PREFORMULATION STUDIES ON THE ANTI-CANCER DRUG IMEXON

By

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Philip Kuehl
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DEDICATION

To Kallie and My Family
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ABSTRACT

Imexon is an aziridine-containing iminopyrrolidone that is of significant interest due to its selective growth inhibitory effect against multiple myeloma. Regrettably, administration of Imexon has proven difficult largely due to its rapid degradation in aqueous medium. The collective aim of this research was to conduct preformulation studies to characterize and understand the stability and solubility of Imexon in both aqueous and non-aqueous systems. Furthermore, these data will be utilized as rational support to create an efficacious formulation for the delivery of Imexon.
CHAPTER 1: INTRODUCTION

1.1 DRUG OVERVIEW

Imexon is a member of a group of 2-cyanoaziridine derivatives, which have been under investigation since the 1970’s due to their anticancer properties (Bicker, 1975; Bicker and Fuhse, 1975). Imexon has shown activity in several tumor cell lines and animal tumor models and does not appear to be susceptible to multiple drug resistance. Preclinical data has shown the efficacy of Imexon to be schedule dependant favoring prolonged exposure times (Dorr et al. 1995; Hersh, 1992; Sagaster et al. 1995). Furthermore, Imexon has proven to be well tolerated in all species studied (Dvorakova et al., 2002a). Current clinical trials for cancer require administration of the drug via IV infusions for five days to maintain therapeutic drug levels for prolonged time periods.

1.2 CANCER BACKGROUND

Cancer is a collection of diseases that share one common theme, that is, the unregulated growth and prospering of mutant cells at the expense of healthy cells. Cell growth and reproduction is regulated by several cellular processes; mutations at the cellular level can result in these processes functioning improperly, or not at all. These mutations can be caused either by external or internal factors. When mutated cells are allowed to proliferate, a mass can arise. This mass of growing, abnormal cells is called a tumor.
Tumors are classified into two different groups; benign or malignant, based on whether or not they enclose the abnormal cells. A benign tumor is defined as a tumor that is encapsulated in a single mass and can be surgically removed. A benign tumor is not cancer. A tumor is only considered cancer if it is malignant; meaning that its cells have the ability to invade other parts of the body. Cancerous cells can spread throughout the body via the bloodstream or the lymphatic system. These circulating cancer cells can metastasize or form tumors in other parts of the body.

Cancers are classified based on the type of tissue or cell type from which they originate. Carcinomas are cancers that develop from epithelial cells; sarcomas develop from muscle cells or connective tissue. Leukemia’s are cancers that arise from nervous tissue and do not fit into the other two categories.

Cancerous cells can be treated by surgery, drug therapy, radiation, or any combination thereof. The complexity in treating cancer lies in the inability of these therapies to destroy all cancer cells without simultaneously destroying healthy cells. New therapies are exploring methods that selectively deliver therapeutic agents to cancerous cells either by providing local drug delivery, thereby decreasing systemic exposure, or utilizing a specific characteristic of cancer cells so that therapeutic agents only attack cancer cells.
1.3 DRUG BACKGROUND

1.3.1 Drug Description

Chemical Name: 4-imino-1,3-diazabicyclo[3,1,0]-hexan-2-one

Chemical Formula: C<sub>4</sub>H<sub>5</sub>N<sub>3</sub>O

Molecular Weight: 111.1

Appearance: white powder, odorless

Chemical Structure:

![Chemical Structure Image]

1.3.2 Mechanism of Action

Imexon has shown activity against cancers of the pancreas, lung, breast and prostate either alone or in combination with other clinical agents. It has been shown to selectively inhibit growth of multiple myeloma. The activity of Imexon is through opening of the aziridine ring moiety and results in binding to the sulfhydryl groups on cytosolic proteins and cellular thiols. This binding causes a depletion of cellular thiols resulting in mitochondrial swelling, leakage of cytochrome C and ultimately oxidative stress and
subsequent apoptosis. The affected tumor cells have their cell cycle stopped and accumulate in either S-phase or G2 phase (Dvorakova et al., 2000; Dvorakova et al., 2001; Dvorakova et al., 2002b; Iyengar et al., 1999; Iyengar et al., 2004; Samulitis et al. 2006).

1.3.3 Preclinical Pharmacokinetics

Preclinical pharmacokinetic parameters detailing absorption, volume of distribution, clearance and area under the plasma concentration-time curve (AUC) have been obtained from studies conducted in mice and dogs. In mice, a single IP dose of 100 mg/kg resulted in a biphasic concentration-time curve with peak plasma concentrations of 100 µg/mL. The terminal half-life for this dose was 29 minutes. Single IV doses of 12.5 and 25 mg/kg in dogs also showed similar biphasic profiles. Peak plasma concentrations were slightly higher and the terminal half-life was about 90 minutes. An oral dose of 100 mg/kg, administered to mice, results in an oral bioavailability of 20%, based on AUC comparison (Dorr et al., 1995). Further preclinical studies indicated that Imexon had a maximally tolerated dose, in mice, of 150 mg/kg (Pourpak et al., 2006).

1.4 SPECIFIC AIMS OF THIS STUDY

Despite the known therapeutic effectiveness of Imexon, little is known about its solubility and stability. The purpose of this study is to first characterize the raw drug material and
subsequently determine the solubility and stability of Imexon in a variety of different media. In order to achieve this goal, the following studies were preformed:

◊ Determination of the solid state characteristics of the raw active pharmaceutical ingredient (API), Chapter II.

◊ Preformulation studies exploring the solubility and stability of Imexon in aqueous medium, Chapter III.

◊ Development and evaluation of an enteric coated oral formulation, Chapter IV.

◊ Exploration of Imexon solubility and stability in a pressurized metered dose inhaler, Chapter V.
CHAPTER 2: SOLID STATE DRUG CHARACTERIZATION

2.1 INTRODUCTION

Characterizing the solid-state properties of a drug is essential prior to any preformulation studies or formulation development. These data establish a solid-state fingerprint that functions as a foundation from which one can obtain subsequent data. Furthermore, this fingerprint allows a research investigator a basis for explaining an unexpected discovery. In this study the following solid-state experiments were conducted: differential scanning calorimetry, thermogravimetric analysis, photomicroscopy, powder x-ray diffraction pattern, single-crystal structural determination (via x-ray diffraction) and nuclear magnetic resonance.

2.2 EXPERIMENTAL

2.2.1 Materials

Imexon was provide by the AmpliMed Corporation and was used as received. Ethanol was received from Aaper (Shelbyville, Kentucky).

2.2.2 Differential Scanning Calorimetry
The melting point of the raw drug substance was determined via thermal analysis with a Q1000 Differential Scanning Calorimeter (DSC) (TA Instruments, New Castle, DE, USA) with an auto sampler. Indium was used for the calibration of the DSC. Samples of 1 to 2 milligrams were weighed and placed into an aluminum pan and crimped with an aluminum lid. Samples were equilibrated and isothermally heated at 30°C for five minutes, followed by heating at 5°C/min up to 300°C. A nitrogen purge was used at 40 ml/min for each sample.

2.2.3 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was performed using a TA Instruments Q50 TGA. Samples of 1 to 2 milligrams were placed into an empty aluminum pan and heated at 5°C/minute up to 300°C. Weight loss as a function of temperature was analyzed under nitrogen at 60 ml/minute purge.

2.2.4 Polarizing Microscope

A Leica DMLP polarizing microscope (E. Licht Co., Denver, CO) was used to visually inspect Imexon crystallites. The powder was examined under light and cross-polarized light; photomicrographs were taken using a Nikon E8000 camera, which was attached to the microscope.
2.2.5 X-ray Powder Diffraction

X-ray powder diffraction (XRD) was conducted at ambient temperature with a PANalytical X’Pert PRO MPD system (Panalytical Inc., Tempe, AZ, USA) with copper (Kα) radiation (λ = 1.54 Å) at 50 KV (40 mA target current). High-resolution scans were conducted along the goniometer axis (θ/2θ) at a step size of ca. 0.0167°. A fixed divergent slit of 1/4° was used in the incident beam optics, followed by an anti-scattering slit of 1/2°. X’Celerator, a RTMS detector (real time multiple strip), was used in the diffracted beam optics. Approximately 4000 scans were taken between 2-Theta of 5.00° and 74.99°. Samples were placed on a silica zero background holder and rotated at 0.25 revolutions per second. Data were collected with an X’Pert Data Collector version 2.0 and analyzed with X’Pert Pro version 1.4 software. The XRD patterns were compared via peak position and relative intensity.

2.2.6 Solid State Analysis of Various Lots of Imexon Active Pharmaceutical Ingredient

In order to determine the robustness of the Imexon synthesis and crystallization process the solid state characteristics of five different lots have been analyzed by DSC, TGA, microscopy and XRD.

Lot numbers were as follows:

◊ Cambrex Lot 15106355
2.2.7 Single Crystal Structural Determination

Single crystal structural determination was conducted using a Bruker SMART 1000 CCD detector X-ray diffractometer. Data collection and cell refinement was conducted with APEX2, data reduction was done on SAINT and SHELX97 was used to solve and refine the structure (Bruker, 2005; Bruker, 1997a; Bruker, 1997b; Sheldrick, 1997). The structure was solved using direct methods on non-overlapped reflections from domain one and was refined using all reflections from both components including overlaps using SHELXS in the Bruker SHELXTL (Version 5.0) software package. Merging of equivalent reflections was not performed due to the overlap of twin components. The correct unit cell and twin law relating two components was determined using the program CELL_NOW (Sheldrick, 2005). Data were corrected for absorption and decay effects with TWINABS (Sheldrick, 2005). Visual aids were produced with MERCURY (Macrae et al., 2006).

Twinning of the single crystals examined was obvious from examining initial diffraction patterns and concurrent difficulty in indexing. The correct unit cell and twin law relating
two components was determined using the program CELL_NOW (Sheldrick, 2005). Twinning occurred via a 180° rotation about the real $a$ axis.

2.2.8 Single Crystal Growth

Pharmaceutical grade Imexon was obtained as a free flowing powder from AmpliMed Corporation (Tucson, AZ, USA). Imexon crystals were grown from absolute ethanol. The ethanol was heated to 313 K and saturated with an excess of Imexon the solution was then vortexed. Following vortexing, the solution was filtered through a 0.2 µm PTFE filter. The filtrate was allowed to cool slowly to room temperature (296 K) and was loosely capped to allow for slow evaporation of solvent.

2.2.9 Nuclear Magnetic Resonance

NMR samples were analyzed on a Bruker AM-250 NMR. Samples were dissolved in DMSO for collection of 64 scans in $^1$H mode.

2.3 RESULTS AND DISCUSSION

2.3.1 Differential Scanning Calorimetry and Thermogravimetric Analysis
Analysis of pure Imexon via DSC showed an exothermal event initiating near 165°C and reaching a maximum at 201°C, (Figure 2.1). Integration of this peak gives an enthalpy for this event to be 949.3 J/g. This thermograph does not indicate an endothermic event indicative of melting and suggests that the API may not be crystalline. However, examination of Imexon crystallites, under magnification with cross-polarized light, showed birefringence, indicating crystallinity, as seen in Figure 2.2.

Figure 2.1 DSC (Heat Flow) and TGA (Weight %) overlay of Imexon raw drug substance.
Figure 2.2 Imexon crystallites, under 400x magnification, with cross-polarized light.

Figure 2.1 also includes TGA analysis of the Imexon raw drug substance. It can be seen that the onset of weight loss is at or near the same temperature of the thermal event seen on DSC (165°C). In order to further investigate this thermal event, Imexon was heated and isothermally held at 155°C for 15 minutes. No weight loss was detected, however, changes in the physical appearance of the material were observed. The resultant material had changed in color from the initial white powder to a brown powder. Upon cooling attempts were made to solubilize the material with a variety of solvents, including water, ethanol, acetonitrile and tetrahydrofuran. The solubility properties of the isothermally held material were different than those of Imexon parent and subsequent HPLC analysis confirmed that the parent Imexon had been decomposed.
2.3.2 X-ray Powder Diffraction

XRD allows for rapid and nondestructive structural analysis of a solid material. A crystalline material will produce sharp peaks caused by diffraction of the x-ray beams. While an amorphous material will produce a halo, this halo is essentially background noise from the detector. While preparing a sample for XRD analysis it is important to ensure that there is a random assortment of crystal orientations (Byrn et al., 1999). This is accomplished by lightly grinding larger crystals, without over grinding and disrupting the repeating lattice.

The XRD data in the case of Imexon showed several shape peaks, indicating that Imexon API is indeed crystalline. This is shown in Figure 2.3.
2.3.3 Solid State Analysis of Cambrex Lot 15106355

Visual examination of the Cambrex lot of API under polarized light microscopy indicated some degree of birefringence, Figure 2.4. Particle size varied widely and was largely dependent on the amount and nature of physical handling of the API. Resultant size ranged from 5 microns to ~20 microns. As a result of the delicate nature of the crystals, no clear crystal habit is present.
As the Cambrex lot 15106355 was used for the majority of the studies detailed herein, it was used as the reference material for the other API lots. As such, the thermal analysis presented in Section 2.3.1 is for this lot and the XRD data are discussed in Section 2.3.2.

2.3.4 Solid State Analysis of IMX024

Light microscopy showed some birefringence of the drug when viewed with cross-polarized light. Particle size varied widely and was largely dependent on the amount and nature of physical handling of the API. Resultant size ranged from 5 microns to ~20 microns, Figure 2.5. Due to the delicate nature of the crystals, there is no clear crystal habit seen in this API lot.
Figure 2.5 Light microscopy photos of IMX024.

Thermal analysis of IMX024 provided the overlay of DSC with TGA shown in Figure 2.6. This lot shows a strong exotherm beginning at ~120°C and reaching its peak at 187.41°C. The integration of this peak indicates that this lot gives off 906 J/g of energy in this process. TGA data indicate significant weight loss beginning near the completion of this process, probably due to decomposition. The specific nature of this process is not known; however, it should be noted that no melt of this lot is seen on DSC.
Figure 2.6 DSC/TGA overlay of IMX024 pure API.

Powder x-ray analysis of IMX024 displayed sharp peaks beginning at ~ 16 θ, which clearly indicates that the sample is crystalline in nature, Figure 2.7. No analysis was conducted to determine the unit cell or crystal structure of these crystals.
Figure 2.7 XRD pattern for IMX024 raw API.

2.3.5 Solid State Analysis of IMX028

Light microscopy showed some birefringence of the solid drug when viewed with cross-polarized light. Particle size varied widely and was largely dependent on the amount and nature of physical handling of the API. Resultant size ranged from 5 microns to ~25 microns, Figure 2.8. Due to the delicate nature of the crystals, there is no clear crystal habit seen in this API lot.
Thermal analysis of IMX028 provided the DSC/TGA overlay shown in Figure 2.9. The DSC exhibits a strong exotherm beginning at \( \sim 130^\circ C \) and reaching its maximum at 188.35°C. The integration of this peak indicates that this lot gives off 1001 J/g of energy in this process. The TGA data indicate significant weight loss beginning near the completion of this process, probably due to decomposition. The specific nature of this process is not known. However, it should be noted that no melt of this lot is seen on DSC.

Figure 2.8 Light microscopy photos of IMX028.
Figure 2.9 DSC/TGA analysis of IMX028 raw API.

Powder x-ray analysis of IMX028 displayed sharp peaks beginning at ~ 16 θ, which clearly indicates that the sample is crystalline in nature, Figure 2.10. No analysis was conducted to determine the unit cell or crystal structure of these crystals.
2.3.6 Solid State Analysis of SCR 84-43

Light microscopy showed some birefringence of the solid drug when viewed with cross-polarized light. Particle size varied widely and was largely dependent on the amount and nature of physical handling of the API. Resultant size ranged from 5 microns to ~25 microns, Figure 2.11. Due to the delicate nature of the crystals, there is no clear crystal habit seen in this API lot.
Figure 2.11 Cross polarized light microscopy of SCR 84-43.

Thermal analysis of SCR 84-43 provided the overlay shown in Figure 2.12. The DSC for this lot shows a strong exotherm beginning at ~140°C and reaching its peak at 194.94°C. The integration of this peak indicates that this lot gives off 860.9 J/g of energy in this process. The TGA data indicates significant weight loss beginning near the completion of this process, probably due to decomposition. The specific nature of this process is not known. However, it should be noted that no melt of this lot is seen on DSC.
Figure 2.12 DSC/TGA overlay of SCR 84-43 raw API.

Powder x-ray analysis of SCR 84-43 displayed sharp peaks beginning at ~ 16 \( \theta \), which clearly indicates that the sample is crystalline in nature, Figure 2.13. No analysis was conducted to determine the unit cell or crystal structure of these crystals.
2.3.7 Solid State Analysis of SCR 84-44

Light microscopy showed some birefringence of the solid drug when viewed with cross-polarized light. Particle size varied widely and was largely dependent on the amount and nature of physical handling of the API. Resultant size ranged from 5 microns to ~20 microns, Figure 2.14. Due to the delicate nature of the crystals, there is no clear crystal habit seen in this API lot.

Figure 2.13 XRD pattern for SCR 84-43 raw API.
Thermal analysis of SCR 84-44 provided the overlay shown in Figure 2.15. The DSC for this lot shows a strong exotherm beginning at ~140°C and reaching its peak at 194.58°C. The integration of this peak indicates that this lot gives off 928.7 J/g of energy in this process. The TGA data indicates significant weight loss beginning near the completion of this process, probably due to decomposition. The specific nature of this process is not known. However, it should be noted that no melt of this lot is seen on DSC.
Figure 2.15 DSC/TGA overlay of SCR 84-44 API.

Powder x-ray analysis of SCR 84-44 displayed sharp peaks beginning at ~ 16 θ, which clearly indicates that the sample is crystalline in nature, Figure 2.16. No analysis was conducted to determine the unit cell or crystal structure of these crystals.
2.3.8 Comparison of Imexon API Lots

Visual examination of all lots of Imexon API indicated that there is some degree of crystallinity in each sample. Furthermore, in all cases the particle size and expression of crystal habit is largely dependent on the degree of physical handling.

Analysis of each of the DSC thermographs indicates similar patterns for all samples, shown in Figure 2.17. While the shape of the exotherm changes slightly for each of the various lots, along with the amount of energy release, the overall motif does not change.
Figure 2.17 DSC overall for all Imexon API lots.

Similarly, the overlay of all the TGA thermographs showed very little difference in the onset or rate of weight loss upon heating, Figure 2.18.
The XRD patterns for each lot of Imexon API indicate that they are all crystalline as supplied at room temperature. Furthermore, no difference is seen when all XRD patterns are overlaid and evaluated, Figure 2.19. The fact that the XRD patterns are the same for all drug sources indicates that the crystal structure is the same for each of the source lots. Therefore, it makes little difference as to the source material for single crystal determination.
Figure 2.19 Overlay of all XRD patterns for Imexon API's.

2.3.9 Single Crystal Structural Determination

The crystal structure of Imexon was determined at 170 K (Kuehl et al., 2006b). The crystals were grown, as described in Section 2.2.8, from a solution of absolute ethanol, saturated with Imexon at 296 K, by slow evaporation of solvent. Analysis of Imexon crystallites, under polarized light microscopy, showed plate like crystals which were very delicate. It was determined that under these conditions Imexon crystallizes in plates with well developed 100 faces. The crystals readily twin by a two-fold rotation about the $a$
axis. Figure 2.20 shows the 50% probability ellipsoids and atom labels for the single crystal, with hydrogen atoms shown as spheres of arbitrary radii.

Adjacent molecules are held together via a hydrogen bond network, Figure 2.21. Each molecule forms a total of four hydrogen bonds with the three adjacent molecules, this hydrogen bond network results in tapes of infinite length.
Figure 2.21 Partial packing diagram of Imexon showing intermolecular hydrogen bonds which form infinite tapes.

There are however, no significant interactions between the adjacent tapes. These tapes are illustrated in Figure 2.22, noting the absence of any significant interactions between the adjacent tapes.
Specifically, Imixon crystallized into a monoclinic cell in space group $P\ 2_1/c$ with 4 molecules in the unit cell and calculated density of 1.54 g/cm$^3$. Unit cell parameters are as follows $a = 10.3612 (12)$ Å, $b = 6.5999 (6)$ Å, $c = 7.2077 (11)$ Å, $\alpha = \gamma = 90^\circ$ and $\beta = 103.685 (9)^\circ$.

Imixon crystallized as a zwitterion with the doubly protonated $N_8$ having a positive charge and a negative charge distributed between $C_2$, $N_3$ and $C_4$, shown in Figure 2.23. The presence of the zwitterion is stabilized by conjugation from the carbonyl to $N_8$. This is further confirmed by the almost equal $C_2$-$N_3$ and $C_4$-$N_3$ bond lengths of 1.36 (1) Å, typical for a delocalized double bond. All other bond lengths were as expected (Lide,
1991). The five membered ring in the molecule is planar (RMSD of all non-hydrogen atoms except C₆ is 0.03 Å) with the aziridine ring (C₆) extending (1.239 (2) Å) above or below the ring equally as the drug is a racemic mixture of enantiomers.

Figure 2.23 Crystal structure of Imexon crystallized from absolute EtOH.

The single crystal atomic parameters were used to calculate a theoretical XRD pattern. Comparison of the calculated and the actual XRD data, Section 2.3.2, indicate that there are only slight differences between the two, Figure 2.24. The observed powder patterns differed little from the patterns calculated using single crystal atomic parameters and a unit cell refined from the observed powder pattern.
Figure 2.24 Overlay of the XRD data collected via XRD (PowderObs) and the calculated XRD pattern from the single crystal data (PowderCalc).

A typical cell refined from the powder data was $a = 10.4200\ (9)\ \text{Å}$, $b = 6.553\ (2)\ \text{Å}$, $c = 7.310\ (2)\ \text{Å}$, $\alpha = \gamma = 90^\circ$, and $\beta = 104.000\ (9)^\circ$, which was only slightly different then those measured from the single crystal. The source of this small variation is due to the fact that the microcrystalline material used for powder x-ray analysis was crystallized by rapid evaporation of ethanol which results in slightly imperfect crystals. While the single crystal utilized for x-ray diffraction data was grown under controlled, slow evaporation of ethanol, resulting in a more orderly arrangement of the molecules in the crystal.
2.3.10 Nuclear Magnetic Resonance

Imexon API was dissolved in D$_2$O and analyzed for 32 scans in $^1$H mode. The resultant data showed a doublet of doublets at $\sim$ 3.65 ppm corresponding to the hydrogens on the CH$_2$ (C$_6$) on the aziridine ring being split by the hydrogen of the adjacent CH (C$_5$) on the five membered ring. Conversely, two doublets were seen, one at 2.6 ppm and one at 2.45 ppm, corresponding to the hydrogen of the CH (C$_5$) in the five membered ring being split by each of the hydrogen’s of the CH$_2$ (C$_6$) in the aziridine ring.

2.3.11 *In Silico* Prediction of the Octanol-Water Partition Coefficient and pKa

The octanol-water partition coefficient for Imexon calculated using CLOGP© software is -1.35 (CLogP, 1999). Imexon is predicted, *in silico*, to have a basic pKa near 7.5, via protonation of N$_8$, see Figure 2.20 for labeling schema (ACD labs, 2003).

2.4 CONCLUSIONS

The solid state characteristics of Imexon were determined in this study. It was determined that Imexon decomposes prior to melting, beginning at $\sim$ 150°C. Examination of several different raw API lots indicated that all the lots were identical, regardless of source. Furthermore, the single crystal structure has been determined. The visually observed delicate nature of the crystals and varied particle size is explained via
the lack of molecular interactions between adjacent tapes in the crystal structure. These structural data will provide insight at the molecular level for further studies. Furthermore, as these studies were conducted over a period of several months it can be deduced that the crystalline material is relatively stable.
CHAPTER 3: PREFORMULATION STUDIES ON IMEXON

3.1 INTRODUCTION

In Chapter II it was shown that the Imexon crystal structure is robust and it was indirectly determined that the solid is stable. These data also show that Imexon’s physiochemical properties, calculated LogP of -1.35 (CLogP, 1999), molecular weight of 111.10 and a relatively high water solubility, are contrary to the current trend, dating back to the 1960’s, of new drugs possessing a increasingly higher LogP and molecular weight along with lower aqueous solubility (Gribbon and Sewing, 2005). Imexon’s relatively high aqueous solubility will allow preformulation studies to focus on the stability under a variety of different conditions as poor solubility and/or stability are often the cause for failure of a molecule in development (Darvas et al., 2002; Di, 2003). In contrast to the stability of the raw API, Imexon undergoes rapid decomposition under aqueous conditions. This situation suggests preformulation studies be modeled after those conducted by Ni et al. 2002, Zhu et al. 2002, and Anderson et al. 1988.

To this end, extensive preformulation studies have been conducted to first characterize the solubility of Imexon as a function of pH and in several aqueous systems. Second, to determine the degradation rate as a function of pH, ionic strength, temperature, buffer species and initial drug concentration.
3.2  BACKGROUND

3.2.1  Solubility of Crystalline Solids

It is known that the aqueous solubility of a crystalline solid is dependent on the ability of
the solid to interact with the aqueous medium (activity coefficient) and the strength of the
crystal (melting point). Yalkowsky and coworkers have, through rigorous evaluation,
shown that the aqueous solubility of an organic nonelectrolyte can be calculated via
Equation 3.1:

$$\log S_w = 0.5 - \log P - 0.01(MP - 25)$$  \hspace{1cm} \text{Eqn 3.1}

where $S_w$ is the solubility in water, $\log P$ is the log of the octanol-water partition
coefficient and $MP$ is the melting point of the crystalline solid (Jain and Yalkowsky,
2001; Yalkowsky, 1999).

In the case of Imexon, calculation of the aqueous solubility is not straightforward. While
CLogP provides an accurate estimate of the $\log P$ (Machatha and Yalkowsky, 2005), the
melting point term is typically deduced from thermal analysis on the DSC. However,
Imexon does not undergo an endothermal event, indicative of melting; rather it is thought
to decompose prior to melting. If it is assumed that the melting point is the same as the
decomposition then the calculated solubility of Imexon is 2.81 molar or 313 mg/mL.
Clearly this value is high, however, from the partition coefficient it is suggested that Imexon be somewhat soluble in water.

3.2.2 The Effect of pH on Solubility

The solubility of an ionizable compound in aqueous media is the sum of the ionized and unionized compound in solution, Equation 3.2.

\[ S_t = S_i + S_u \]  
Eqn 3.2

where \( S_t \) is the total solubility, \( S_i \) is the solubility of the ionized species and \( S_u \) is the solubility of the unionized species (Yalkowsky, 1999). The value for \( S_u \) is defined as the intrinsic solubility of the compound and can either be experimentally determined, at a pH where there is no ionization, or it can be calculated via Equation 3.1. The value of \( S_i \) is equal to the amount of the compound that is ionized. In the case of a weak base, as Imexon is proposed to be, the fraction ionized can be calculated from the Henderson-Hasselbach equation:

\[ pH = pK_a + \log \frac{B}{BH^+} \]  
Eqn 3.3
where the pH is the pH of the solution, B is the concentration of the unionized base (in our case $S_u$) and BH\(^+\) (in our case $S_i$) (Martin, 1983). Substitution for our example yields the following “H-H” equation:

$$pH = 7.5 + \log \frac{S_u}{S_i}$$  \hspace{1cm} \text{Eqn 3.4}

which can be rearranged and substituted into Equation 3.2 to yield:

$$S_i = S_u \left(1 + 10^{(7.5-pH)}\right)$$  \hspace{1cm} \text{Eqn 3.5}

which can be used to calculate the solubility of Imexon at any pH, assuming the drug to be a base with a pKa of 7.5. Utilizing Equation 3.5, a plot of the solubility of Imexon as a function of pH would look like Figure 3.1. Note the log-linear increase that is expected when the pH decreases below the pKa, 7.5.
Figure 3.1 Theoretical plot of the solubility of Imexon as a function of pH, assuming the calculated pKa of 7.5.

3.2.3 Chemical Stability in Aqueous Medium

3.2.3.1 pH-Rate Determination

When evaluating the effect of pH on the degradation rate of a drug, one will typically be presented with a pH-rate profile, that is, a plot of the log of the degradation rate constant versus the pH of the solution. An example of a pH-rate profile is seen in Figure 3.2. This figure shows the pH-rate profile for a drug that undergoes degradation in basic conditions and is increasingly stable under acidic conditions for 12 pH values between 2 and 13.
Figure 3.2 An example pH-rate profile for a compound that is susceptible to base-catalyzed degradation.

It is important to appreciate the extent of work that one pH-rate profile entails. A pH-rate profile is specific for the conditions of the experiment, e.g. temperature, buffer strength, ionic strength, etc. Also, it must be understood that each data point represents one pH condition, typically conducted in duplicate for the specific conditions.

In order to generate a pH-rate profile the investigator must hold all variables constant, with the exception of pH. Variables such as light, initial concentration, temperature, ionic strength and buffer concentration must be maintained. Once these are controlled,
samples can be assayed, over time, for drug content. The amount of drug remaining can then be plotted as a function of time. The majorities of drug molecules undergo apparent first-order degradation and follow the mathematical relationship in Equation 3.6.

\[
\log \frac{A^\circ}{A} = -\frac{k \times t}{2.303}
\]  \hspace{1cm} \text{Eqn 3.6}

where \( A^\circ \) is initial drug concentration, \( A \) is drug concentration at any time, \( k \) is the apparent first-order rate constant and \( t \) is time. A typical plot for the percent of drug remaining versus time, for first-order degradation, is shown in Figure 3.3

![Graph showing log percent drug remaining vs time](image)

Figure 3.3 Example analysis of first-order drug degradation.
Assuming first-order kinetics, linear regression analysis of the data will determine a slope that can be used to calculate the degradation rate constant, $k$, from Equation 3.7.

\[ k = -Slope \times 2.303 \quad \text{Eqn 3.7} \]

It is the logarithm of this $k$ that is represented by one data point on Figure 3.2. Evaluating the degradation of a drug molecule in this manner at 12 pH values between 2 and 13 would result in the example pH-rate profile shown in Figure 3.2.

Knowing the degradation rate constant also permits the calculation of half life ($T_{50}$) and usage life ($T_{95}$) defined as the time at which 5% of the API has degraded, Equation 3.8 and 3.9, respectively.

\[ \frac{0.693}{k} = T_{50} \quad \text{Eqn 3.8} \]

\[ \frac{0.0515}{k} = T_{95} \quad \text{Eqn 3.9} \]

3.2.3.2 Acid/Base Degradation

As stated in Section 3.1, poor stability of an API is often one of the reasons for failure to advance the molecule to market. Furthermore, the rate of degradation varies drastically
for different drug molecules. As such, it is important to determine the chemical stability as a function of numerous variables prior to formulation development. Some variables that are known to have an effect on degradation include pH, temperature, buffer species, ionic strength, initial drug concentration and different aqueous medium. Forcing degradation in order to study degradation rates provides vital information that is utilized in development of a formulation (Carstensen and Rhodes, 2000).

The pH of a solution can have a catalytic effect on the degradation rate of many drug molecules. This catalysis can either be specific acid/base catalysis or general acid/base catalysis. Specific acid/base catalysis is characterized by the fact that only the concentration of H⁺ or OH⁻ has an effect on the degradation of the drug molecule. In this case, the slope of the pH-rate profile, in a range where degradation occurs, is equal to unity, Figure 3.4. An example of a generic rate equation for specific acid/base catalysis is seen in Equation 3.10.

\[- \frac{dC}{dt} = k_0 + k_H[H^+][D] + k_{OH^{-}}[OH^{-}][D]\]  

Eqn 3.10

where \(k_0\) is the degradation rate constant in water, \(k_H\) is the degradation rate constant from \(H^+\), \(k_{OH}\) is the degradation rate constant from \(OH^-\) and \(D\) is the concentration of the drug (Martin, 1983). By definition, the only factors that affect the overall rate are the concentrations of either acid or base and the concentration of the drug.
Specific acid/base catalysis is contrasted by general acid/base catalysis. In general acid/base catalysis involves not only the concentration of acid and base effects on the overall rate, but other factors such as buffer species and buffer concentration can affect the degradation rate. A generic example of a rate equation for general acid/base catalysis is shown in Equation 3.11.

\[
-\frac{dC}{dt} = k_0 + k_a[HA][D] + k_b[A^-][D] + k_H[H^+][D] + k_{OH}[OH^-][D] \quad \text{Eqn 3.11}
\]

where \(k_0\) is the degradation rate constant from water, \(k_a\) is the rate constant due to the protonated acid, \(k_b\) is the rate constant due to the ionized acid, \(k_H\) is the degradation rate
constant from $H^+$, $k_{OH}$ is the degradation rate constant from $OH^-$, $HA$ is the concentration of the protonated acid, $A^-$ is the concentration of the ionized acid and $D$ is the concentration of the drug (Martin, 1983).

3.2.3.3 Temperature Effect on Degradation

Temperature may also affect the rate of a reaction. Typically, the rate of a reaction doubles with each 10° increase in temperature (Martin, 1983). Several elevated temperatures are often used to force degradation in early preformulation. These degradation rates, for elevated temperatures, can be used to calculate the degradation rates at other temperatures, provided the degradation mechanism doesn’t change.

The effect of temperature on the degradation rate is usually assessed via the Arrhenius equation, Equation 3.12.

$$k = Ae^{-E_a/RT} \quad \text{Eqn 3.12}$$

where $k$ is the degradation rate constant, $A$ is a constant called the frequency factor, $E_a$ is the activation energy for the reaction, $R$ is the gas constant and $T$ is the temperature. This equation is typically rearranged into Equation 3.13
\[ \log k = \log A - \frac{E_a}{2.303 RT} \]  
Eqn 3.13

Taking the natural logarithm of Equation 3.13 gives Equation 3.14, which is typically plotted as Ln K as a function of 1/T.

\[ \ln k = -\frac{E_a}{R} \frac{1}{T} + \ln[A] \]  
Eqn 3.14

The utility of a plot of Ln K versus 1/T is two-fold; first, the presence of two different linear portions in the plot can suggest differences in mechanisms. Second, linear regression affords simple calculation of the activation energy, \( E_a \), through Equation 3.15.

\[ E_a = -Slope \times 8.314 \]  
Eqn 3.15

The most facile method to understand the importance of \( E_a \) is through an example. In the following example the degradation rates constants have been determined at 55°, 70° and 85°C. By conducting regression analysis for a plot of Ln K versus 1/T, for these data, the activation energy can be determined by Equation 3.15. This activation energy can then be substituted into Equation 3.14 along with any temperature to mathematically determine the rate constant for that temperature. The underlying assumption is that the mechanism of degradation does not change over the temperature range where the rate constant is calculated. Furthermore, the calculated rate constant can then be substituted
into Equation 3.8 and 3.9 to calculate the $T_{50}$ or $T_{95}$, respectively, as described in Section 3.2.3.1.

3.3 EXPERIMENTAL SECTION

3.3.1 Materials

Imexon was provided by AmpliMed Corp., Tucson, AZ, USA. Ammonium acetate ($\text{C}_2\text{H}_7\text{NO}_2$), monosodium phosphate ($\text{NaH}_2\text{PO}_4$), dipotassium phosphate ($\text{K}_2\text{HPO}_4$), sodium chloride ($\text{NaCl}$), boric acid ($\text{H}_3\text{BO}_3$) and hydrochloric acid ($\text{HCl}$) were obtained from Sigma Aldrich (St. Louis, MO, USA). Citric acid ($\text{C}_6\text{H}_8\text{O}_7$) was obtained from Spectrum Chemical (New Brunswick, NJ, USA). Sodium hydroxide ($\text{NaOH}$) was obtained from EM Science (Darmstadt, Germany). Hydrogen peroxide ($\text{H}_2\text{O}_2$), 30%, stock solution was obtained from Mallinckrodt (Hazelwood, MO, USA). Sterile water for Injection USP, 0.9% NaCl USP (normal saline), 0.45% NaCl USP (0.5 normal saline) and 5% dextrose USP for injection (D$_5$W) were obtained from Baxter (Deerfield, IL, USA). HPLC grade acetonitrile (ACN) was obtained from EMD (Gibbstown, NJ, USA). A Millipore (Billerica, MA, USA) Milli-Q Ultrapure Water purification system with a 0.22 µm filter was utilized for water.

3.3.2 HPLC Assay
The HPLC system consisted of a Waters 2695 separation module (Waters, Milford, MA, USA) coupled with a Waters 996 Photodiode array (PDA) detector. Analysis was performed by a normal phase HPLC assay, using a 150 mm x 4.6 mm, Apollo Silica 5 μ column (Alltech Associates, Deerfield, IL), maintained at 30 ± 2°C. Ultraviolet detection was done at 234 nm. Mobile phase conditions were 90:10 (v/v) ACN:H₂O at a flow rate of 0.6 mL/min. Water was buffered with ammonium acetate at 0.1 M, with a pH of approximately 6.50. The injection volume was 5 µL. The parent compound had a retention time of 7.8 minutes.

Quantification was conducted via linear regression analysis of the five point standard curve, which was prepared daily. Standards were prepared by volumetric dilution in purified water and stored at 4°C.

3.3.3 Solubility Determination

The solubility of Imexon, as a function of pH, was conducted at seven pH values between 5.1 and 8.5 using phosphate buffer. Buffers were 0.1 M and NaCl was utilized to adjust each to an ionic strength of 0.2 M. Each sample was saturated with Imexon raw drug substance and allowed to agitate for at least 30 minutes. Samples were visually inspected to ensure that solid drug remained in excess and then filtered through a 0.2 μm PTFE filter. The final pH of the buffered samples was then measured. The filtrate was diluted appropriately with sterile water and assayed via the previously described HPLC assay,
Section 3.3.2. The solubility’s were also determined in 0.5 normal saline and sterile water for injection, at 12°C, 22°C and 34°C. Prior to any solubility measurement studies were conducted to determine appropriate agitation time.

3.3.4 Aqueous Stability

3.3.4.1 Effect of pH

The influence of pH on the stability of Imexon, at 0.12 mg/mL, was studied with a citrate buffer at pH 2.0 and 5.0, a phosphate buffer at pH 5.0, 7.2, and 9.0 and a borate buffer at pH 9.0 and 10.0. All buffers were made to be 0.1 M concentration and the pH was adjusted with NaOH or concentrated HCl. Ionic strength was adjusted to 0.2 M with NaCl. The influence of ionic strength was evaluated at pH 2.0 with a citrate buffer, pH 7.2 with a phosphate buffer and pH 10.0 with a borate buffer by adjusting the ionic strength to 0.5 M with NaCl.

3.3.4.2 Effect of Temperature

Temperature influence on the degradation rates of Imexon was determined at four temperatures (4°C, 25°C, 37°C and 48°C) at pH 5.0 with citrate buffer, pH 7.2 with phosphate buffer and pH 9.0 with borate buffer. Analysis of the degradation rates of Imexon at
different temperatures affords the assessment of activation energy, as shown in Section 3.2.3.3.

3.3.4.3 Effect of Other Aqueous Solvents

In order to determine the susceptibility of Imexon to oxidation, experiments were performed with a 6% solution of hydrogen peroxide. In addition, degradation rates were evaluated in normal saline and D5W.

3.3.4.4 Effect of Initial Drug Concentration

The stability of Imexon as a function of concentration was conducted at 0.12, 1, 5, 10 and 18.5 mg/mL with phosphate buffers at pH 7.2 and 8.8. Phosphate buffers were prepared at 0.1 M and the ionic strength was adjusted to 0.2 M with NaCl. The effect of ionic strength was evaluated at 10 mg/mL by adjusting the ionic strength to 0.3 and 0.5 M with NaCl. Samples to determine the effect of drug concentration on Imexon stability were stored at 19°C.

In order to accurately determine the effect that pH, temperature, other aqueous solutions and initial drug concentration have on the degradation of Imexon, samples were prepared in duplicate and assayed for a minimum of six samples, spanning three to six half lives. In order to minimize the potential for self catalysis (Waterman and Adami, 2005) samples
were prepared at a dilute concentration of 0.12 mg/mL, and stored at 25°C, unless otherwise stated. Sampling time varied due to large differences in degradation rates. The pH of each sample was measured at the beginning and completion of each stability trial.

3.3.5 Degradation Product Structural Determination

3.3.5.1 Mass Spectroscopy

LC-MS analyses were performed with the Finnigan MAT TSQ 7000. The initial screening of Imexon was made in Q1 MS mode with the following parameters: scan type --full; polarity--positive; mass range: 50–500 m/z. Atmospheric pressure chemical ionization (APCI) source operating parameters: heated capillary temperature: 250°C; vaporizer temperature: 400°C; corona discharge current: 4.0 µA. The sample was introduced into the mass spectrometer via a Hewlett Packard 1050 HPLC system. Chromatographic separation was performed using the Apollo Silica column, previously described, with 12 minute isocratic conditions of 80:20 (v/v) ACN:H2O with a flow rate of 0.6 ml/min.

Individual fractions were collected utilizing the Waters HPLC system and method mentioned above with 100% isopropyl alcohol (IPA) at 0.6 ml/min. Fractions were analyzed in both Q1MS and MS/MS modes. The samples were reconstituted with methanol and introduced via flow shot injection.
As to Q1 MS, all the settings were the same as above. The instrument parameters for the MS/MS mode were as follows: scan type: full; polarity: positive; mass range: 10--150 m/z; set mass: 112.0; collision energy: 30V. APCI source settings remained the same as in Q1 mode.

3.3.5.2 Nuclear Magnetic Resonance

NMR samples were analyzed on a Bruker AM-250 NMR. Samples were dissolved in DMSO for collection of 64 scans in $^1$H mode.

3.4 RESULTS AND DISCUSSION

3.4.1 Chromatography

The isocratic conditions, described in the Section 3.3.2, were maintained for 25 minutes in order to achieve separation of Imexon from one degradation peak and a second unknown peak. All peaks were well resolved and were of gaussian shape. Figure 3.5 shows a chromatograph of parent Imexon, 0.12 mg/mL, overlaid with a sample obtained at pH 2.0 after 10.0 minutes. The degradation peak elutes at ~ 6.8 minutes (Degradation Peak A) and another peak at ~ 20.2 minutes. As seen in Figure 3.5, the peak at ~ 20.2 minutes is present both in the standard and in the degraded sample.
3.4.2.1 pH-Solubility of Imexon

The solubility of Imexon, as a function of pH, was investigated to explore the hypothesized presence of a basic pKa at or near pH 7.5. As discussed in Section 3.2.2,
the solubility of a basic compound will increase as the pH of the solution decreases below the pKa.

Since it is known that Imexon can undergo rapid degradation as a function of pH, the required equilibration time needed for solubility determination was balanced with degradation considerations. Fortunately, studies revealed that solid Imexon dissolves rapidly and that the solubility does not change appreciably after 30 minutes of agitation. As a result, solubilities were determined for all of the desired conditions, utilizing this relatively short sampling time. The solubility of Imexon at each of the pH values studied is overlaid with the predicted theoretical plot from Figure 3.1 and shown in Figure 3.6.

Figure 3.6 Experimental and calculated solubility values for Imexon as a function of pH.
From these data it was found that the solubility of Imexon is constant for pH’s 5.1, 6.5, 6.85, 7.54, 8.05 and 8.51 with an average solubility of 25.12 mg/mL (S.D. = 0.71). Due to the fact that the solubility of Imexon does not change in this pH range it is concluded that there is not a pKa in this pH range.

3.4.2.2 The Effect of Temperature on the Solubility of Imexon

The solubility of Imexon was also determined in water at three different temperatures, shown in Figure 3.7. Specifically, the solubilities at 12°, 22° and 34°C are 19.9, 25.12 and 55.14 mg/mL, respectively.

![Figure 3.7 Solubility of Imexon at three different temperatures in both sterile water and 0.5 normal saline.](image)
The solubility of Imexon was also determined in 0.5 normal saline showed similar results, Figure 3.7. As expected for a crystalline solid, the solubility of Imexon increases as a function of temperature.

3.4.2.3 Back Calculation of Melting Point

As discussed in Section 3.2.1, a priori calculation of the solubility of Imexon using Equation 3.1 was not possible due to the absence of a true melting point. Utilizing Equation 3.1 with the experimental solubility value we can back calculate the melting point and determine if it is indeed above the decomposition temperatures. Equation 3.1 calculates a theoretical melting point of 274.6°C, which supports the fact that Imexon crystals decompose prior to melting.

Manipulation of Equation 3.1 can yield Equation 3.16 which allows the solubility to be calculated at temperatures other then 25°C.

\[
\log S_w = 0.5 - \log P - \frac{3(T_m - T)}{T}
\]

Eqn 3.16

where \( T_m \) is the melting point of the compound and \( T \) is the temperature of interest, both in Kelvin. Using the aqueous solubility values determined at 12°C and 34°C theoretical melting points of 258°C and 254°C are obtain, respectively. These theoretical melting
points are similar to the one obtained for the 22°C temperature and again confirm that Imexon decomposes prior to melting.

3.4.3 Aqueous Stability

3.4.3.1 Effect of pH

It is known that the pH of a solution can affect the degradation rate of many drugs. This change in degradation rate can be attributed to the catalytic effect of either the hydrogen ions or hydroxide ions in solution. The log percent of Imexon remaining versus time, for several pH values, is shown in Figure 3.8.

![Figure 3.8: Average (n=2) Log % drug remaining vs. time. Citric acid buffer represented for pH 5.0 and borate buffer represented for pH 9.0](image-url)
These results suggest that Imexon undergoes apparent first-order degradation (Carstensen and Rhodes, 2000). Degradation rates constants \( k \) were calculated, as describe in Section 3.2.3.1, from the slope of the linear best-fit line for the relationship between the logarithm percent drug remaining versus time. The rate constant was then used to calculate \( T_{50} \), and \( T_{95} \), Equation 3.8 and 3.9, respectively. These data are summarized in Table 3.1; note these values are all at 0.2 M ionic strength.

<table>
<thead>
<tr>
<th>pH</th>
<th>( k ) (per hour)</th>
<th>SD</th>
<th>( T_{50} ) (Hours)</th>
<th>( T_{95} ) (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 (Citrate)</td>
<td>10.381</td>
<td>2.9058</td>
<td>0.067</td>
<td>0.005</td>
</tr>
<tr>
<td>5.0 (Citrate)</td>
<td>0.095</td>
<td>0.0016</td>
<td>7.33</td>
<td>0.55</td>
</tr>
<tr>
<td>5.0 (Phosphate)</td>
<td>0.048</td>
<td>0.0017</td>
<td>14.41</td>
<td>1.07</td>
</tr>
<tr>
<td>7.2 (Phosphate)</td>
<td>0.010</td>
<td>0.0017</td>
<td>68.67</td>
<td>5.10</td>
</tr>
<tr>
<td>9.0 (Phosphate)</td>
<td>0.015</td>
<td>0.0002</td>
<td>47.15</td>
<td>3.50</td>
</tr>
<tr>
<td>9.0 (Borate)</td>
<td>0.011</td>
<td>0.0001</td>
<td>62.10</td>
<td>4.61</td>
</tr>
<tr>
<td>10.0 (Borate)</td>
<td>0.029</td>
<td>0.0007</td>
<td>23.70</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Table 3.1 Degradation rate constant, half life and usage life for Imexon at various pHs.

Preliminary studies on the degradation of Imexon indicated an increase in pH as the samples degraded. As a result, the buffer species described in Material and Methods Section was employed for stability samples. These buffers were found to hold pH constant throughout degradation studies. Figure 3.9 shows the pH-rate profile for Imexon at 25°C and 0.12 mg/mL.
Analysis of the pH-rate profile indicates that the slope of this line deviates from negative one in the acid range and one in the basic range, suggesting general acid/base catalysis, Section 3.2.3.2.

3.4.3.2 Effect of Buffer Species

The effect of buffer species was determined at pH 5.0 and 9.0 (0.2 M ionic strength). Imexon was found to undergo apparent first-order degradation kinetics for each of the buffer species evaluated. Table 3.1 lists the $T_{50}$ values at pH 5.0 (citrate and phosphate buffers) and at pH 9.0 (phosphate and borate buffers). At pH 5.0 the $T_{50}$ for the citrate buffer is about one-half of the value of the $T_{50}$ for phosphate buffer, indicating that at lower pH, Imexon is more stable in a phosphate buffer. When buffered at pH 9.0,
Imexon’s $T_{50}$ in the phosphate buffer is ~25% lower than that of the borate buffer. The differences in degradation rates for different buffers systems, at the same pH, further supports general acid/base catalysis, Section 3.2.3.2.

### 3.4.3.3 Effect of Ionic Strength

The effect that ionic strength has on the degradation of Imexon was determined at three different pH values (2.0, 7.2 and 10.0) by adjusting the ionic strength of the buffers from 0.2 M to 0.5 M. Table 3.2 shows that the degradation of Imexon is accelerated by an increase in the ionic strength, at all three pH values.

<table>
<thead>
<tr>
<th>pH 2.0</th>
<th>Ionic Strength</th>
<th>$k$ (per hour)</th>
<th>$T_{50}$ (Hours)</th>
<th>$T_{95}$ (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
<td>10.38</td>
<td>0.067</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>19.63</td>
<td>0.035</td>
<td>0.003</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 7.2</th>
<th>Ionic Strength</th>
<th>$k$ (per hour)</th>
<th>$T_{50}$ (Hours)</th>
<th>$T_{95}$ (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
<td>0.010</td>
<td>68.7</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.017</td>
<td>41.9</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 10.0</th>
<th>Ionic Strength</th>
<th>$k$ (per hour)</th>
<th>$T_{50}$ (Hours)</th>
<th>$T_{95}$ (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
<td>0.029</td>
<td>23.7</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.038</td>
<td>18.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 3.2 Effect of ionic strength on the degradation rate of Imexon at 25°C for three pH values, 0.12 mg/mL initial concentration.
While the degree of acceleration is greater at low pH values, the trend is apparent for each of the pH values studied.

3.4.3.4 Effect of Temperature

The natural logarithms of the degradation rates were plotted as a function of $1/T$, as described in Section 3.2.3.3, for three pH values. From these data three linear plots are shown, Figure 3.10, indicating that the degradation of Imexon increases as the temperature increases.

Figure 3.10 Arrhenius plot (pH 5.0 citrate, pH 7.2 phosphate and pH 9.0 borate) showing the dependence of degradation rate at four temperatures for three different pH’s.
The activation energies were calculated with Equation 3.15 for the degradation of Imexon at pH 5.0, 7.2 and 9.0 with a citrate buffer, phosphate buffer and a borate buffer, respectively, and presented in Table 3.3.

<table>
<thead>
<tr>
<th>pH</th>
<th>Slope</th>
<th>Ea (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>-7.20</td>
<td>59.87</td>
</tr>
<tr>
<td>7.20</td>
<td>-10.10</td>
<td>83.95</td>
</tr>
<tr>
<td>9.00</td>
<td>-10.18</td>
<td>84.60</td>
</tr>
</tbody>
</table>

Table 3.3 Activation energies for the degradation of Imexon at three pH's.

In aqueous solutions of pH 7.2 and 9.0 the activation energy for the degradation of Imexon is 83.95 kJ/mol and 84.60 kJ/mol, respectively, while the activation energy at pH 5.0 is 59.87 kJ/mol. The equivalent activation energies for pH 7.2 and 9.0 are in alignment with the stability data in Figure 3.9 and indicate the region in which Imexon is most stable. The decrease in activation energy at pH 5.0 can be attributed to the increase in the hydrogen ion catalysis of Imexon as well as the citrate buffer system utilized for the study. From Figure 3.9, pH clearly has an affect on degradation. In addition, buffer species has been shown to have some importance on degradation (Table 3.1), with Imexon degrading faster in citrate buffer as compared to phosphate buffer.

3.4.3.5 Effect of Other Aqueous Solvents
Analysis of Imexon degradation in 6% H₂O₂, normal saline and D₅W showed degradation occur via apparent first-order degradation. Table 3.4 shows the effect of three aqueous solvents (6% H₂O₂, normal saline and D₅W) on the stability of Imexon at room temperature.

<table>
<thead>
<tr>
<th></th>
<th>k (per hour)</th>
<th>SD</th>
<th>T₅₀ (Hours)</th>
<th>T₉₅ (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>0.252</td>
<td>0.0514</td>
<td>2.753</td>
<td>0.203</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>0.008</td>
<td>0.0018</td>
<td>87.86</td>
<td>6.466</td>
</tr>
<tr>
<td>D₅W</td>
<td>0.002</td>
<td>0.0007</td>
<td>290.1</td>
<td>21.35</td>
</tr>
</tbody>
</table>

Table 3.4 Rate constant (K), standard deviation (SD), T₅₀ and T₉₅ for Imexon at 25°C and 0.12 mg/mL in H₂O₂, normal saline and D₅W.

These data indicate the effect of ionic strength on the degradation rate of Imexon. Normal saline, with an ionic strength of 0.154 M, has a T₉₅ of 6.46 hours while D₅W, with an ionic strength of 0.0 M, has a T₉₅ of 21.35 hours. Since both solutions are at or near physiological pH and were kept at room temperature, the ionic strength is the main difference between the two solutions. Based on the effect changes in ionic strength had on the buffered samples, it is hypothesized that it is also the cause of the increase in degradation rate in normal saline. H₂O₂ at 6% (pH 5.2) showed an increased degradation rate when compared to pH 5.0. This is indicative of the fact that Imexon is susceptible to oxidation catalyzed by peroxide.

3.4.3.6 Effect of Initial Drug Concentration
In order to determine the effect initial concentration has on the degradation rate of Imexon, samples were evaluated at 0.12, 1, 5, 10 and 18.5 mg/mL, at pH 7.2 and 8.8. These data are shown in Figure 3.11.

![Figure 3.11 Concentration rate profile of Imexon, indicating a decrease in stability of Imexon as concentration increases, conducted at 19°C.](image)

Interestingly, all samples undergo apparent first-order degradation for each pH, however, the rates of the different initial concentrations are different from one another (p value <0.05 (Stata 7.0, 2001)). A similar situation was observed by Fubara and Notari (1998), and alludes to the possibility that Imexon (or a moiety on the molecule) acts as a catalyst in the degradation pathway. No pH changes were noted for any of the samples, regardless of initial concentration or ionic strength. In addition, the data presented in
Figure 3.11 confirm the pH-stability study, Figure 3.9, in which the stability of Imexon is the similar for pH 7.2 and 8.8.

The effect of ionic strength was concurrently studied at 10 mg/mL (pH 7.2) by adjusting buffer concentration to 0.3 M and 0.5 M, data shown in Table 3.5.

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>k (per hour)</th>
<th>T\textsubscript{50} (Hours)</th>
<th>T\textsubscript{95} (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M</td>
<td>0.0130</td>
<td>53.49</td>
<td>3.98</td>
</tr>
<tr>
<td>0.3 M</td>
<td>0.0153</td>
<td>45.42</td>
<td>3.38</td>
</tr>
<tr>
<td>0.5 M</td>
<td>0.0206</td>
<td>33.72</td>
<td>2.51</td>
</tr>
</tbody>
</table>

Table 3.5 Degradation rate, T\textsubscript{50} and T\textsubscript{95} for pH 7.2 at varied ionic strength.

As with the 0.12 mg/mL studies (Table 3.2), the degradation rate increased as the ionic strength of the buffer solution increased. Further analysis of the effect of ionic strength indicated that increasing ionic strength from 0.2 M to 0.5 M results in a ~37% decrease T\textsubscript{50}, independent of initial concentration.

3.4.4 Degradation Product Structural Determination

3.4.4.1 Mass Spectrometry
Mass spectrometry was used to determine the m/z of Imexon and the degradation products, actual m/z are m+1 since positive ion spray was used to ionize samples. Under conditions described in Section 3.4.4.1, an Imexon standard at 0.12 mg/mL was introduced to the mass spectrometer via flow shot and analyzed. The Imexon standard showed a clean mass spectrum with an m/z of 111.8 for the parent, Figure 3.12.

![Flow shot mass spectrograph of Imexon parent.](image)

Throughout MS analysis it was discovered that Imexon interacts with several of the solvents used in either the HPLC conditions or in sample preparation. Interactions with
ACN, IPA and MeOH were observed. The ACN, IPA and MeOH showed m/z peaks of 152.8, 171.7 and ~ 142, respectively. When the same standard was analyzed in MS/MS mode, described in Section 3.4.4.1, Figure 3.13 was seen, containing m/z values of 42, 69, 84 and 95.

Figure 3.13 MS/MS analysis of Imexon parent.

The explanation for the fragmentation is as follows: m/z of 95 is the loss of the oxygen, m/z of 42 and m/z of 69 are complementing fragments resulting from fragmentation of
two nitrogen’s, one carbon, and two hydrogen’s, and the m/z of 84 is the result of the loss of the carbonyl group.

Analysis of the potential degradation products was conducted in both MS mode and MS/MS mode. Initial characterization attempts were conducted through LC/MS analysis of the degraded samples and analysis only of the retention time around the degradation peak. Analysis of Degradation Peak A showed two meaningful m/z peaks, 111.8 and 152.8, Figure 3.14.

Figure 3.14 MS analysis of Imexon Degradation Peak A.
The m/z of 111.8 is an isomer of the parent while the 152.8 is the isomer forming the same complex with ACN that is seen with the parent. Analysis of the peak at 20.2 minutes yielded no meaningful m/z peaks. MS/MS analysis of the same degraded sample, injected via the HPLC system, displayed the same fragmentation ions seen in the flow shot injection of the standard for the Degradation Peak A and again no meaningful peaks for the peak at 20.2 minutes were observed.

In order to ensure accurate sampling of each component, a degraded sample of Imexon was separated using the Waters HPLC with 100% IPA as a mobile phase. While a mobile phase of 100% IPA provided better resolution of the degradation product from the parent the peak, symmetry was substantially decreased. Five injections were collected as separate fractions and the IPA was evaporated. Fractions were reconstituted with methanol and injected via flow shot for MS/MS analysis. Degradation Product A and the parent drug gave the same mass as seen with the standard. Analysis of the peak at 20.2 minutes showed the similar mass fragments; however, its signal was over three times lower than that of the other products and therefore was not meaningful.

Based on these data, the structure of Degradation Product A is proposed to be a constitutional isomer of the parent drug. It is hypothesized to have the structure shown in Figure 3.15. Mechanism of degradation coincides with that proposed by Den Brok, et al. (2005a and 2005b).
Concurrent with the proposed structure is the nature of the peak seen via chromatography, assuming that the only interaction taking place on the column is the hydrophilic interaction of the molecule to the column then a molecule with a more nonpolar LogP would elute before a molecule with a more polar LogP. Noting that the proposed degradation product has a LogP of -1.21 and Imexon’s LogP of -1.35 it would be expected that the proposed degradation product would elute before Imexon, Figure 3.5.

3.4.4.2 Nuclear Magnetic Resonance

Fractions of Degradation Product A were collected via HPLC separation with 100% IPA as the mobile phase. The organic solvent was evaporated off and the sample was reconstituted with DMSO. Resultant $^1$H NMR data showed two doublets at 5.2 and 5.8 ppm. Theses peaks are consistent with the alkene shown in Figure 3.15.
In addition to the previous results, visual inspection of the high concentration stability samples (pH 7.2 and 8.8 at 10 and 18.5 mg/mL) revealed the formation of a precipitate as Imexon degraded. The precipitate was found to be more soluble in both high pH (~11.0) and low pH (~1.5), suggesting that the degradation product is a zwitterion. Structural analysis of the proposed Degradation Product A (Figure 3.15) suggest two potential ionizable functional groups; the nitrogen at position “1” being acidic and the nitrogen at position “2” being basic. However, due to the rapid degradation of Degradation Product A, confirmation of these pKa’s via potentiometric titration was not possible. This observation further supports the structure of the Degradation Product A.

3.5 CONCLUSIONS

Imexon has been shown to undergo apparent first-order degradation via general acid/base catalysis in aqueous conditions independent of all variables studied. The stability of Imexon is dependant upon pH, ionic strength, low temperature and low initial concentration. Maximal stability of Imexon would be achieved in aqueous conditions at a pH between 7.2 and 9.0 with low ionic strength, temperature and initial concentration. Further analysis has shown stability to be increased in D$_5$W, as compared to normal saline, presumably due to the lower ionic strength. The solubility of Imexon has been determined in 0.5 normal saline, sterile water and as a function of pH. Aqueous solubility has been shown not to change with pH in the range of 5.1 to 8.5. Analyses
have been conducted and a constitutional isomer of Imexon has been proposed as the primary degradation product.
CHAPTER 4: DEVELOPMENT AND EVALUATION OF AN ENTERIC COATED ORAL FORMULATION

4.1 INTRODUCTION

The primary route of administration for drug delivery is oral. In order to prepare an efficacious oral formulation for Imexon the preformulation data presented in Chapter III and Kuehl et al. (2006a) must be revisited. These data indicate that the solubility of the Imexon is not a function of pH and therefore use of a buffer to alter physiological pH will not affect solubility. The intrinsic aqueous solubility is sufficient and an oral formulation will not require the addition of any solubilizing agents. Furthermore, dissolution studies indicate that the drug dissolves rapidly; therefore, absorption should not be dissolution rate-limited. Based on these data the solubility of Imexon is sufficient and should not require any excipients to increase the solubility when creating a formulation for oral administration.

The difficulty in formulating Imexon orally will be related to its rapid degradation when exposed to acidic conditions. The pH of the human stomach varies between 1 and 3.5 and gastric emptying can range between 1 and 4 hours. Near the pH of the stomach Imexon has an approximate half-life of 0.067 hours (~ 4 minutes). Based on these data, Imexon would be completely degraded in ~24 minutes. Research has shown that the stability of drugs that undergo hydrolysis can be improved by formulation into soft
gelatin capsules (Maconachie, 1977; Ebert, 1977). Furthermore, dosage forms of drugs that are susceptible to degradation in the stomach, due to the acidic medium, generally are protected via the application of an enteric polymer.

4.2 BACKGROUND

4.2.1 Use of Enteric Polymers

Enteric coating polymers have been employed for years for several different reasons. Some of these include; decreasing the incidence of gastric irritation (Petroski, 1989), to deliver drugs to the small intestine (Sherif et al., 1969; Cole et al., 2002) and to improve the stability of drugs susceptible to degradation (Felton et al. 1995; Felton and McGinity, 2003).

There are currently several different polymers that are used to coat pharmaceutical dosage forms; these include cellulose, vinyl and acrylic derivatives. Derivatives of these polymers are usually weak acids, which ionize at elevated pH. When ionization occurs the polymers are soluble and allow release of the drug. Functionally, this is advantageous because at the low pH of the stomach the polymers are not soluble and protect the drug from degradation, while at the higher pH of the small intestine the coating will dissolve and present the drug to the small intestine, where it can be absorbed.
The fact that the pH of the small intestine varies, duodenum pH 5.5 - 6.0, jejunum pH 6.0 – 6.5 and ileum pH 6.5 – 7.0, is also advantageous in that it allows for targeted release of drug molecules through selection of acid functional groups. For example, if a formulation is desired to be released in the duodenum the pKa of the acid should be at or near pH 5.0, whereas, for delivery to the ileum the pKa should be at or near 6.5.

While selection of pKa allows for targeted delivery to a specific region of the small intestine, the primary objective for an enteric coated formulation of Imexon will be to protect it from the acid-catalyzed degradation in the stomach. Therefore, the pKa of the polymer will be such that it will not ionize in the stomach, e.g., greater then ~ 4.5. As such, Eudragit L 30 D-55, a commercially available enteric coating product designed to dissolve at or near pH 5.5, was selected for exploration.

4.2.2 Eudragit L 30 D-55

Eudragit is an aqueous dispersion of an anionic copolymer based on methacrylic acid and ethyl acrylate. The structure is show in Figure 4.1. The expected pKa of the carboxylic acid is at or near 5.0. The ratio of the carboxyl groups to the ester is about 1:1 (Rohm GmbH & CO, 2004). With an acidic pKa at or near 5.0 the polymer matrix will ionize once it is presented into the duodenum, of the small intestine.
Figure 4.1 Molecular structure of Eudragit L 30 D-55.

According to the Degussa website, the release profiles of their commercially available polymers are shown in Figure 4.2. These data confirm that L 30 D-55 should protect the Imexon API from the acidic conditions of the stomach and present the API to the proximal regions of the small intestine.
Figure 4.2 Release rates of different Eudragit polymers as a function of pH.

Research has shown Eudragit L 30 D-55 to be an effective enteric coating polymer for coating gelatin capsules (Felton et al. 1995; Felton et al. 1996; Zheng and McGinity 2003; Felton and McGinity, 2003). Furthermore, Eudragit L 30 D-55 is a well-established coating technology that has been used in a number of FDA approved formulations.

4.3 EXPERIMENTAL SECTION
4.3.1 Materials

Imexon was provided by AmpliMed Corp., Tucson, AZ, USA. Eudragit L 30 D-55 was received from Rohm Tech, Maiden, MA, USA. Triethyl Citrate (TEC) was obtained from Morflex, Inc. (Greensboro, NC, USA). Hydrochloric acid (0.1N, HCl) was purchased from Sigma Aldrich (St. Louis, MO, USA). A Millipore (Billerica, MA, USA) Milli-Q Ultrapure Water purification system with a 0.22 μm filter was utilized for water.

4.3.2 Coating Preparation

The preparation of the coating material was modeled after those described and tested by other researchers (Felton et al., 1995). Fifty milliliters of Eudragit L 30 D-55 was mixed with 3 mL of TEC. Mixing was initiated via a magnetic stir plate. While mixing 47 mL of water was added to the mixture. The solution was allowed to mix for at least 30 minutes prior to application to the gelatin capsules.

4.3.3 Spray Coating Procedure

The coating material was sprayed, as an aerosol, onto tumbling gelatin capsules for approximately five minutes. Enteric coating medium was applied onto a limited number of capsules at one time to enhance uniformity of coating and to ensure that application of the aqueous based material did not dissolve the gelatin capsule itself. After application of
one coat the capsules were allowed to completely dry. This procedure was repeated four times for complete and uniform coverage of the capsules.

4.3.4 Visual Inspection of Enteric Coated Capsules

A Leica DMLP polarizing microscope (E. Licht Co., Denver, CO) was used to visually inspect enteric coated capsules. Photomicrographs were taken using a Nikon camera, which was attached to the microscope.

4.3.5 Visual Evaluation of Enteric Coated Capsules

The integrity of the enteric coating was evaluated on coated gelatin capsules by dissolution testing in 0.1 N HCl. The coated capsules were agitated in 0.1 N HCl for a period of four hours, as these conditions should mimic those of the stomach. Visual inspections were conducted throughout agitation to evaluate dissolution/disintegration of the coated capsules.

Upon determination that the coating prevented dissolution/disintegration in the acidic medium, coated capsules were agitated in 0.1 N HCl for a period of four hours and subsequently transferred to a phosphate buffer at pH 7.2 and agitated. This study was conducted to mimic oral administration of the capsule, as the capsule is first exposed to the acidic environment of the stomach, for ~ 4 hours, and then transferred to the small
intestine, pH 5 and above. If the capsule remains viable in the acidic medium and is then dissolved in the pH 7.2 buffer it can then be deduced that it would protect its contents from the stomach and release them to the small intestine.

4.4 RESULTS AND DISCUSSION

4.4.1 Visual Evaluation

The spray application of the coating material resulted in coated capsules that maintained principle shape and capsule integrity. Examination of the coated capsules under magnification revealed that the surface aesthetics of the coating procedure may not be ideal, Figure 4.3.

Figure 4.3 Magnified photomicrograph of an enteric coated soft gelatin capsule.

However, the coating procedure did result in complete coverage of the capsule with the enteric matrix. Furthermore, application of the aqueous material did not disrupt the
integrity of the gelatin capsule. Based on these data the coating procedure appears viable for application onto a gelatin capsule and appropriate for evaluation via dissolution.

4.4.2 Evaluation of Enteric Coating via Dissolution

Initial evaluation of the integrity of the enteric coating was determined by dissolution testing of both coated and uncoated gelatin capsules in 0.1 N HCl. Capsules were agitated, by a magnetic stir bar, in 0.1N HCl for a period of four hours. Under these conditions the uncoated capsules disintegrated in ~ 15 minutes while the coated capsules remained intact for the entire four hour period, Table 4.1.

<table>
<thead>
<tr>
<th>Capsule</th>
<th>0.1N HCl protection</th>
<th>pH 7.2 protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated (n=5)</td>
<td>~ 15 minutes</td>
<td>~ 30 minutes</td>
</tr>
<tr>
<td>Coated (n=5)</td>
<td>&gt;4 hours</td>
<td>~ 25-30 minutes</td>
</tr>
</tbody>
</table>

Table 4.1 Comparison of coated and uncoated gelatin capsules disintegration time in different media.

Subsequently, the coated capsules, that had remained intact in the 0.1 N HCl, were transferred to a pH ~ 7.2 phosphate buffer. Under these conditions the coated capsules dissolved in ~ 30 minutes, nearly the same time as the uncoated capsules.

4.5 CONCLUSIONS
As suggested by previous researchers, the application of Eudragit L 30 D-55 provides an effective enteric coating for protection to acidic medium, typically in the stomach. This is followed by dissolution when exposed to the higher pHs typically experienced in the small intestine. Experimental data for the capsules in these studies indicate that while the aesthetics of the coating procedure are not ideal, the coating is efficacious. The coated capsules, based on *in vitro* analysis, will protect Imexon from the acidic conditions of the stomach and present the drug to the small intestine. This should improve the bioavailability of oral Imexon.
CHAPTER 5: DETERMINATION OF THE SOLUBILTY AND STABILTY OF IMEXON IN A PRESSURIZED METERED DOSE INHALOR

5.1 INTRODUCTION

Imexon has shown *in vivo* activity against a number of different cancer cells lines including non-small-cell lung cancer (NSCLC) (Salmon and Hersh, 1994; Salmon and Hersh, 1995). The administration of a drug via inhalation to the lung may be therapeutically advantageous for the treatment of many lung diseases. By targeting the lung, the drug is delivered directly to the intended site of action. This can afford a rapid biological response, an increase in drug concentration at the site of action, a decrease in systemic exposure and the elimination of first-pass affects. The most common and convenient method to delivery therapeutic agents to the lung is via a pressurized metered dose inhaler (MDI) (Gupta and Myrdal, 2004a). Pressurized metered dose inhalers use a propellant to aerosolize and disperse drug particles for inhalation into the respiratory system.

An MDI can either be formulated as a suspension, where the API is dispersed in the propellant system, or a solution, where the API is dissolved in the propellant system (Smyth, 2003). In order to formulate an MDI the solubility of the API in the propellant is critical, as it dictates what type of formulation is selected (suspension or solution). Once a formulation type is selected the chemical and physical stability of the formulation must
be assessed. For a solution formulation the chemical stability will be the predominate focus, while in a suspension formulation both physical and chemical stability will be required.

The preformulation work detailed in Chapter 3 indicates that Imexon is soluble in water and the degradation rate has been shown to be a function of pH, initial drug concentration, temperature, ionic strength and buffer species. While not all of the conditions evaluated in Chapter 3 apply to an MDI environment, these data will be utilized to assist in determining what conditions should be evaluated for an MDI formulation of Imexon. Initially, the solubility of Imexon will be determined in propellant and as a function of two cosolvents; EtOH and water. Once solubility values have been determined the stability of Imexon will be determined as a function of temperature, EtOH concentration, water concentration and initial drug concentration.

5.2 BACKGROUND

5.2.1 Pressurized Metered Dose Inhalers

Since the introduction of MDIs in the mid-1950s by Riker laboratories (3M), chlorofluorocarbons (CFCs) have been the propellants of choice in pressurized MDIs. However, scientific research has concluded that CFCs deplete the stratospheric ozone layer through a UV induced, free radical cascade with ozone. This discovery prompted...
the Montreal Protocol, in which 46 countries signed an international treaty to phase out the production and use of CFC products by the year 2000 (Montreal Protocol, 1987). This date was eventually advanced to 1996 due to the severity of the environmental issue. The use of CFCs for medicinal MDIs was granted an exempt due to their therapeutic need and the lack of appropriate alternatives to treat asthma and COPD. While a number of industries have been able to transition from CFCs to HFAs, the conversion of MDIs to HFA-based propellant systems has been complex and slow. The slow conversion has been largely due to reformulation issues relating to the new HFA propellants.

There are currently two HFA propellants that are under development in the pharmaceutical industry, HFA-134a and HFA-227. While these two propellants have physical properties that are acceptable for MDI use, they are significantly different than those of the CFCs. A compilation of physical-chemical properties for CFC and HFA propellants is given in Table 5.1. From the data in Table 5.1, it can be seen that the boiling points and vapor pressures differ significantly, with HFAs having relatively high vapor pressures. While this increase in vapor pressure has proven useful in making more efficient delivery systems, there are limited options for reducing vapor pressure through propellant blending.
<table>
<thead>
<tr>
<th>Propellant</th>
<th>Formula</th>
<th>MW</th>
<th>Boiling Point (°C)</th>
<th>Density (20°C)</th>
<th>Vapor Pressure (psig, 20°C)</th>
<th>Solubility Parameter (cal/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFC-11</td>
<td>CCl₃F</td>
<td>137.4</td>
<td>24</td>
<td>1.49</td>
<td>-1.8</td>
<td>7.5</td>
</tr>
<tr>
<td>CFC-12</td>
<td>CCl₃F₂</td>
<td>120.9</td>
<td>-30</td>
<td>1.33</td>
<td>67.6</td>
<td>6.1</td>
</tr>
<tr>
<td>CFC-114</td>
<td>C₂Cl₂F₄</td>
<td>170.9</td>
<td>4</td>
<td>1.47</td>
<td>11.9</td>
<td>8.0</td>
</tr>
<tr>
<td>HFC-134a</td>
<td>CF₃CH₂F</td>
<td>102</td>
<td>-27</td>
<td>1.21</td>
<td>81</td>
<td>6.6</td>
</tr>
<tr>
<td>HFC-227</td>
<td>CF₃CHF₂F₃</td>
<td>170</td>
<td>-17</td>
<td>1.41</td>
<td>56</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Table 5.1 Physical-chemical properties of different propellants.

For the studies conducted herein HFA-134a was utilized as the propellant. The structure of HFA-134a is shown in Figure 5.1.

![Figure 5.1 Chemical structure of HFA-134a.](image)

The most significant problem in reformulating HFA-based systems has been in the differing solvency properties of CFCs and HFAs. Ethanol can be used as a cosolvent in HFA-134a systems. Its use however, is limited by stability and product performance concerns (Gupta and Myrdal, 2003).

5.2.2 Solubility Determination
5.2.2.1 Traditional Method

As highlighted in the background of MDIs, the differences in the solvency properties of CFCs and HFAs has caused great difficulty in formulating new CFC free MDIs. The traditional methodology employed to determine the solubility of compounds in propellants is a technique that employs significant time and labor. The traditional method involves equilibration of the compound in the propellant (Dalby et al., 1991; Williams et al. 1999). The liquid propellant, in the equilibrated vial, is then transferred to a chilled receiving vial, schematic shown in Figure 5.2. Both vials must be crimped with continuous flow valves in order for the cold transfer to take place. The two vials are connected via couplers and the contents of the donor vial are filtered as they pass into the receiving vial to ensure only the dissolved compound is transferred. After the transfer is complete, the weight of the receiving vial is recorded and the vial is chilled below its boiling point. Once chilled, the vial is either decrimped or punctured and the contents are analytically transferred to a chilled volumetric flask. The propellant is then allowed to boil and the compound is dissolved in a known amount of a suitable solvent. The diluted sample can then be assayed via HPLC.
Figure 5.2 Schematic of the traditional MDI solubility method.

This method not only requires considerable amounts of time to generate solubility data, but it also has significant possibility for experimental error related to human error. Furthermore, this method allows for only one solubility measurement to be taken from each equilibrated MDI vial.
5.2.2.2 Online Direct Inject Method

Due to the inherent disadvantages of the traditional method a new method has been developed and validated for the analytical quantification of pharmaceutical ingredients in an MDI environment (Gupta and Myrdal, 2004a; Gupta and Myrdal, 2004b; Gupta and Myrdal, 2005). The new online direct inject method utilizes direct injection of the MDI into the injection port of a manual injector on an HPLC, schematic shown in Figure 5.3. In this method, the MDI vial is crimped with a continuous valve fitted with a coupler, filter and an injection needle. The injection needle is seeded into the injection port of the manual injector and the valve is actuated to allow sample to flow into the sample loop. A backpressure regulator is maintained on the overflow of the injection port to ensure that the propellant is maintained in the liquid state when filling the sample loop.

This method has been shown to be sensitive and accurate while not requiring the time of the traditional method. Furthermore, this method allows for an MDI vial to be repeatedly assayed for concentration of the pharmaceutical ingredient of interest, affording multiple solubility samples or stability of the API as a function of time. As such, this method was employed for both solubility determination and stability assessment of Imexon in an MDI environment.
Figure 5.3 Schematic of the new online direct inject method

5.3 EXPERIMENTAL
5.3.1 Materials

Imexon was provided by AmpliMed Corp., Tucson, AZ, USA. Pressure resistant glass aerosol vials and continuous valves were provided by 3M Drug Delivery Systems (St. Paul, MN, USA). 1,1,1,2-Tetrafluoroethane (HFA-134a) and ethanol (200 proof) were obtained from DuPont Chemicals (Wilmington, DE, USA) and Aaper Alcohol and Chemical Company (Shelbyville, KY, USA), respectively. Ammonium acetate ($C_2H_7NO_2$) was obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (ACN) was obtained from EMD (Gibbstown, NJ, USA). A Millipore (Billerica, MA, USA) Milli-Q Ultrapure Water purification system with a 0.22 µm filter was utilized for water.

5.3.2 HPLC Method

The online direct inject method detailed in Section 5.2.2.2 was employed to allow for direct injection of the MDI into a Waters 600E multisolvent delivery module (Waters, Milford, MA, USA) coupled with a Waters 2487 diode array (Dual) detector. Analysis was performed by a normal phase HPLC assay, using a 150 mm x 4.6 mm, Apollo Silica 5 µ column (Alltech Associates, Deerfield, IL). Ultraviolet detection was done at 234 nm. Mobile phase conditions were 90:10 (v/v) ACN:H_2O at a flow rate of 0.6 mL/min. Water was buffered with ammonium acetate at 0.1 M, with a pH of approximately 6.50.
The injection volume was 5 µL. The parent compound had a retention time of 7.8 minutes.

5.3.3 Solubility Determination

The solubility of Imexon was determined in pure HFA-134a propellant at 23°C. The solubility of Imexon has also been determined as a function of the cosolvent EtOH at 5, 10, 15, 20 and 25 % (w/w). Preformulation work has also shown that Imexon has favorable water solubility (~25 mg/mL, at 23°C), thus the solubility of Imexon was also explored as a function of water. Using ethanol as a cosolvent for water, solubility was determined for formulations containing 0.5 and 1% H₂O (w/w) with 10% EtOH, and 0.5, 1, 1.5, 2, and 3% H₂O (w/w) with 20% ethanol (w/w).

5.3.4 Stability of Imexon in an MDI

The stability of Imexon, in an MDI environment, was determined as a function of temperature at 11°, 23° and 37°C, with a MDI containing 80:20 HFA-134a:EtOH and 80 µg/g of Imexon. The effect of EtOH was evaluated at 80:20 and 75:25 HFA-134a:EtOH with a drug concentration of 80 µg/g. The stability as a function of H₂O was determined with compositions of 80:20:0, 79:20:1 and 78:20:2 (HFA-134a:EtOH:H₂O, % w/w) with 80 µg/g of Imexon. Because initial drug concentration had an effect on stability in aqueous studies, the effect of initial drug concentration on stability was determined at
concentrations of 80, 150 and 250 µg/g with an inhaler composition of 78:20:2 (HFA-134a:EtOH:H₂O, % w/w).

5.4 RESULTS AND DISCUSSION

5.4.1 Solubility

The solubility of Imexon in pure HFA-134a was determined to be 0.00022 % (w/w). The solubility of Imexon as a function of the cosolvent EtOH is presented in Figure 5.4. As the figure indicates, the solubility of Imexon increases linearly as a function of EtOH up to 25%.

![Figure 5.4 Solubility of Imexon in a MDI as a function of EtOH in HFA-134a.](image)
Given that Imexon is relatively polar, the use of water to increase the solubility of Imexon was evaluated. In view of the fact that water is relatively immiscible with HFA-134a alone, the presence of either 10 or 20% (w/w) ethanol was incorporated to solubilize the water. Figure 5.5 displays the solubility increase for Imexon with 10% ethanol and water concentrations of 0, 0.5, and 1% (w/w).

![Figure 5.5 The solubility of Imexon as a function of water with 10% EtOH in HFA-134a.](image)

Figure 5.6 shows the solubility of Imexon as a function of water at 0, 0.5, 1, 1.5, 2 and 3% (w/w) with 20% ethanol. Both Figure 5.5 and 5.6 indicate that the solubility of Imexon increases linearly as a function of H₂O concentration regardless of the amount of EtOH present. This is as would be expected based on the solubility of Imexon in water at ambient conditions.
Figure 5.6 Solubility of Imexon as a function of water with 20% EtOH in HFA-134a.

These solubility data, along with preformulation data collected under aqueous conditions, were utilized to determine the formulation combinations for evaluating the chemical stability of Imexon in a solution MDI. Specifically, these were the effect of temperature, H₂O, EtOH and initial drug concentration on the degradation of Imexon in a MDI.

5.4.2 Stability

Prior to formulation of stability vials, the effectiveness of the direct inject method to function as a stability indicating method was established. The HPLC conditions (column, mobile phase, flow rate, etc.) previously used to characterize the stability of Imexon under aqueous conditions were combined with the direct injection method (Kuehl et al., 2006). Importantly, the mobile phase contains a high percentage of organic (90:10, ACN: H₂O) which facilitates the direct inject of the non aqueous formulation (Gupta and
Myrdal, 2004b). For an initial screen, a representative formulation was prepared (80:20 HFA-134a:EtOH, 80 µg/g) and crimped with a continuous valve. The vial was stored at 37°C to facilitate the degradation of the Imexon parent drug. The chromatography of the Imexon and degradant from the non aqueous system was similar to that observed from analysis of Imexon under aqueous conditions. As can be seen from the four sequential injections represented in Figure 5.7, the Imexon peak is well resolved from Degradation Peak A. The degradation product is proposed to be the same degradation product observed in aqueous media, Section 3.4.4.

Figure 5.7 Chromatograph of four repeat injects of a degraded Imexon MDI formulation.

The overall method capabilities were in alignment with aqueous analysis, having a linear range from 5 to 500 µg/mL. Repeated analyses from the same vial have an RSD of 4%. From these data it was concluded that the direct inject method is feasible for use in preformulation stability studies. In order to accurately determine the concentration at
each time point, each vial was injected four times. The reported concentrations are averages of these four injections.

Similar to all degradation studies conducted in aqueous conditions MDI vials were assayed for a minimum of six sample points, spanning three to six half lives. In order to minimize the potential for self catalysis (Waterman and Adami, 2005) samples were prepared at a dilute concentration of 80 μg/g, and stored at 23°C, unless otherwise stated. Sampling times, again, varied due to large differences in degradation rates.

5.4.2.1 Effect of Temperature

The effect of temperature on the degradation of Imexon was evaluated with formulations conditions of 80:20 (HFA-134a:EtOH), 80 μg/g initial Imexon concentration and stored at three different temperatures (11°, 23° and 37°C). To maintain consistency, the inhalers were brought to room temperature for analysis and then immediately returned to the appropriate storage condition. Analysis of Log percent drug remaining as a function of time (Figure 5.8) indicate that Imexon undergoes apparent first-order degradation in an MDI environment, which was ultimately observed to be the same for all MDI conditions evaluated.
Figure 5.8 Log percent remaining for Imexon in an MDI environment, indicating apparent first-order degradation.

As Imexon undergoes apparent first-order degradation equations described in Section 3.2.3.1 were utilized to calculate relevant degradation parameters. Similarly, equations described in Section 3.2.3.3 were utilized to calculated relevant degradation parameters for the effect of temperature on degradation.

Analysis of the effect of temperature on the degradation of Imexon indicates that the degradation rate of Imexon increases as the temperature increases. An Arrhenius plot for the three temperatures evaluated is shown in Figure 5.9, which results in a calculated activation energy of 110.04 kJ/mol for Imexon in the propellant system.
y = -13.2x + 35.3

$E_a = 110.04 \text{ kJ/mol}$

Figure 5.9 Arrhenius plot for Imexon in an MDI environment.

Relevant degradation parameters, for all conditions evaluated in an MDI, are shown in Table 5.2.

<table>
<thead>
<tr>
<th>HFA-134a (%)</th>
<th>EtOH (%)</th>
<th>H₂O (%)</th>
<th>Imexon (µg/g)</th>
<th>Temp (°C)</th>
<th>k (Hours⁻¹)</th>
<th>T₅₀ (Hours)</th>
<th>T₉₅ (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>20</td>
<td>0</td>
<td>80</td>
<td>23</td>
<td>0.000184</td>
<td>3761</td>
<td>279.5</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>0</td>
<td>80</td>
<td>11</td>
<td>0.000009</td>
<td>75228</td>
<td>5591</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>0</td>
<td>80</td>
<td>37</td>
<td>0.000336</td>
<td>2061</td>
<td>153.2</td>
</tr>
<tr>
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<td>0.000576</td>
<td>1204</td>
<td>89.4</td>
</tr>
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<td>20</td>
<td>2</td>
<td>80</td>
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<td>1067</td>
<td>79.3</td>
</tr>
<tr>
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<td>0.000157</td>
<td>4425</td>
<td>328.9</td>
</tr>
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<td>0.000415</td>
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<td>2</td>
<td>250</td>
<td>23</td>
<td>0.000394</td>
<td>1760</td>
<td>130.8</td>
</tr>
</tbody>
</table>

Table 5.2 Calculated degradation parameters for Imexon in an MDI environment
5.4.2.2 Effect of EtOH

The effect of EtOH concentration on the degradation of Imexon in an MDI environment was evaluated at EtOH concentrations of 20 and 25% (w/w). Referring to Table 5.2, it can be seen that increasing the concentration of EtOH decreases the degradation rate of Imexon.

5.4.2.3 Effect of H₂O

In order to elucidate the effect water concentration has on the degradation of Imexon, in an MDI, formulations were prepared with water levels of 0, 1 and 2% (w/w). As would be expected based on aqueous preformulation stability, an increase in the concentration of water results in an increase in the degradation rate of Imexon.

5.4.2.4 Effect of Initial Drug Concentration

Under aqueous conditions the initial concentration of Imexon was determined to have a direct correlation to the degradation rate. In order to determine if this was true in an MDI, formulations were prepared at 80, 150 and 250 µg/g. Analysis of these formulations indicates that when the concentration is increased the degradation rate is decreased, data shown in Table 5.2.
5.5 CONCLUSIONS

Preformulation solubility studies on Imexon indicated that Imexon has low solubility in HFA-134a alone. However, both EtOH and H₂O proved to be effective cosolvents for increasing the solubility in HFA-134a. The direct inject method proved to be an effective method for determining the stability of Imexon in an MDI environment. The stability data for Imexon in an MDI indicates that the Imexon undergoes apparent first-order degradation under all conditions studied. The degradation of Imexon is accelerated with increase temperature and the calculated activation energy for Imexon is 110.04 kJ/mol. The degradation rate of Imexon is directly related to the concentration of water and inversely related to initial drug concentration. The degradation was determined to decrease as the concentration of EtOH was increase from 20 to 25%.
SUMMARY

Imexon is a member of a group of 2-cyanoaziridine derivatives that has been under investigation since the 1970’s due to its anticancer properties. Prior to the studies detailed herein Imexon had shown activity in several tumor cell lines and animal tumor models and did not appear to be susceptible to multiple drug resistance. Preclinical data had shown the efficacy of Imexon to be schedule dependant, favoring prolonged exposure times (Dorr et al. 1995; Hersh, 1992; Sagaster et al. 1995) and Imexon is well tolerated in all species studied (Dvorakova et al., 2002a). However, Imexon undergoes rapid decomposition in aqueous medium that can be affected by numerous variables. As such, the preformulation studies were conducted to gain a more complete understanding of Imexon’s degradation. These preformulation data provide a basis of knowledge to be utilized in formulation development.

Imexon is a crystalline solid that is stable at room temperature. The melting point of Imexon is not known as it decomposes at ~ 165°C, prior to melting. Characterization of several different lots of Imexon indicated no differences in the crystal of the API. In the crystal, adjacent molecules are held together via a hydrogen bond network that forms tapes of infinite length. There are however, no interactions between adjacent tapes, which cause the crystals to be delicate in nature. While Imexon crystallizes as a zwitterion, no pKa was found in the pH range of 5.0 to 8.5. The solubility of Imexon was determined to be 25 mg/mL in water at 23°C and showed no dependence on the solution pH.
Imexon was determined to undergo apparent first-order degradation under all conditions evaluated. Imexon undergoes general acid/base catalysis with maximal pH stability in the range of 7.5 to 8.5. The degradation rate of Imexon has been shown to increase as the ionic strength of the solution is increased. This was found to be true at both 0.12 mg/mL and 10 mg/mL. An increase in temperature results in an increase in the degradation rate of Imexon, regardless of the pH of the solution. In contrast to typical first-order kinetics an increase in initial drug concentration has also been shown to increase the degradation rate of Imexon.

Based on the preformulation data an oral formulation of Imexon would be rapidly degraded in the acidic conditions of the stomach, therefore, an enteric coated formulation has been developed and evaluated in vitro. The enteric coated formulation will protect the drug from the stomach, low pH, and then be released in the duodenum for absorption by the small intestine.

Preformulation work in a MDI has shown Imexon to have a solubility of 0.00022 % (w/w) in HFA-134a alone. Both EtOH and H2O function as effective cosolvents to increase the solubility of Imexon in the propellant system. The stability of Imexon in the MDI was determined to decrease as the temperature increased. As would be expected from the aqueous stability, the degradation rate of Imexon, in an MDI, increases as the
concentration of H$_2$O is increased. Differing from the aqueous stability, the degradation rate of Imexon decreases as the initial concentration is increased.
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