve for all the standards analyzed was obtained which provided the relation between the concentration of ifosfamide (C in µg/mL) and the peak height of the detector response which is described as:

Peak Height = 333.41 C + 496.22; r² > 0.999

The coefficient of determination (r²) of value > 0.99 is acceptable for an analytical procedure. A high r² value suggests that there is a strong relationship between the peak height and the
Figure 3.1 A representative chromatogram of the mobile phase without drug (ifosfamide).

Figure 3.2 A representative chromatogram of the mobile phase containing ifosfamide (250µg/mL).
concentration in the given concentration range. **Figure 3.3** shows a linear standard curve obtained by injecting ifosfamide standards.

![Graph showing linear standard curve](image)

\[ y = 333.41x + 496.22 \]
\[ R^2 = 0.9999 \]

**Figure 3.3** Standard curve for ifosfamide found to be linear over the concentration range of 5-500 µg/mL

3.6.3. Precision

The USP defines precision of an analytical method as the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample [81]. It is the measure of reproducibility or repeatability of the analytical method under normal operating conditions. Repeatability is defined as the use of the analytical method by the same analyst using the same instrument in same laboratory over a short period of time [81]. The higher the precision, the closer are the values to each other on repeated measurements in identical conditions. For the current assay validation, within day precision and day to day precision were reported. For performing within day precision, a set of ifosfamide
standard solutions was prepared and injected five times on the same day. Day to day precision was performed by injecting a set of ifosfamide standard solutions on four different days over a period of thirty days. A new set of ifosfamide standard solutions was prepared each time for day to day precision since ifosfamide has been reported to degrade in aqueous solutions. The relative standard deviation (RSD) values were calculated for within day (< 10 %) as well as day to day precision (< 15 %) and were found to be within acceptable limits.

**Table 3.1** Within day and day to day precision for HPLC analysis of ifosfamide

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Within day</th>
<th>Day to day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean peak height</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>5.000</td>
<td>1593.60</td>
<td>9.21</td>
</tr>
<tr>
<td>7.800</td>
<td>3087.40</td>
<td>4.70</td>
</tr>
<tr>
<td>62.50</td>
<td>23386.40</td>
<td>2.12</td>
</tr>
<tr>
<td>125.0</td>
<td>46320.00</td>
<td>1.61</td>
</tr>
<tr>
<td>500.0</td>
<td>179715.80</td>
<td>1.68</td>
</tr>
</tbody>
</table>

3.7.4. Accuracy

The USP defines accuracy of an analytical method as the closeness of test results obtained by that method to the true value [81]. The accuracy of an analytical method should be established across its range. Hence, by checking the accuracy of the analytical method, one can make sure that the measured concentration is close enough to the actual concentration of the analyte we want to measure. The accuracy of the HPLC method was determined by analyzing three quality control samples (15.60 µg/mL, 31.25 µg/mL, and 250.00 µg/mL). These samples
were selected to represent the entire range of the standard curve i.e. lower, middle and higher range of the standard curve. These quality control samples were analyzed a total of four times by injecting along with the ifosfamide standards over a period of 30 days. The accuracy of the assay was determined by comparing the theoretical concentration with the actual concentration value obtained by transforming peak height into concentration through the regression equation. The accuracy was measured as percentage and given by the formula:

\[
\% \text{ Accuracy} = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100
\]

**Table 3.2** Accuracy of the HPLC assay method for ifosfamide

<table>
<thead>
<tr>
<th>Theoretical concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.60</td>
<td>14.48±0.42</td>
<td>92.83±2.66</td>
</tr>
<tr>
<td>31.25</td>
<td>30.60±0.66</td>
<td>97.93±2.12</td>
</tr>
<tr>
<td>250.0</td>
<td>255.06±2.59</td>
<td>102.03±1.04</td>
</tr>
</tbody>
</table>

The accuracy values were calculated and found to be within acceptable limits (80 % - 120 %).

3.8. Conclusion

A rapid analytical method for quantification of ifosfamide was successfully developed and validated using reverse phase high performance liquid chromatography. The chromatographic method used water: acetonitrile (70: 30) as the mobile phase with a flow rate of 1.5 mL/min and the separation was carried out on C 18 Luna column (250 x 4.6 mm, 5 µ). The detection of eflluents was carried out at 195 nm using a photo diode array detector. This method
was specific for detection of ifosfamide. The method exhibited a strong linear correlation ($r^2 > 0.999$) between the concentration of ifosfamide and peak height obtained upon ifosfamide elution in the concentration of 5-500 µg/mL. The precision of the analytical method was tested using within day and inter-day precision and was found to be within acceptable limits. The accuracy of the analytical method was determined using quality control samples of concentration 15.6, 31.25 and 250 µg/mL and the RSD was found to be within acceptable limits. This method was used to determine the drug load, drug loading efficiency in the delivery system as well as to evaluate the *in vitro* release characteristics of the drug from the delivery system.
CHAPTER 4

Development and Characterization of the Delivery system
4.1. Introduction

Preparation of nanoparticles for hydrophilic drugs remains a challenge for formulation scientists due to rapid diffusion of the drug to the external aqueous phase and its low entrapment efficiency [82]. The methods used to prepare these nanoparticles include emulsion-diffusion, double emulsification, emulsion coacervation, nanoprecipitation and polymer coating [83]. Many hydrophobic polymers like poly (lactide-co-glycolide) (PLGA), poly-ε-caprolactone (PCL) and polylactide (PLA) have been used previously to encapsulate hydrophilic drugs but drug loading in these polymers has been found to be very low due lack of interaction between hydrophilic drug and the hydrophobic core material [84]. Preparation of solid lipid nanoparticles (SLNs) is one of the approaches used to encapsulate hydrophilic drugs to obtain good sustained-release and better entrapment efficiency [85,86]. Their significance in the field of drug delivery is increasing [32]. SLNs consist of solid lipids in which drugs are dispersed or dissolved and they are stabilized by surfactants. The lipids used for preparing SLNs are solid at room temperature and even body temperature. The lipids mainly used for formulation of SLNs are highly purified triglycerides, complex mixtures of glycerides or even waxes [87]. SLNs prepared by double emulsion method have been used to entrap hydrophilic drugs [88]. Surface modified SLNs have been prepared by coating particles with hydrophilic polymers like chitosan which enhance their cellular uptake [29].

Chitosan is used along with other hydrophilic polymers like alginate for oral delivery of hydrophilic drugs [89,90]. Chitosan is a naturally occurring biodegradable polymer derived by deacetylation of chitin which is obtained from the exoskeletons of crustaceans and insects. It is a co-polymer of D-glucosamine and N-acetyl D-glucosamine which are linked through β 1-4 glucosidic bonds. Some of the N-acetyl groups in chitosan are deacetylated which imparts
different grades of chitosan having different solubility profiles. The degree of deacetylation and molecular weight of chitosan are some of the important factors affecting its solubility, viscosity, and encapsulation efficiency of drugs [36,37]. Due to its ability to open tight junctions and its mucoadhesiveness, chitosan serves the role of absorption enhancer across the intestinal epithelium [51]. Chitosan is generally regarded as biodegradable because some enzymes of the body, such as α-amylase or lysozyme can degrade chitosan [37]. Chitosan has a pKa of 6.5 which makes it insoluble at neutral pH but it is soluble and positively charged at acidic pH [91]. Hence, it can react with negatively charged ions like tripolyphosphate (TPP) and this property is used to form particles of chitosan ionically cross linked with TPP. TPP is capable of providing negatively charged phosphate ions in the physiological pH range which makes this ionotropic gelation feasible at a wide range of pH. Chitosan has also been chemically cross-linked with glutaraldehyde, d-glyceraldehyde and glyoxal [92,93] to obtain microparticles but their toxicity limits their use. The particles formed by cross-linking of chitosan with TPP are biocompatible and hence can be used for oral as well as parenteral delivery of drugs. Chitosan cross linked with TPP has also been shown to provide better acid resistance for controlled release of acid labile drugs than the non cross linked chitosan [94]. Role of cross-linking of chitosan to provide sustained-release of active therapeutic agents, especially hydrophilic drugs and peptides has been widely acknowledged [95,96]. SLNs prepared by Trickler et al, 2010, using Glyceryl monooleate and chitosan have already been demonstrated to sustain release of hydrophilic drug gemcitabine as well as hydrophobic drug paclitaxel. These paclitaxel loaded nanoparticles have been found to be efficacious in vivo to treat breast cancer in mice [97]. Cross-linking of chitosan coated over SLNs could be one of the approaches to sustain the release of hydrophilic drugs from these SLNs.
Glyceryl monooleate (GMO) is an ester of glycerol and oleic acid. When GMO comes in contact with water, it forms lamellar and bicontinuous cubic liquid crystalline phases which act as a reservoir for sustained-release of drugs [98]. It has been used previously to form cubosomes which act as sustained-release matrices for both hydrophilic and hydrophobic drugs [99,100]. It is capable of taking up considerable quantities of water which helps in diffusion of drugs dispersed in it [101]. GMO is a self emulsifying material thus forming different liquid crystalline phases and it can also be used as a lipid phase for dispersing hydrophilic drugs, in which case, it has to be emulsified with other surfactants to form a stable emulsion. This property of GMO has been used to form a stable emulsion which is then lyophilized to produce solid lipid nanoparticles. It has been used to prepare solid lipid nanoparticles to sustain the release of hydrophilic drugs like gemcitabine and hydrophobic drugs like paclitaxel [10,29]. Due to its lipid nature, it can sustain the release of lipid soluble drugs more than it can for hydrophilic drugs. But it can considerably control the release of water soluble drugs when combined with hydrophilic polymers like chitosan. Ifosfamide, being a hydrophilic drug has been used in this study, to formulate SLNs for sustained-release.

Ifosfamide is a DNA-alkylating agent and a structural analogue of cyclophosphamide. It acts as a prodrug, its metabolism occurring mainly through CYP 3A4 and CYP 2B6 enzymes, which are present predominantly in the hepatocytes [26]. It undergoes a series of reactions to form Ifosforamide mustard which is the terminal alkylating metabolite that forms covalent bonds with the nucleophilic moieties in the DNA. This leads to cross-linking of DNA strands which inhibits DNA replication and ultimately leads to apoptosis due to activation of caspases in the cells [75]. Ifosfamide is most frequently administered as solution through i.v. route in a clinical setting. A large fraction of the administered dose of ifosfamide undergoes side-chain
dealkylation to yield N-2-dechloroethylifosfamide (2-DCEIF) and N-3- dechloroethylifosfamide (3-DCEIF). These metabolites lead to the formation of chloroacetaldehyde which is responsible for the nephrotoxic and neurotoxic effects associated with ifosfamide administration [102]. Ifosfamide has a solubility of 100 mg/mL in water which suggests it is a hydrophilic compound [60]. It degrades in the acidic media with its rate of degradation depending on the pH of the solution. The lower the pH of the solution, the shorter is the half-life of ifosfamide [103]. Acid hydrolysis has also been shown to yield 2-DCEIF [104]. Hence, it was sought to develop a delivery system which could stabilize ifosfamide in acidic environment and reduce the formation of the toxic metabolites by preventing its exposure to the acidic medium. Ifosfamide is mostly delivered as continuous intravenous infusion with each cycle of therapy being 3-5 days. This increases the cost of therapy and could lead to patient incompliance. A sustained-release oral formulation of ifosfamide which can sustain its release over a longer period of time and with better stability could prove helpful in decreasing the dosing frequency. Therefore, SLNs of ifosfamide have been developed and characterized to sustain the release of as well as overcome the instability of the drug in acidic environment during oral administration.

4.2. Materials

Ifosfamide (≥98 %) and Rhodamine 6 G were obtained from Sigma Aldrich (St. Louis, MO). Low molecular weight chitosan (MW 10000-12000 Daltons) and polyvinyl alcohol (MW 30000-70000 Daltons) were acquired from Aldrich chemical Co. (Milwaukee, WI). Glycerol monooleate was purchased from Spectrum Chemical (New Brunswick, NJ). Tocopheryl PEG 1000 Succinate NF (TPGS) was sourced from PCCA, Houston, TX. Oleic acid was sourced from Acros Organics, NJ. Spectra/Por® dialysis membrane having a molecular weight cutoff of 1000 Daltons was obtained from Spectrum labs. Acetonitrile for HPLC analysis (HPLC grade),
sodium tripolyphosphate (TPP), sodium phosphate monobasic, sodium hydroxide, hydrochloric acid (reagent grade), Optima grade water; methanol and acetonitrile for LC/MS analysis were obtained from Fisher Scientific (Fair Lawn, NJ). Formic acid (50 % v/v, HPLC grade) was acquired from Fluka analytical (Buchs, Switzerland). Caco-2 cell line was purchased from American Type Culture Collection (Manassas, VA). The Eagle’s Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, L-glutamine, sodium pyruvate, and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA).

4.3. Methods

4.3.1. Formulation of the SLN systems

Chitosan solution was prepared by dissolving 2.4 g in 100 mL water containing 2% (w/v) acetic acid. TPGS solution was prepared by dissolving 150 mg of TPGS in 100 mL water by gentle heating and stirring. A 1.5 % (w/v) solution of sodium TPP was prepared in water. SLNs were prepared by multiple emulsion, followed by a lyophilization process. Briefly, crystalline ifosfamide was dispersed in 300 µl molten GMO at 40°C. To this mixture, 30 µL oleic acid was added, which acts as an (w/o) emulsifier. This dispersion was emulsified with 9 mL TPGS solution (0.15 % w/v) by sonication at 36 W for 2 min (Sonicator 3000, Misonix, Farmingdale, NY). To this primary emulsion, 1.8 mL PVA solution (0.5 % w/v) and 6.25 mL Chitosan solution (2.4% w/v) were added one after the other and emulsified at 30 W for additional 2 min. The chitosan was then ionically cross-linked by adding 10 mL of TPP (1.5 % w/v) and stirred for 30 minutes using a magnetic stirrer. The emulsion was frozen at -80°C, and lyophilized at -52°C under two different conditions of chamber pressure: ~0.04 mBar (high vacuum) and ~0.10 mBar.
(low vacuum). SLN Particles with different ifosfamide loading (5%, 7.5 % and 10 % w/w) were prepared.

4.3.2. Characterization of the delivery system

4.3.2.1. Particle size and surface charge analysis

The particle size and the zeta potential of drug loaded and blank particles were determined using zetameter (ZetaPlus, Brookhaven Instruments Corporation, Holtsville, NY). Briefly, the lyophilized powder was suspended in deionized water at a concentration of 0.2mg/mL and both particles size and zeta potential were determined in triplicate.

4.3.2.2. Scanning electron microscopy (SEM)

The samples for scanning electron microscope (SEM) were mounted on metal stubs and the surface and surface morphology of the particles were examined by a Hitachi S4800 Field Emission Scanning Electron Microscope (Hitachi, Gaithersburg, MD). The analytical parameters included an accelerating voltage of 10 KeV, a working distance of 13.5 mm, and a vacuum 40 Pascals.

4.3.2.3. Karl Fischer titrimetry

Sample (approximately 15 mg) was weighed and analyzed using Karl Fischer titrimetry for the moisture content. The sample was added to the titrating chamber and titrant was added till the end point was reached, which was determined by biamperometric titration. The percentage moisture content of the delivery system was reported.

4.3.2.4. Thermogravimetric analysis (TGA)
For thermogravimetric analysis, approximately 5 mg sample was weighed into aluminum pans and heated from room temperature to 300°C at a heating rate of 10°C/min under nitrogen purge.

4.3.2.5. Attenuated total reflectance infrared spectroscopy

FTIR analyses of chitosan powder, chitosan-TPP particles without drug, and ifosfamide loaded chitosan-TPP nanoparticles were carried out using IR Prestige-21 (Shimadzu, Columbia, MD). The sample was placed in direct contact with ATR crystal ensuring good contact. All the spectra were recorded as a mean of 20 scans, with a resolution of 4 cm\(^{-1}\) and in the range of 800-4000 cm\(^{-1}\).

4.3.2.6. Differential scanning calorimeter

A differential scanning calorimeter (DSC) (Shimadzu DSC-60, Columbia, MD, USA) was used to analyze pure ifosfamide, all the components of the delivery system and the formulations. The sample to be analyzed (3-5 mg) by DSC was crimped nonhermetically in an aluminum pan and heated from room temperature (23°C) to 300°C at a rate of 10°C/minute under nitrogen purge (20 mL/min).

4.3.2.7. X-ray diffraction analysis

X-ray diffraction analysis of the blank and drug loaded nanoparticles as well as blank and drug loaded matrix system was performed at ambient temperature. The sample was filled in copper holder and exposed to Cu K alpha radiation (40kVx40mA) in a wide angle X-ray diffractometer (Bruker, model D8 advance, Madison, WI).
The instrument was operated in a step-scan mode, in 0.05 degree two theta increments, and counts were accumulated for 1.0 second at each step over the angular range of 5 to 40 degree two theta.

4.3.3. HPLC analysis

As previously described, the HPLC separation of ifosfamide was achieved using C 18 Luna column (250 x 4.6 mm, 5 µ; Phenomenex, Torrance, CA). The mobile phase consisted of water: acetonitrile (70:30 v/v). The flow rate was maintained at 1.5 mL/min and the column was maintained at 40⁰C. The column effluents were monitored at 195 nm and quantified using area under the peak for the unknown drug and compared to peak height for the standard solutions prepared in the mobile phase. The method was validated using validation parameters such as linearity, accuracy, within day and inter-day precision.

4.3.4. In vitro release studies

The in vitro drug release profile of the formulation was determined by measuring the cumulative amount of drug released with time. The in vitro release studies were carried out in water-jacketed side-by-side 3 mL glass diffusion chambers containing both donor and receiver compartments. Three mL of Phosphate buffer (PB) (pH 7.4) was placed in both donor and receiver compartments. About 15 mg of formulation was dispersed in the donor compartment. The release media in both chambers were stirred using magnetic stirrers. The donor and receiver compartments were separated by dialysis membrane having a molecular weight cut-off of 1000 Daltons (Spectrum labs) and maintained at 37⁰C by circulating water through the jacketed system. For sample collection, the whole contents of the receiver compartment were removed at specific time intervals and were replaced with equal volume of PB to maintain the sink
conditions. The samples were analyzed for ifosfamide, using the HPLC method. The in vitro release study of the delivery systems was also carried out at pH 6.8 to simulate the intestinal pH.

4.3.5. Determination of drug loading efficiency

The theoretical drug load of the formulation was calculated by dividing the weight of the drug added to the formulation divided by the total weight of the formulation ingredients. The drug loading efficiency of the formulation was calculated experimentally. Briefly, 10 mg of the formulation was dispersed in 10 mL PB in triplicate and this suspension was centrifuged at 4000 rpm for 30 minutes. The supernatant was removed, filtered, diluted with the mobile phase and analyzed for the drug content. The drug loading efficiency (%) of the formulation was determined as follows:

\[
\text{Drug Loading Efficiency} = \frac{\text{Amount of drug extracted from formulation}}{\text{Amount of drug initially incorporated in the formulation}} \times 100
\]

4.3.6. Degradation of ifosfamide

A solution of pH 2.5 was prepared by drop wise addition of formic acid to two hundred mL of LC/MS grade water. This solution was used to dissolve free ifosfamide and suspend the delivery systems. A stock solution of ifosfamide of concentration 1 mg mL\(^{-1}\) was prepared. Solutions of 100 \(\mu\)g mL\(^{-1}\) were prepared in triplicate by diluting 1 mL of the stock solution to 10 mL with pH 2.5 solutions in scintillation vials and degradation kinetics of ifosfamide was studied. To study the degradation pattern of the encapsulated drug, seven milligrams of the formulation was weighed accurately and dispersed in pH 2.5 solutions in triplicate. The vials were kept on a shaker bath at 37°C shaking at 100 rpm. Samples (200 \(\mu\)L) were collected at
regular time intervals using tuberculin syringes with syringe filters and analyzed for ifosfamide using LC/MS.

4.3.6.1. Preparation of sample and standards for LC/MS

The samples were collected in eppendorf tubes and centrifuged at 13000 rpm for 10 minutes in aacuSpin Micro R and diluted with methanol and were analyzed using the LC/MS method as described below. A stock standard solution (1 mg mL\(^{-1}\)) of ifosfamide was prepared in 0.1 % (w/v) formic acid. From this stock solution, standards were prepared in concentration range of 0.1-50 µg mL\(^{-1}\) after appropriate dilution with methanol. A set of newly prepared standards was injected each time with the samples.

4.3.6.2. LC/MS Analysis

Ifosfamide separation was achieved using the Agilent 1200 series liquid chromatograph consisting of a binary pump, an autosampler and a vacuum degasser. The detection and quantitation was carried out using Applied Biosystems 3200 Q TRAP mass spectrometer with electrospray ionization (ESI). Analyst ver. 1.5.1 software (Applied Biosystems) was used for instrument control and data analysis. The separation was achieved using Phenomenex Luna C-18 (250 mm x 4.6 mm x 5 µm) reversed phase column maintained at room temperature. The mobile phase composition was 0.1 % aqueous formic acid (A) and acetonitrile (B). The flow rate was kept at 1.5 mL min\(^{-1}\) and ifosfamide was eluted in isocratic mode having composition 70 % A and 30 % B. The injection volume was kept at 30 µL for samples as well as standards. The effluents were injected directly to the ESI source maintained at 500\(^{\circ}\)C, without any flow splitting. The samples were ionized in positive mode. The spectrometer was used in the MS/MS mode with various Multiple Reaction Monitoring (MRM) fragments obtained. Due to the
isotopic distribution of the two chlorine atoms present in ifosfamide, [MH$^+$] and [MH+2]$^+$ ions were obtained having m/z of 261.022 and 263.023 in Q1 mode. The multiple reaction monitoring (MRM) fragments obtained from Q1 261.022 (m/z) were 92.000 and 63.000 (m/z) and MRM fragments obtained from Q1 263.023 (m/z) were 92.000 and 94.100 (m/z). Amongst these MRM transitions, the transition of 261.022 m/z to 92.000 m/z was chosen for purposes of quantitation. Turbo spray was used as the ion source and the optimized parameters were as follows: declustering potential 71 eV, collision cell entrance potential 38 eV, collision energy 41 eV, collision cell exit potential (CXP) 4 eV and ion spray voltage 5500 eV.

4.3.6.3. Data analysis

All the data was analyzed using Analyst 1.5.1 software. Calibration curves of peak area vs. drug concentration were constructed using standards of known concentration (0.1-50 µg mL$^{-1}$). The degradation rate constants were determined by graphical method.

4.4. Results

4.4.1. Physical appearance of the delivery systems

As described earlier, the multiple emulsions were lyophilized under two different conditions of chamber pressure; high vacuum (< 0.040 mBar) and low vacuum (< 100 mBar). Both these lyophilized formulations exhibited two distinct product morphologies. Product formed at high vacuum had a well defined cake structure. It was found to be fluffy, with low density and floated when dispersed in water. This system was named as the “Matrix system”. The product formed at a low vacuum was found to have a collapsed cake like structure and was in the form of free flowing particles and are called as the “Nanoparticulate system”. The density of these nanoparticles was found to be higher than that of the matrix system and they settled
rapidly when dispersed in water. Both these terminology of matrix and nanoparticulate systems would be used in the rest of the manuscript for these delivery systems and both these systems were characterized further.

4.4.2. Particle size, zeta potential, and drug loading

Both the blank as well as drug loaded nanoparticles exhibited particle sizes in the nano range. The size of the drug loaded particles were $222.8\pm12.4$ nm with a zeta potential of the drug of $-25.3\pm2.1$ mV. Blank nanoparticles without any drug were found to have particle sizes of $217.8\pm3.1$ nm. The blank nanoparticles had a zeta potential of $-38.2\pm1.3$ mV. The matrix system was obtained in the form of a cake which could not be dispersed and therefore, the actual particle size and zeta potential of the matrix system could not be determined.

Three drug loaded (5%, 7.5% and 10% (w/w)) formulations were investigated to determine the optimal drug load in the formulation. Both drug loads of 5 % and 7.5 % (w/w) yielded stable matrix systems while at 10 % drug loading, destabilization of emulsion during the lyophilization was evidenced. This was apparent by the sticky nature of the product obtained after lyophilization. Hence, a maximum drug load of 7.5 % (w/w) was selected for further studies.

4.4.3. Scanning electron microscopy analysis

The surface topography of the matrix as well as the nanoparticulate system was studied using scanning electron microscopy. Figure 4.1 (a) represents the SEM of the matrix system. The micrograph reveals that the surface of the matrix system has a smooth and glass-like appearance. However, Figure 4.1 (b), the SEM of nanoparticulate system showed a granular appearance with a porous structure.
Figure 4.1 SEM images of (a) the matrix system at a magnification of 2000x showing a well-formed cake showing cross-linked chitosan, (b) the nanoparticles system at a magnification of 10000x showing a collapsed cake with agglomerates of nanostructures.
4.4.4. Moisture content and thermogravimetric analysis

The moisture content (% w/w) of the delivery systems as well as the weight loss on heating is shown in Table 4.1. The moisture content of the nanoparticles was found to be significantly higher than the matrix system. The TGA analysis of the nanoparticles showed a weight loss of about 14.6 % up to a temperature of 150°C which could be attributed partially to loss of moisture from the system. The matrix system, however, showed a weight loss of about 9 % when heated up to 150°C as shown in Figure 4.2. The weight loss detected by TGA in both the delivery systems was higher than the moisture content determined by KFT. This suggests that the higher weight loss in the TGA accounts for both the dehydration and loss of other volatile components in the formulation during heating.

Table 4.1 Percentage moisture content and weight loss of the particulate and the matrix system.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Karl Fischer Titrimetry (% Moisture content)</th>
<th>TGA (% Weight loss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate system</td>
<td>11.5±0.2</td>
<td>13.5±1.2</td>
</tr>
<tr>
<td>Matrix system</td>
<td>5.9±0.6</td>
<td>8.9±0.8</td>
</tr>
<tr>
<td>Difference</td>
<td>5.6</td>
<td>4.6</td>
</tr>
</tbody>
</table>
Figure 4.2 TGA curves of the nanoparticles and the matrix system showing weight loss at around 120°C for particulate system while matrix system shows gradual weight loss over a range of 100 to 150°C.
4.4.5. Differential scanning calorimetry analysis

DSC thermograms of all the components of the delivery system and the drug are shown in Figure 4.3 (a). Ifosfamide shows a sharp endothermic peak at 48°C which corresponds to its melting point. GMO also shows a melting endotherm at about 27°C. TPP shows an endotherm at 121°C. The DSC analysis of the delivery systems showed three endothermic peaks at around 60°C, 120°C and 200°C, respectively as shown in Figure 4.3 (b). These peaks were diffuse in blank and drug loaded matrix systems but were well defined in case of blank and drug loaded nanoparticles. Both the DSC and powder XRD analysis of ifosfamide confirms that it is crystalline in nature and its melting point was confirmed to be 48°C by DSC. The DSC analysis of the nanoparticles and the matrix system containing the highest drug load (7.5% w/w) did not show any melting peak for ifosfamide at around 48°C, indicating the drug to be present in the non-crystalline state. This was further confirmed by the powder x-ray diffraction studies as shown in Figure 4.4.

4.4.6. X-ray diffraction (XRD) analysis

The plot of the powder x-ray diffraction patterns of the blank matrix, drug loaded matrix, blank nanoparticulate system and drug loaded nanoparticles is shown in the Figure 4. The XRD analysis of the blank nanoparticles was shown to have a unique fingerprint pattern. The drug loaded nanoparticles was shown to have identical fingerprints to the blank nanoparticles. The
Figure 4.3 DSC thermograms of (a) all the components of the formulations and ifosfamide and (b) all the lyophilized formulations including both blank and drug loaded systems.
**Figure 4.4** An overlay plot of XRD patterns of (a) Blank matrix system, (b) Drug loaded Matrix system, (c) Blank nanoparticles and (d) Drug loaded nanoparticles system
blank matrix and drug loaded matrix systems, on the other hand, showed an amorphous halo patterns.

4.4.7. Fourier transform infrared spectroscopy analysis

The FTIR spectra for chitosan, cross-linked chitosan particles with and without ifosfamide are shown in the Figure 4.5. Cross linked blank and drug loaded particles show a peak for P=O bond at 1151 cm\(^{-1}\). Chitosan gives characteristic peak at 1563 cm\(^{-1}\) which shifts to 1541 cm\(^{-1}\) in cross-linked particles and a new peak appears at 1643 cm\(^{-1}\) after cross-linking with TPP. A strong band occurs in the region from 3200 cm\(^{-1}\) to 3500 cm\(^{-1}\) which becomes very strong bands in the cross linked particles. Peaks at 2924 cm\(^{-1}\) and 2852 cm\(^{-1}\) were also noticed.

4.4.8. In vitro release of ifosfamide

The drug loading efficiency of the particles was experimentally determined to be 98.37±10.9 % (n=3). The delivery systems showed an initial burst release with about 70% drug being released at the end of 24 hours. The release of ifosfamide was gradual thereafter with a plateau being reached after 60 hours Figure 4.6 (a). To investigate the mechanism of drug release, the release data was fitted to Higuchi’s square root equation. A plot of cumulative amount of drug release vs. square root of time was found to be linear [Figure 4.6 (b)]. The release profile of ifosfamide at pH 6.8 was found to be significantly different from that at pH 7.4. Only about 25 % and 28 % of the drug being released from the nanoparticles and the matrix system, respectively at the end of 48 hours in pH 6.8 buffer compared to about 80% and 76 % in pH 7.4 buffer.
Figure 4.5 FTIR analysis of chitosan, blank and ifosfamide loaded nanoparticles systems
Figure 4.6 (a) The *in vitro* release profiles of nanoparticles and matrix systems in Phosphate Buffer (pH 7.4), and (b) a plot of the cumulative amount of drug released vs. square root of time.
4.4.9. Degradation kinetics of ifosfamide in acidic medium

The acid stability studies of ifosfamide incorporated in both the nanoparticulate and matrix systems were carried out at pH 2.5. Figure 4.7 (a) shows the plot of the ifosfamide concentration vs. time. Figure 4.7 (b) gives the ln concentration vs. time, the slope of which was used to determine the degradation rate constant. The degradation rate constant for ifosfamide degradation in solution was calculated in the same way since the degradation rate constant for ifosfamide at pH 2.5 is not available. The concentration of ifosfamide in solution decreased with time in all the three cases. Figure 4.8 shows degradation rate constants of ifosfamide in the solution and the delivery systems.

4.5. Discussion

4.5.1. Formulation of delivery system

The formulation was prepared by a double emulsion method as described by Trickler et al., 2008 where GMO was used as an internal phase. However, this method of preparation has utilized TPGS as an additional surfactant and TPP as a cross-linking agent. GMO is a lipid which can incorporate large amount of water in it and form liquid crystalline phases. Due to this property of GMO, it has been used for sustained-release of both hydrophobic and hydrophilic drugs [105]. The purpose of using oleic acid was to act as stabilizer for water in oil emulsions [106]. It has been previously used to form (w/o) emulsions for oral use [107]. TPGS is another surfactant that was used which acts as an (o/w) emulsifier. It has a HLB value of 13 [108]. PVA is a stabilizer for (o/w) emulsions and was used in the final step of sonication to stabilize the emulsion. Low molecular weight chitosan was used having 85% degree of deacetylation. A
Figure 4.7 (a) A plot of ifosfamide concentration vs. time, and (b) A plot of the ifosfamide concentration (natural log scale) vs. time.
Figure 4.8 First-order degradation rate constants for ifosfamide in free drug solution, nanoparticles system and matrix system
lower molecular weight and higher degree of deacetylation are responsible for improving the solubility of chitosan [109]. Low molecular weight chitosan also helps achieve smaller particle size and better redispersibility of particles in water [29]. Hence low molecular weight chitosan was used for the preparation of delivery systems. An attempt to prepare only GMO nanoparticles without chitosan was unsuccessful. The nanoparticles formed when lyophilized produced a viscous semisolid mass. Incorporation of chitosan onto this lipid surface on the other hand produced a free flowing powder after lyophilization which was well dispersed when no cross-linker were used. Introduction of chitosan also produced nanoparticles with positive surface charge and addition of TPP changed the surface charge to negative. Furthermore, ifosfamide is a highly hydrophilic drug, therefore, a high drug loading efficiency by using only the lipid phase is very difficult. Therefore, the rationale behind coating of chitosan over solid lipid nanoparticles is to: improve free flowing and well dispersed nanoparticles enhance the drug loading efficiency, and modify the drug release profile from these nanoparticles.

4.5.2. Particle size, zeta potential and drug loading

No significant difference was observed in the size of drug loaded and the blank nanoparticles. The size of the particles did not increase significantly in spite of incorporation of drug. Chitosan when dissolved in acidic medium bears a net positive charge due to protonation of free amino groups. On addition of TPP, the protonated amino groups of chitosan interact with \( \text{OH}^- \) and \( \text{P}_3\text{O}_{10}^{5-} \) ions provided by sodium tripolyphosphate. The hydroxyl ions are ionically bonded to the amino groups which causes deprotonation of amino groups [36]. This leaves negatively charged phosphoric ions, which have not been utilized for neutralization of positive charge on chitosan, to provide a net negative charge on to the particles. Adsorption of the negatively charged phosphate ions on to chitosan particles could be the underlying mechanism of
these particles bearing a negative charge. Negative charge on the particles has been shown to cause particles to have a better interaction with intestinal cells [110]. This could be attributed to the ifosfamide and that adsorbed at the surface of the particles. The secondary nitrogen attached to the phosphorus atom of the phosphamide group in ifosfamide could get protonated to give it a net positive charge which causes an increase in the zeta potential of drug loaded particles.

Drug loading is a critical parameter to be optimized in order to efficiently deliver therapeutic amounts of drug to the site of action. Entrapment of hydrophilic molecules like peptides and other low molecular weight compounds is a major challenge for a formulation scientist. It has been shown that as the drug load of hydrophilic compound increases in a polymeric matrix, its entrapment efficiency decreases [111,112]. Moreover, the loading of hydrophilic drugs into a hydrophobic core is severely limited by thermodynamic interactions at the interface of the drug and the lipid [113]. Excess amount of hydrophilic drug in the emulsion leads to emulsion instability; therefore a limited amount of drug can be incorporated.

4.5.3. Scanning electron microscopy, moisture content, thermogravimetric analysis and x-ray diffraction analysis

Lyophilization is considered to consist of three stages; freezing, primary drying and secondary drying. During freezing, most of the water is separated from the solutes to form ice. Due to this, the solute phase becomes highly concentrated. During primary drying, the chamber pressure is reduced and the shelf temperature is increased which facilitates sublimation process. The third step is secondary drying during which desorption of water from the freeze concentrate takes place [114]. Optimization of the process of freeze drying is an important requirement for obtaining the desired product quality. Though, lyophilization helps in increasing product
stability, many problems like particle aggregation are encountered [114]. Lyophilization of emulsions has been shown to produce aggregation of particles. This could be reasoned due to adherence of ice crystals to the particles which on sublimation causes aggregation of the particles left behind [115]. Various stresses are encountered by the formulation during the freezing and drying steps of lyophilization. These stresses cause a significant increase in particle size due to agglomeration [116].

The process of freeze drying has been shown to affect the particle size [117]. The pressure in the lyophilization chamber was found to be a critical parameter controlling the morphology of the particles. The chamber pressure dictates the rate of removal of water from the system and also affects the cake formation. A lower chamber pressure is associated with higher vacuum and a higher chamber pressure is considered a low vacuum. Ideally, a sponge like cake formation having a highly porous structure is the desired product during lyophilization [57]. A sponge like cake was obtained with a high vacuum when the chamber pressure was < 40 µ bars. This cake was fluffy and having a low density which imparts it a property to float when dispersed in the pH 2.5 and pH 7.4 media. The scanning electron micrographs of the matrix system showed a glassy appearance. The nanoparticulate system which is granular in appearance was found to have a high density and settled rapidly when dispersed in the two media. The matrix system was shown to exist in a non-crystalline state whereas the nanoparticulate system was confirmed to have a crystalline nature by the powder X-ray diffraction analysis. It has been shown earlier that absorption of moisture by amorphous solids could promote crystallization. Absorbed moisture has a plasticizing effect on amorphous solids which lowers their glass transition temperature (T_g). A decrease in T_g can increase the molecular mobility which leads to nucleation and crystal growth [58]. The XRD analysis showed that the nanoparticulate system
had a unique fingerprint demonstrating their crystalline nature. The blank nanoparticles showed identical fingerprint pattern to the drug loaded nanoparticles and this observation confirms that the drug in the delivery system was present in a non-crystalline state. Hence, it was deduced that the crystalline nature of the nanoparticles was due to the components of the delivery system with no contribution from drug. The drug loaded matrix system as well as the blank matrix, on the other hand, showed an amorphous halo indicating non-crystalline nature of the matrix systems. The higher moisture content of the nanoparticles was demonstrated by Karl Fischer titration. The presence of higher moisture in the nanoparticulate system could be a result of the low vacuum used during lyophilization which could cause incomplete moisture removal from the product. The matrix system, which was non-crystalline, was found to have low moisture content owing to application of high vacuum during lyophilization. The weight loss determined for both the systems by TGA and the water content determined by Karl Fisher Titrimetry were compared as shown in Table 1. The data clearly indicates that the nanoparticulate system had twice higher moisture content as compared to the matrix system. Interestingly, the weight loss shown by the TGA was found to be higher than the moisture content determined by KFT which indicates the loss in weight within the temperature range was attributed by water loss as well as other volatile components of the delivery system.

4.5.4. Differential scanning calorimetry analysis

The endothermic peak in the DSC thermogram of TPP could be attributed to loss of moisture. This peak disappeared on reheating the sample showing the irreversibility of this peak. The moisture content determined by Karl Fischer titrimetry is in agreement with the weight loss shown in TGA (data not shown). The DSC thermograms of the matrix system further confirmed that it exists in a non-crystalline state which was further reinforced by SEM micrographs and
powder XRD studies. The DSC thermogram of the physical mixture (not shown) was identical to that of the drug loaded nanoparticles but different from the matrix system. Therefore, these studies further confirm that the crystallinity of the materials in the formulations was changed during lyophilization at a high vacuum which leads to formation of the matrix system. The peak at around 200°C in drug loaded nanoparticles was also observed in the physical mixture. Thermal microscopy of the physical mixture provided evidence that crystalline components in the system started melting at this temperature. A possible explanation could be that crystallinity could be disturbed at high temperatures leading to melting of these crystal structures.

4.5.5. Fourier transform infrared spectroscopy analysis

The sharp peak occurs at 1563 cm\(^{-1}\) indicates the bending vibration of amino groups present in chitosan which shift slightly to 1541 cm\(^{-1}\) and a new peak appears at 1643 cm\(^{-1}\) after cross-linking with TPP. A characteristic band between 3500- 3200 cm\(^{-1}\) is seen which is due to O-H and N-H stretching was seen in chitosan. This band is further intensified in the cross-linked particles due to interaction between hydroxyl ions of TPP and amino groups of chitosan. This further confirms the cross-linking of chitosan with TPP in the formulation. TPP molecules cross-link chitosan molecules by either deprotonation or ionic cross-linking [36]. In the process of deprotonation, there is formation of hydrogen bonds between the positively charged amino groups of chitosan and negatively charged hydroxyl groups donated by TPP. Thus, this extensive hydrogen bonding intensifies the peak at 3500- 3200 cm\(^{-1}\). A new peak appears in cross-linked particles at 1151 cm\(^{-1}\) which could be attributed to P=O bond that is derived from TPP. Peaks at 2924 cm\(^{-1}\) and 2852 cm\(^{-1}\) were observed which denote C-H stretching in alkanes. GMO is an ester of glycerol and oleic acid. Oleic acid is a straight chain carboxylic acid composed of 18 carbons. Furthermore, TPGS and oleic acid present in the formulation too have alkyl chains. C-H
stretching in alkanes is seen in the range of 3000- 2850 cm\(^{-1}\). Thus, the peaks at 2924 cm\(^{-1}\) and 2852 cm\(^{-1}\) could be attributed to TPGS, oleic acid and GMO present in the formulation.

4.5.6. *In vitro* release of ifosfamide

During preparation of SLNs, the particles were neither washed nor centrifuged prior to the estimation of drug loading efficiency. The process of washing or centrifuging removes surface adsorbed drug. Hence, the theoretical drug loading efficiency should be close to a 100 % since no drug is removed from the system. This takes into account any free drug present on the surface of the particles that was not entrapped into the nanoparticles. This explains the high drug loading efficiency of the SLNs which is close to a 100 %.

The *in vitro* release studies of ifosfamide nanoparticles and matrix system were carried out at pH 7.4 in order to correspond to the pattern of release of ifosfamide at the intestinal pH. The drug release pattern was plotted as a percentage cumulative drug released vs. time plot. The initial burst release in the delivery system could be accounted for by any free or surface bound drug. To investigate the mechanism of drug release from the delivery system, the cumulative drug release was plotted against square root of time. The linear nature of this plot indicates that the mechanism of release of drug from these delivery systems follows a Higuchi square root type of release, which is a matrix diffusion controlled mechanism of release. The time dependent pattern of release from an insoluble matrix was further confirmed by suspending the delivery system in pH 7.4 buffer up to 24 hours and any degradation of the delivery system was visually observed. For this study, the nanoparticles were compressed into small discs with a die and a punch at a pressure of 1000 psi and dwell time of 10 seconds. These discs were suspended in pH 7.4 buffer in scintillation vials kept shaking at 37°C. The integrity of these compressed disks was
observed over a period of 24 hours. No significant change in the integrity of the delivery system was observed under the experimental conditions.

At pH 6.8, ifosfamide showed a different release pattern as compared to that shown at pH 7.4. There was a significant reduction in the amount of drug released. This significant reduction in the release of drug from the delivery systems could be explained on the basis of interaction between chitosan and TPP. The interaction between chitosan and TPP is a pH dependent reaction [36]. With a decrease in the pH, the degree of ionization of chitosan increases with nearly 50% of it being ionized at a pH around 6.8 and it bears a positive charge. This value significantly drops to less than 10% at a pH 7.4 [118]. Hence, even a small change in the pH causes huge difference in the degree of ionization of chitosan. Greater degree of ionization of chitosan leads to a better cross-linking with negatively charged TPP. The stronger cross-linking could lead to reduction in diffusion of drug from the matrix and hence decrease in drug release.

4.5.7. Degradation kinetics of ifosfamide in acidic environment

Ifosfamide has been reported to be unstable in acidic conditions [103]. The purpose of selection of ifosfamide was to act as a model hydrophilic drug which could successfully be incorporated in SLNs to provide sustained-release and possibly enhance its stability in acidic media. The prepared matrix system and the nanoparticles are intended for oral administration after meals. The pH of the stomach in fed state is around 2.5. Therefore, the degradation of the drug in the free as well as in the nanoparticulate system at this pH was used during the degradation studies under acidic condition. Ifosfamide The degradation rate constant for ifosfamide in solution as well as in the delivery systems were calculated by graphical method from a plot of ln concentration vs. time as shown in Figure 7 (b). These plots were linear in all
the three cases. Thus, the pattern of degradation of ifosfamide in solution as well as in the delivery systems was found to follow first-order degradation rate kinetics. For ifosfamide in solution, first-order degradation rate constant was found to be $0.022 \pm 0.002 \text{ hr}^{-1} (k_f)$. This value of degradation rate constant reported by us for degradation at pH 2.5 lies in between the degradation rate constant values of $0.007 \text{ hr}^{-1}$ and $0.132 \text{ hr}^{-1}$ which have been reported by Gilard et al., 1996 for degradation of ifosfamide at pH 3 and pH 2 respectively. The degradation rate constants for ifosfamide in nanoparticulate and matrix systems were $0.017 \pm 0.000 \text{ hr}^{-1} (k_n)$ and $0.018 \pm 0.001 \text{ hr}^{-1} (k_m)$, respectively. Single factor ANOVA was performed to find if there was any significant difference between the rate constants obtained for degradation of free ifosfamide and ifosfamide in the delivery systems. It was observed that the degradation of ifosfamide in solution was significantly higher ($P$ value < 0.05) than the rate of degradation of ifosfamide in the delivery systems. However, no significant difference was found between $k_n$ and $k_m$ as shown in Figure 4.8 above.

When both the delivery systems were exposed to acidic pH, two kinetic processes are supposed to occur simultaneously. The first one is the release of the drug from the delivery system to the release medium followed by the degradation of the released drug in the medium. These two processes could have different rates. When the rate of release of the drug is higher than the degradation rate, one should expect a possible increase in the concentration of ifosfamide in the release medium with time. However, the concentration of the drug in the release medium decreased when the rate of degradation of drug is higher than the rate of its release. We observed a similar phenomenon in case of ifosfamide released from the delivery systems, where the concentration of ifosfamide increased during the initial stages while during the later stages, a decrease in concentration was observed as shown in Figure 4.7 (a).
4.6. Conclusions

In summary, a sustained-release delivery system for oral delivery of ifosfamide was successfully developed. The delivery system was characterized as nanoparticles and matrix system, depending on the morphology of the product obtained after lyophilization. The matrix system, formed from intact cake while the nanoparticles formed from a collapsed cake. Thus, we were able to demonstrate that different chamber pressures during lyophilization led to formation of products having different morphology. The drug in delivery systems was found to be in a non-crystalline state. Both the delivery systems provided sustained-release of drug at pH 6.8 and pH 7.4. These delivery systems were efficient in retarding the degradation of ifosfamide under acidic conditions by increasing the stability of ifosfamide in acidic medium.
CHAPTER 5

Cellular uptake and Sub-cellular localization
5.1. Introduction

The focus of this study was to study the permeability of ifosfamide solution and ifosfamide loaded delivery systems across the Caco-2 cells. Caco-2 cells are commonly used as an in vitro model for intestinal absorption [119]. Preparatory phase of this study involves growing Caco-2 cell monolayers (obtained from ATCC) on transwell plates. These plates have six wells which are composed of nylon membranes on which the cells are grown. The apical side of the cells faces up while the basolateral side of the cells faces down. The growth media is provided to both the apical as well as the basolateral sides of the cells. The cells are evaluated for transepithelial electrical resistance (TEER) values in order to ensure that the membranes are intact and there are no leakages in them. When the TEER values are consistently above 300 $\Omega \text{cm}^2$, the membranes are considered to be intact, which could be used for performing permeability studies. These membranes are mounted in diffusion apparatus and equilibrated for about 30 minutes with Tyrode buffer before the experiment begins. The delivery systems for ifosfamide are intended for oral delivery and hence, it is very important to study their permeability across intestinal cells.

Mucoadhesive polymers like chitosan can interact with the mucous membranes present on the epithelial cells [97]. This phenomenon is primarily due to the interaction between positive charges present on chitosan and the negative charges on mucin. Apart from the interaction dependent on charges, endocytosis is one of the most common ways of uptake of nanoparticles by the cells. These endocytotic pathways include phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis [120]. These pathways could be useful for uptake of negatively charged particles into the cells. The nanoparticles taken up by the cells are normally internalized into the lysosomes where they could be broken up to release their contents.
5.2. Materials

Caco-2 cell line was purchased from American Type Culture Collection (Manassas, VA). The Eagle’s Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, L-glutamine, sodium pyruvate, and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA).

5.3. Caco-2 permeability studies

Caco-2 cell monolayers were cultured on a tissue-culture-treated polycarbonate filter (diameter 24.5 mm, growth area 4.7 cm$^2$) in BD Falcon Transwell 6 wells/plate (BD, NJ) and were used for permeability experiments approximately 15 days after seeding (TEER values in the range of 380–400 Ωcm$^2$). On the day of the experiment, these membranes were mounted between the donor and receiver compartments of the diffusion chambers. Three mL of Tyrode buffer (pH 7.4) was placed in the receiver compartment. A solution of ifosfamide having concentration of 500µg/mL was prepared in Tyrode buffer. This constitutes about 0.5 % of the saturation solubility of ifosfamide. A suspension of the delivery system at a concentration of 2.33 mg/mL was prepared in Tyrode buffer. The drug solution and nanoparticulate suspensions were placed separately in the donor compartments of the diffusion chambers. Samples (200 µL) were collected at fixed intervals and replaced with Tyrode buffer. The analysis of the samples was done using UPLC.

The apparent permeability coefficients (Papp, cm/s) of ifosfamide were calculated according to the following equation [121]:

$$P_{app} = \frac{Q}{Act}$$
Where, “Q” is the total amount of ifosfamide permeated (µg), “A” is the diffusion area of the cell monolayers (cm²), “c” is the initial concentration of the ifosfamide in the donor compartment (µg/cm³) and “t” is the total time of the experiment. The permeability coefficient was calculated for diffusion of drug from apical to basolateral membrane as well as from basolateral to the apical side.

5.4. Sub-cellular localization studies

The *in vitro* cellular association and sub-cellular localization of the delivery systems were evaluated in Caco-2 cell line. In these studies, cells were cultured on Falcon multiwell slides. Briefly, the cells were seeded in a multiwell cell culture slide at a density of 10,000 cells per well and incubated overnight in a humidified chamber at 37°C in the appropriate growth medium. The cells were treated with the delivery system loaded with rhodamine 6 G (fluorescent laser dye) as a function of time (2 and 10 min) in Dulbecco’s Phosphate Buffered Saline (DPBS) spiked with lysotracker green (50 nM). The adherent cells were washed three times in DPBS and fixed with glutaraldehyde (1%). The wells were removed and the cells were further stained with mounting media consisting of DAPI (1.5 μg/mL) and sealed with coverslips. The slides were viewed on a multi-photon confocal microscope (Carl Ziess, Germany) at the Nebraska Center for Cell Biology at Creighton University.

5.5. Results

5.5.1. Caco-2 Permeability studies

Permeability of free drug from apical to basolateral (A to B) and basolateral to apical (B to A) was studied. Similarly, ifosfamide encapsulated in particulate and matrix systems was also
studied. The apparent permeability coefficient of free drug from A to B was found to be (Papp x 10^{-4} \text{ cm/s}) 36.75±8.84 while its permeability from B to A was found to be (Papp x 10^{-4} \text{ cm/s}) 14.29±2.06. For ifosfamide encapsulated in particulate and matrix systems, the apparent permeability coefficient from A to B were (Papp x 10^{-4} \text{ cm/s}) 14.59±0.86 cm/s and 12.51±4.68 cm/s respectively while Papp values for B to A were (Papp x 10^{-4} \text{ cm/s}) 12.21±0.59 cm/s and 11.9±3.31 cm/s, respectively. **Figure 5.1** shows the A to B and B to A permeability of ifosfamide free drug, nanoparticles and the matrix system. Statistical analysis using Single Factor ANOVA was performed to compare the A to B and B to A permeability of the drug in all three cases.

5.5.2. Subcellular localization studies

The subcellular localization of the delivery systems was evaluated in Caco-2 cells using confocal microscopy techniques. The nanoparticles containing Rhodamine 6 G appear red, the nuclei stained by DAPI appear blue and the lysosomes stained by lysotracker green appear green in color. The nanoparticles appear to accumulate in the cells in a time dependent manner (**Figure 5.2**). The delivery systems were found to internalize in the lysosomes which was confirmed by co-localization of the red fluorescent dye with lysotracker green.

5.6. Discussion

5.6.1. Caco-2 Permeability studies

Clinically, ifosfamide is mostly given as intravenous injection. Hence, its permeability across the intestinal epithelium has not been studied to a significant extent. Therefore, it was attempted to study the permeability of ifosfamide using Caco-2 cells which are colorectal adenocarcinoma cells. Permeability studies of drugs across Caco-2 monolayers could be used as
an *in vitro* model for intestinal absorption of drugs [119]. Compounds with Papp of > $10^{-5}$ cm/s are considered to have a high permeability which suggests that ifosfamide is highly permeable in

**Figure 5.1** Permeability of free drug, the nanoparticles system, and the matrix system through Caco-2 cell monolayers.

![Caco-2 Permeability studies](chart.png)
Figure 5.2 Cellular accumulation and subcellular localization of the fluorescent nanoparticles in the Caco-2 cells (a) 2 min and (b) 10 min
both the directions [122]. It was found that the permeability of ifosfamide in solution was significantly higher from A to B than B to A. This suggests that there is lack of efflux mechanisms which could drive ifosfamide from basolateral to apical side. The high permeability of ifosfamide could be attributed to its small size and partition coefficient (0.57) suggesting it has some degree of lipophilicity which helps it to partition into cells. Thus, passive diffusion could be the mechanism underlying permeability of ifosfamide across Caco-2 cells.

For ifosfamide encapsulated in the matrix system, no significant difference was observed in permeability of drug from A to B compared to its permeability from B to A. However, in the case of drug encapsulated in the particulate system, the permeability was found to be significantly higher from A to B compared to B to A. This finding suggests that the drug encapsulated in the particulate system could be selectively taken up by cells. Nanoparticles, due to their small size can easily enter cells and various cellular compartments by the process of endocytosis. It is interesting to note however, that B to A transport was significantly less which could be due to a greater extent of cellular efflux from the basolateral side. It has been shown that P-glycoprotein, which are efflux transporters present in Caco-2 cells are mitigated well by ifosfamide [123]. Hence, it could be hypothesized that other efflux mechanisms could be involved in the ifosfamide efflux from basolateral side.

5.6.2. Subcellular localization studies

It is very important that the dye-loaded nanoparticles used during the sub-cellular localization studies do not leach out the dye or release their contents during the period of the experiment otherwise a high background is obtained while taking pictures in the confocal microscope. Rhodamine 6G is a highly hydrophobic dye which would be embedded in the lipid core of the nanoparticles, and hence, was used to prepare the nanoparticles. The subcellular
localization of nanoparticles was evaluated in Caco-2 cells. Several mechanisms have been described for internalization of nanoparticles inside the cells. These include the clathrin and caveolae mediated endocytosis and endo-lysosomal mechanism [124]. In the present study, the mechanism of internalization of nanoparticles in the cells appears to be endo-lysosomal supported by the fact that the red fluorescent dye is co-localized with the lysotracker green dye. However, no red fluorescent dye was seen to be co-localized with the DAPI stain which suggests that the particles did not internalize in the nuclear compartments of these cells. Nanoparticles are accumulated in the cells by energy driven processes like endocytosis [124]. The size of particles plays an important role in the mechanism of uptake. Particles with sizes less than 700 nm are suitable for uptake by endosomal route and internalized in the cells [125]. The uptake of the nanoparticles by the cells confirms that the drug may be transported across the Caco-2 cell monolayer by transcellular pathway.

5.7. Conclusions

The particulate system was found to be efficient in enhancing permeability of the drug across Caco-2 cell monolayer and dye-loaded nanoparticles were found to be rapidly accumulated and internalized in Caco-2 cells.
CHAPTER 6

Summary and Future Directions
6.1. Summary

A sustained-release solid lipid nanoparticulate (SLN) formulation was developed for the sustained-release of hydrophilic drug ifosfamide. The chitosan in the SLNs was cross-linked with sodium tripolyphosphate. The formulation was prepared using double emulsion solvent evaporation method. The formulation lyophilized at different chamber pressures gave products having different product morphologies. Product lyophilized at high chamber vacuum was obtained as matrix system while nanoparticles were formed at low chamber vacuum.

The nanoparticles were crystalline in nature while the matrix system was non-crystalline as confirmed by XRD. The nanoparticles had higher moisture content as compared to the matrix system. Presence of cross-linked chitosan in both the matrix system and the nanoparticles was showed using FTIR. The melting endothermic peak of the drug was absent in both the delivery systems suggesting it to be in a non-crystalline state.

A rapid, precise and accurate reverse phase HPLC method was developed and validated for the quantitative estimation of ifosfamide. The separation was achieved on a C 18 Luna column. The mobile phase used had a composition of water:acetonitrile (70:30 v/v). This HPLC method was utilized to determine the drug loading efficiency and in vitro release characteristics of the drug from the delivery system. LC/MS was used to evaluate the degradation of the drug both in solution and in the delivery systems.

The in vitro release studies carried out in phosphate buffer of pH 7.4 showed a sustained-release of the drug up to 5 days when almost 90% of the drug release was achieved. The delivery systems were found to be capable retarding the degradation of ifosfamide as compared to free drug in acidic medium of pH 2.5.
The nanoparticles showed higher permeability from apical to basolateral membrane as compared to basolateral to apical membrane in Caco-2 cells. The nanoparticles were also seen to be taken up by the Caco-2 cells in a time dependent manner and accumulated in the lysosomes.

6.2. Future directions

Future continuation of the work could focus on development of animal models to test the \textit{in vivo} efficacy of the formulation. Animal (rodent) models for germ cell cancer and cervical cancer could be developed to carry out safety and efficacy studies of this formulation since ifosfamide is used in treatment of testicular and cervical cancer. Toxicity studies of blank formulation could be carried out to assess the toxicity of the components of the formulation. These could include local tissue and whole organ toxicity studies.

Since the formulation is able to sustain the release of a highly hydrophilic drug, this formulation could be tried for encapsulation of other hydrophilic drugs in order to achieve sustained-release. The formulation has demonstrated protective effect on acid labile drugs. Hence it could be used for encapsulating other acid labile drugs to enhance their stability in acidic media.


