The Separation and Fragmentation of 15-deoxy, $\Delta^{12,14}$-prostamide J_2 as an Anti-Tumor Therapeutic Using ESI-MS/MS

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ABSTRACT

According to the American Cancer Society, colon cancer is the third most diagnosed cancer in people of all ages and the second leading cause of death due to cancer, in the United States. Most cancer treatments involve chemotherapy, which kills the rapidly growing cancer cells, along with healthy cells including blood-forming cells. A class of prostaglandins and prostamides was found to have the capability to cause endoplasmic-reticulum stress-induced apoptosis in cancer cells with minimal effects on non-cancerous cells. This study focuses on 15-deoxy, $\Delta^{12,14}$-prostamide J_2, or 15d-PMJ_2, an effective molecule shown to eliminate cancerous colon cells. The 15d-PMJ_2 will be delivered through micelles due to its nature of being hydrophobic and simply injecting the drug intravenously would be problematic due to solubility issues. To better characterize the drug for future studies, mass spectrometry is being used to analyze and assign the fragments of the molecule when subjected to collision-induced fragmentation into smaller ions. These fragments can be used as a fingerprint for 15d-PMJ_2 which provide high selectivity for detecting 15d-PMJ_2 within a complex mixture.

INTRODUCTION

1.1 Cancer Therapeutics

1.1.1 Cancer Treatments. Cancer is the second leading cause of death in the United States with over 600,000 deaths in the previous year, according to the Centers for Disease Control and Prevention [1]. Furthermore, the second leading cause of death due to cancer is colorectal cancer, which is responsible for approximately 9% of all cancer deaths [2]. Cancer is the rapid and uncontrollable growth of abnormal cells, which invade the surrounding healthy cells, causing damage to the organ. Many different therapies exist and are used to kill cancer cells; however, many treatments are associated with harmful side effects that cause damage to healthy cells. Those include surgical removal of tumors, radiation therapy, and chemotherapy. Some of the common side effects of current cancer treatments are fatigue, anemia, bleeding and bruising, edema, alopecia, nausea, vomiting, and many more issues involving nutrition, sleep, and fertility [3]. Consequently, the treatments target rapidly growing tumorigenic cells. With no means to tell the difference between healthy and cancerous cells, the therapies end up dividing and slowing the growth of non-cancerous cells, along with the cancerous cells.

1.1.2 Eliminating Therapy Side Effects. Chemotherapy works by inducing cell death at different stages of the cell's life cycle [4]. One type of chemotherapy is alkylating agents, which work to stop cell reproduction by damaging its DNA sequence. This is a common type of therapy as it stops the production of the cancer cells, slowing down the spread of the tumor; however, as the medication cannot differentiate between abnormal and normal cells, it typically causes significant damage to bone marrow cells, which affects the production of blood cells, and in rare cases leads to leukemia [5]. All current cancer treatments have adverse effects on healthy cells,

leading to a multitude of side effects which differ magnitude depending on the case. New research of the J-series prostaglandins and prostamides studies the use of this class of molecules in the targeted treatment of cancer cells, by endoplasmic reticulum stress-induced apoptosis [6]. This paper studies a specific prostamide shown to reduce tumor size and cause apoptosis on colon tumor cells, with minimal harm to non-cancerous cells.

1.1.3 J-Series Prostamides. Prostamides are lipid-like compounds that are similar to prostaglandins in structure and hormone-like behavior. A subset of prostamides is prostaglandin-ethanolamides, which involve ethanolamides, which work as surfactants and emulsifying agents. They are synthesized from the ethanolamide of arachidonic acid (ARA), in the same manner as prostaglandins, and can be made from the latter in through a metabolic process involving cyclooxygenase-2 (COX-2) and prostaglandin D synthase (PGDS) [7]. Prostaglandins are pharmacologically significant in the case of their receptor, or multiple receptors, which are able to mediate ER stress-induced apoptosis, ultimately targeting only the tumorigenic cells with the targeted COX-2 compound [8]. The prostamide studied in this research is 15-deoxy, $\Delta^{12,14}$-prostaglandin J_2-ethanolamide or 15-Deoxy, $\Delta^{12,14}$-Prostamide J_2 (15d-PMJ_2), the structure of the molecule is shown in Figure 1.1, which preferentially induces cell-death in tumorigenic keratinocytes in melanoma and colon cancer cells [9]. The 15d-PMJ_2 is synthesized in the lab from prostaglandin, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ_2). Furthermore, no research has been completed on the interaction of the prostamide on the target, however, previous studies show that it does not operate through a receptor similar to how a prostaglandin would.

When targeting colon tumor cells, the issue of drug delivery arises, as 15d-PMJ_2 has a low water solubility, which makes it difficult to make solutions that can be used intravenously to treat these cells. Micelles have been selected as a potential method to deliver the drug to the

targeted site of tumorigenic cells, as they are able to hold the medication in the hydrophilic region, while its hydrophobic exterior does not allow the entrance of water. Once at the absorption site, the micelle breaks down, and the drug interacts with the tumor. In order to fully characterize different micelle formulations of 15d-PMJ_2, it is imperative to be able to identify the molecule by chromatography, which enables properties such as drug-loading and encapsulation efficiency to be measured. A characteristic fingerprint of the molecule can be identified using fragmentation in ESI-MS/MS.

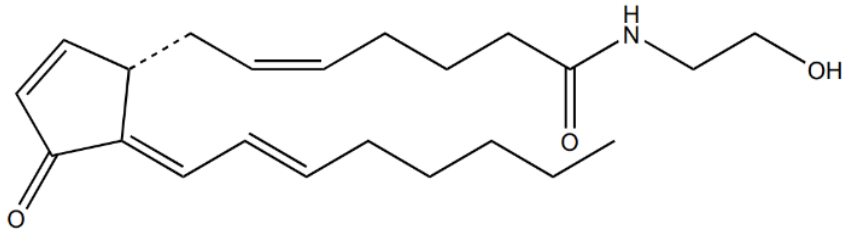

Figure 1.1: The structure of 15-Deoxy, $\Delta^{12,14}$-Prostamide J_2 or 15d-PMJ_2, a J-series prostamide used for ER stress-apoptosis of tumorigenic cells.

1.2 Mass Spectrometry

1.2.1 How Does Mass Spectrometry Work? Mass spectrometry (MS) is a powerful analytical technique that measures the mass-to-charge ratio of ions and enables the identification and quantification of different chemical compounds in a sample. This technique generates a mass spectrum, which displays the mass-to-charge ratio of the ions in the sample based on the ionization mode it is run in. From this, the molecules in the samples can be identified and, in some cases, their concentrations determined. An advanced form of MS is tandem mass

spectrometry, MS/MS or MS^n, which involves multiple steps of mass analysis, the sample goes through multiple analyzers and gets fragmented more. In MS/MS, ions that are produced in the first stage are isolated and fragmented in a collision cell, and the resulting fragments are then analyzed in a second step. This provides a more detailed analysis of structural information on the sample, making it valuable for analyzing complex compounds, elucidating molecular structures, and finding a fingerprint for the molecule. Having a unique fingerprint of the molecule allows for the verification of the presence of a desired analyte in a complex mixture such as when in a micellar formulation. When running drug-loaded samples, it will be desirable to identify the fragments related to the molecule and those of other molecules and impurities.

The mass spectrometer works by passing a sample with a gas phase ion source through to the mass analyzer, where ions are separated by mass-to-charge ratios and output to the ion detector; this is all done under vacuum [8]. The ion detector records the mass and charge data and allows for a system to analyze and present the data in a mass spectrum (Figure 1.2).

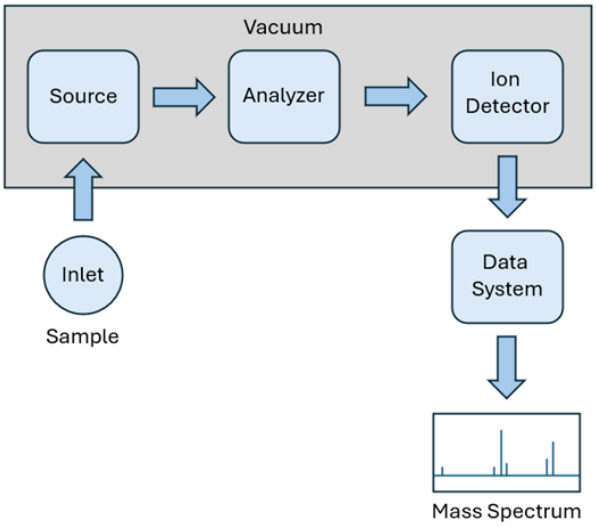

Figure 1.2: The path of operation of a sample passes through a mass spectrometer to produce a spectrum for analysis.

1.2.2 ESI-MS. A commonly used ionization technique in MS is electrospray ionization (ESI). This is categorized as a soft ionization technique, meaning that the initial ions formed usually experience minimal fragmentation which allows for the molecular ion to be observed. ESI involves applying a high voltage to a liquid sample, producing a fine aerosol of charged droplets that pass through the instrument. As the solvent evaporates, the sample droplets shrink and their charge density increases, leading to the ejection of ions in the gas phase for mass analysis. ESI is especially effective for analyzing large biomolecules, such as proteins, prostaglandins, and nucleotides, as it allows for the generation of multiply charged ions, enabling the analysis of these larger, multiple-charge molecules within the instrument's mass range [10]. As the droplets pass through the mass analyzers and are subjected to high-energy electrons, the molecules can break apart in predictable and reproducible patterns, providing insight into their structural features. In MS/MS, a second mass analyzer is used to collide the ion selected from the first mass analyzer with a gas, in this case argon; resulting in a multitude of smaller fragments characteristic of the molecule. Understanding these fragmentation patterns is essential for interpreting mass spectra and identifying compounds produced in a mixture, making it a key component of molecular analysis in MS/MS. Compared to MALDI and DESI, ESI is the most efficient for multiplying charge molecules and therefore used to study 15d-PMJ_2 fragmentation.

1.2.3 Fragmentation Pathways. Molecular fragmentation in mass spectrometry occurs at specific sites on the molecule, influenced by the structure of the molecule, the ionization method, and the collision energy. Typically, fragmentation happens at the weakest bonds of a molecule, however, if the energy is high enough, the molecule can fragment by breaking stronger bonds. Most common fragmentation sites include carbon-carbon single bonds; these bonds can easily break with low collision energies. Another common fragmentation site is heteroatom bonds, which are

bonds between carbon and heteroatoms, as the heteroatoms can hold a charge and stabilize the resulting fragments. In 15d-PMJ_2, one fragment that should be present is a broken carbon-nitrogen or carbon-oxygen bond. Finally, functional groups like alcohols, amines, carboxylic acids, and carbonyls, usually lead to characteristic fragmentation [11]. An alcohol can undergo dehydration, leading to the loss of a water molecule, and a change in the mass of the molecule. In MS/MS, fragmentation is induced by collision-induced dissociation (CID), where the ions collide with argon gas molecules, causing them to break; it depends on the colliding ions' kinetic energy and the target gas' structure and pressure [12].

EXPERIMENTAL

2.1 Materials

The prostamide studied was synthesized from 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 ($\geq$ 95% purity) which was purchased from Cayman Chemicals (Ann Arbor, MI). The solvents used for ESI-MS/MS include LC-grade acetonitrile, formic acid, and ultrapure deionized water (18 MΩ·cm). The 15d-PMJ_2 sample was diluted to 25 μM for MS/MS analysis and was prepared with acetonitrile and 0.1% formic acid solution.

2.2 Instrumentation

2.2.1 ESI-MS/MS. The instrument used for ESI-MS/MS analysis was the AB SCIEX MicroMass Quadrupole/Time-of-Flight mass spectrometer (Milford, MA). The electrospray source was set to positive-ion mode. The flow rate used was 0.200 mL/min and the IonSpray voltage was set at 5000.0 V. Source gas 1 (GS1) nebulizes a stream of liquid at the tip of the electrospray capillary,

while source gas 2 (GS2) transfers heat from the two side heaters into the center providing the heat to evaporate solvent and ions, called desolvation; both set at 50.0. The temperature of the heaters applied to GS2 is set at 500 K. The collision energy was set to 20 eV in the hexapole collision cell during runs, with argon being used as the collision gas. The maximum counts per second range from 2.6×10^6 cps to 7.9×10^6 cps. Data was collected and analyzed using the Analyst software version 1.7.3 and the MultiQuant software.

2.3 Techniques

2.3.1 MS/MS Analysis. The purpose of the research is to find a unique fingerprint for 15d-PMJ_2 to allow for its identification when loaded into a micelle. Tandem mass spectrometry was used to analyze the fragmentation of 15d-PMJ_2 at a collision energy of 20 eV, which is done by examining the parent ion of the molecule (m/z 360, $[M+H]^+$) in the triple quadrupole analyzer and colliding the ions to induce the breaking of bonds. The collision energy accelerates the precursor parent ions at the neutral argon gas atoms, which allows for the collision-induced fragmentation to occur. The more energy used to accelerate the ions, the more fragments and the smaller the fragments produced. These fragments are analyzed and assigned for the characterization of 15d-PMJ_2.

RESULTS AND DISCUSSION

3.1 Mass Spectrometry Results

3.1.1 MS Spectra. The purpose of running mass spectrometry on the molecule 15d-PMJ_2 is to determine the characteristic ions formed so that it can be distinguished from other compounds

that may be present in future experiments; this is done by fragmenting the structure and analyzing the product peaks, the fragment m/z values and their intensity serve as the fingerprint used for subsequent analyses. The collision-induced fragmentation was run over a large range of m/z values, 50 to 500 m/z, to view the smaller fragments of the molecule that may appear after being subjected to electron spray at a 20 eV collision energy. As well as being run with different ranges, the spectra were run multiple times to ensure the presence of the same fragments within different samples of 15d-PMJ_2. The resulting spectra all had the same parent ion peak present at 360 m/z (Figures 3.1 and Figure 3.2).

Other product ions m/z that were consistently present over spectra include 382 (associated with the addition of sodium), 398 (addition of potassium), 342 (dehydration product of $[M+H]^+$), 317 (loss of ethanol), 299 (loss of ethanolamine), 261 (loss of heptene), and 60 (ethanolamine) as illustrated in Figure 3.1 and Figure 3.2. Furthermore, the presence of sodium and potassium are abundant and difficult to rid of from the instrument and the sample, as shown in Figures 3.1 and 3.2, a large intensity peak is associated with the addition of sodium and potassium to our molecule. One possibility for the assignment of an unassigned peak of 442 m/z is the addition of both sodium and potassium on the 15d-PMJ_2, at two different sites. Some peaks remained present in all the spectra; however, they were not associated with any fragments, those being m/z 442, 365, 337, and 122 as shown in Figure 3.1. The fragmentation scheme for 15d-PMJ_2 is shown in Figure 3.3, indicating where the bonds were broken to form the product ions; based on the fragments present in the MS spectra, in Figure 3.1.

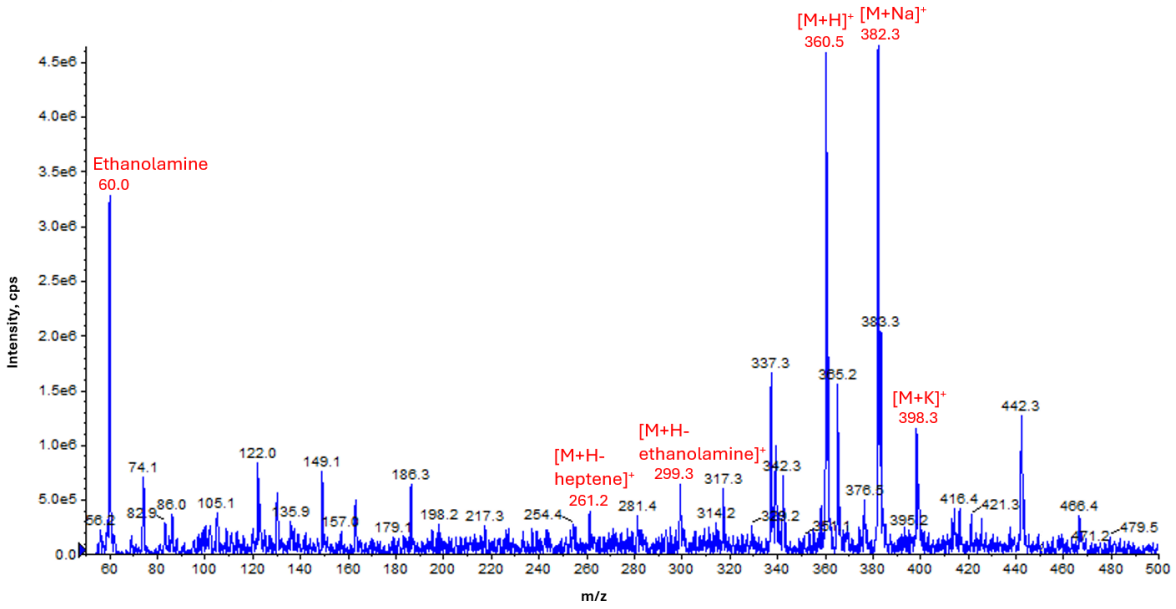

Figure 3.1: Sample MS spectrum for 15d-PMJ$_2$ at a collision energy of 20 eV, with specific product ion peaks exhibiting fragmentation scheme.

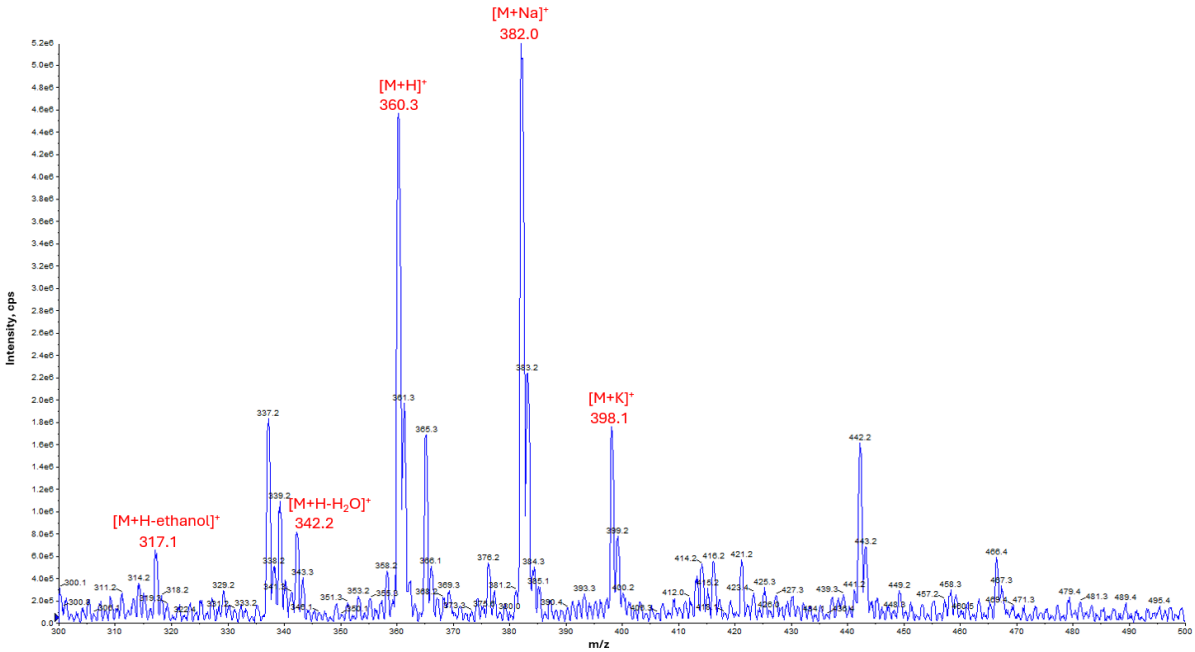

Figure 3.2: Sample MS spectrum for 15d-PMJ$_2$ at a collision energy of 20 eV, re-run at a smaller range, 300 to 500 m/z, presenting the same peaks.

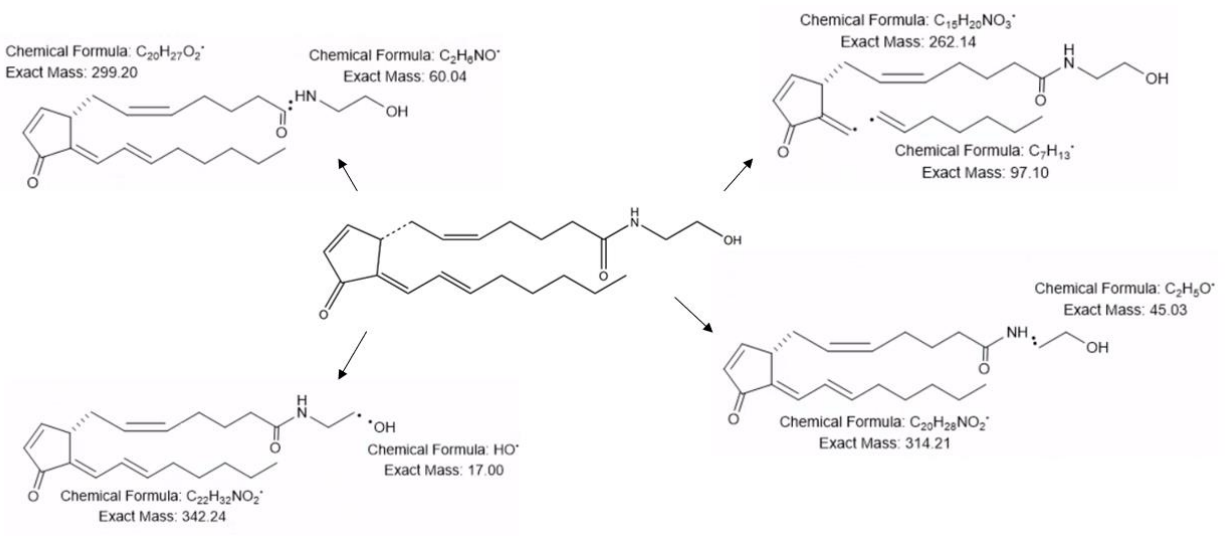

Figure 3.3: The fragmentation scheme for 15d-PMJ_2, showing where the bonds were broken to form each fragment, m/z shown for each charged fragment above.

There are no previous literature spectra to reference for this molecule, the MS spectra were run enough times to determine and analyze the persistent peaks. Spectra for other similar prostaglandins were studied to determine possible fragment patterns that can happen to 15d-PMJ_2, including PGJ_2 and $\Delta^{12}PGJ_2$ [8]. The dissertation shows the potential loss of a hexanal, carboxylate, and water; as mentioned previously, bonds near functional groups tend to fragment more easily as they allow for the product ions to hold charges and remain stabilized [11]. This research also showed the fragmentation of the molecule when analyzed using MS^3 or MS^4. This type of analysis was used when running MS/MS and colliding the parent ion peak, 360 m/z, with argon gas to induce CID.

3.1.2 MS/MS Spectra. After the sample hits the initial mass analyzer, a parent ion peak is selected and is subjected to collision by argon gas, this is then fragmented a second time to

produce a fingerprint pattern that is characteristic of 15d-PMJ$_2$. The parent ion peak selected for analysis is m/z 360.27. The MS^2 spectrum for the molecule is displayed in Figure 3.4, with intense peaks at m/z 78.9, 81.1, 91.1, 104.7, 115.1, 116.8, 118.8, 127.9, 130.9 and 132.7. It is a more difficult task to analyze the fragments after the second mass analyzer, as there are infinite possibilities as to where the bonds can break. Considering the higher collision energy used in this experiment, the bonds can truly break anywhere, and multiple bonds can break simultaneously in the same molecule. The MS/MS spectra collected resulted in an array of the same peaks, which indicates these peaks are associated with the 15d-PMJ$_2$ molecule. This is the fingerprint of 15d-PMJ$_2$, when analyzing samples that contain the compound, it is important to see the following peaks, or similar peaks in the spectrum as they would indicate the presence of the molecule.

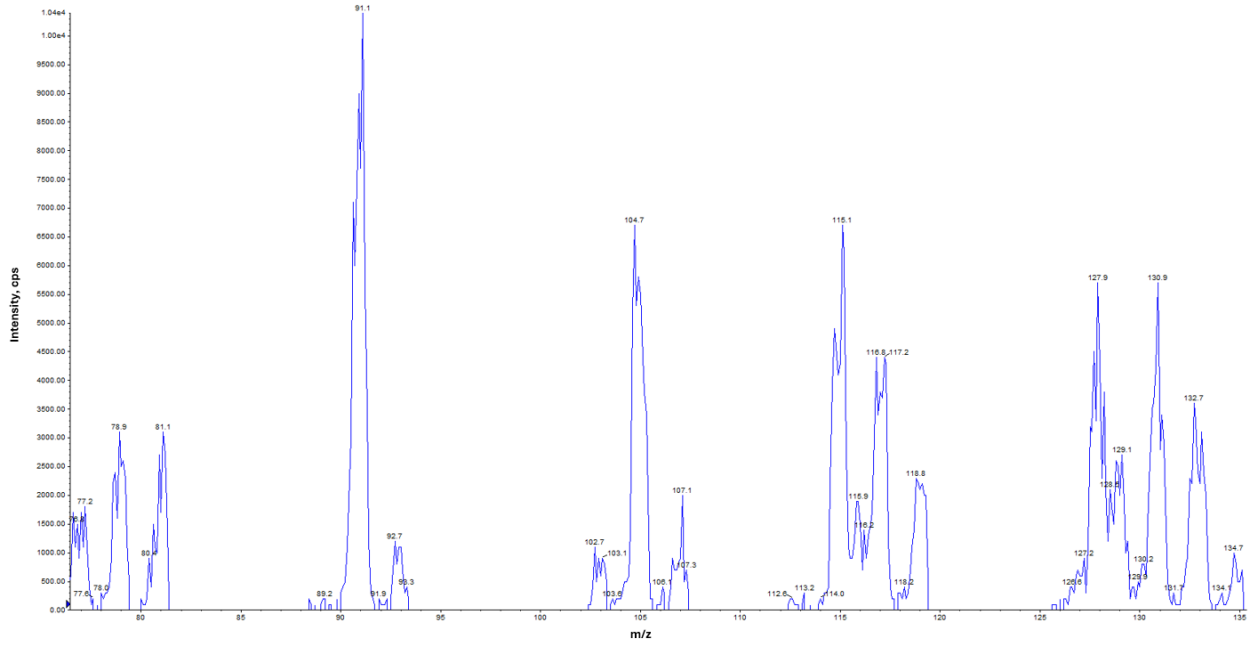

Figure 3.4: MS/MS spectrum for 15d-PMJ$_2$ at a collision energy of 20 eV with argon gas; fragmenting parent ion peak m/z 360.27.

The goal of fragmentation is to assign each peak a fragment in the structure of 15d-PMJ_2, which allows for the characterization of the molecule and obtaining a unique identity that can be used in future analyses. This is most important to ensure the peaks appearing on the spectra belong to the molecule in question or are associated with other ions and coincidently happen to have the same mass.

CONCLUSION

The aim of this study was to find a characteristic identity for the prostamide, 15d-PMJ_2, to be able to identify it in a drug-loaded micelle, or in a mixture with other compounds. This was done by mass spectrometry due to its specificity and sensitivity which allows for more detailed information about the mass, structure, and fragmentation pathways of the molecule. More importantly, the fragmentation of a molecule is specific to the bonds present, and the collision energy being used to break the molecule. 15d-PMJ_2 was fragmented at a collision energy of 20 eV and showed peaks with high intensity at product ions m/z 382, 398, 342, 317, 299, 261, and 60, this is consistent with the predicted fragments displayed in Figure 3.3. The peaks resulting from the second mass analyzer collision with the molecule are shown in Figure 3.4. The presence of these peaks is indicative of the prostamide's addition to a sample, deeming the MS/MS experiments successful in characterizing the molecule. However, these fragments are associated with a specific collision energy, meaning at a lower collision energy, not all the fragments would be present, as more energy might be required to break stronger bonds. Similarly, at higher energies, the peaks would show smaller m/z values, as different bonds can be broken, and smaller fragments can form.

As the study continues, a standard calibration curve of 15d-PMJ_2 needs to be computed, this will be used for quantification of the molecule in a drug-loaded micelle. An optimized method is needed to run the 15d-PMJ_2 and to ensure the drug peak does not overlay a micelle peak. Further fragmentation runs need to be held to understand the characteristics of the molecule further and determine which bonds break at which collision energy. The runs should be completed with a lower collision energy of 5 eV and a higher collision energy of 35 eV, the higher the collision energy the more fragmented the molecule should be, and the smaller the peak m/z values are. This will allow for more specified identification of the molecule with the knowledge of fragments at different induced energies.

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