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Stability studies of MOMIPP, an inducer of methuosis

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University of Toledo

A Thesis

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Stability Studies of MOMIPP, an Inducer of Methuosis

by

Zhaoqi Zhang

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Medicinal Chemistry

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December, 2014

An Abstract of

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Recently, "methuosis" has been identified and labeled as a novel type of cell death. One of the most potent inducers of methuosis is 3-(5-methoxy-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one which is abbreviated as "MOMIPP". MOMIPP is being developed as a treatment for glioblastoma. As part of MOMIPP's development, we are evaluating its stability under several storage and exposure conditions. These include storage in DMSO or chloroform:methanol (2:1) from -80 °C to room temperature for up to 6 months. Likewise, the effects of UV/visible light exposure and storage under high levels of oxygen were assessed. We also evaluated the possibility that MOMIPP might be subject to change when undergoing several freeze-thaw procedure steps or when placed on an HPLC autosampler for prolonged periods of time. Results indicate MOMIPP is stable in DMSO at most temperatures but evaporation is a problem in chloroform-methanol (2:1) except at the lowest temperature. Continuous UV light exposure appears to cause a small amount of breakdown, while visible light does not yield any significant change. Freeze-thawing should be acceptable for up to 5 cycles and placement of samples on an HPLC autosampler should not extend past 2 days. Based on these results, optimal

storage and handling conditions have been establish for MOMIPP solutions that will be used during its potential development as a new drug candidate.

Acknowledgements

It would not have been possible to write this thesis without the help and supporting from the professional and helpful friends around me.

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List of Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
API	Atmospheric Pressure Ionization
$CHCl_3$	Chloroform
DMSO	Dimethyl Sulfoxide
FA	Formic acid
FDA	the Food and Drug Administration
GBM	Glioblastoma multiform
HCl	Hydrochloric Acid
HPLC	High-performance Liquid Chromatography
ICH	International Conference of Harmonization
IND	Investigational New Drug
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
LLOQ	Lower Limit of Quantification
MeOH	Methanol
MOMIPP	3-(5-methoxy-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one
MRM	Multiple Reaction Monitoring
NaOH	Sodium Hydroxide
PDA	Photo Diode Array
SIR	Selected Ion Recording
TEA	Triethylamine
USP	the United Stated Pharmacopeia
UT	University of Toledo
UV	Ultraviolet
WHO	World Health Organization

Chapter 1

Introduction

Glioblastoma, technically glioblastoma multiform or GBM, is one of the more prevalent primary brain tumors occurring in humans. There are about 10,000 new patients diagnosed in the United States each year. Usually, GBM occurs among people, above 45 years in age [1]. GBM is a highly malignant cancer that can grow rapidly when there is vascular support. Tumor cells originate from the brain's astrocyte star shaped cells. However, the exact cause for their transformation is still not known. A complicating feature of the latter is that GBM can demonstrate high resistance toward chemotherapeutic drugs that rely upon apoptotic mechanisms to kill cancer cells. To date, radiation and chemotherapy treatments can only reduce the growth of tumors temporarily. They generally do not accomplish prolonged remission and the prognosis for patients with this disease remains poor [2].

Recently, Maltese et al. at the University of Toledo (UT) identified a new mechanism to kill cancer cells that does not rely upon the apoptosis pathway [3]. This novel mechanism involves the formation of numerous cell vacuoles which build up until the cell's integrity is compromised. Dr. Maltese calls this new form of cell death "methuosis". In collaboration with the Maltese group, UT's Center for Drug Design and Development

(CD3) has recently synthesized a series of small chalcone-like molecules that can induce methuosis in cancer cells. 3-(5-Methoxy-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one, abbreviated as "MOMIPP", is one of the most potent inducers of methuosis when studied in the Maltese lab's *in vitro* cell culture assays. Its chemical structure is shown in Figure 1-1. This novel compound has a basic pyridine ring and an uncommon indole moiety on each side of the α, β-unsaturated ketone portion that is otherwise characteristic of numerous natural product chalcones. MOMIPP represents an exciting drug for the potential treatment of glioblastoma cancer [4,5].

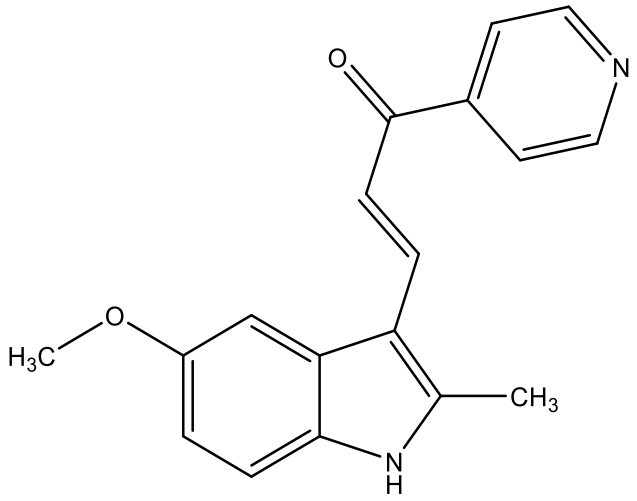


Figure 1-1. Chemical structure of MOMIPP.

In order to confirm the potential of MOMIPP as an effective chemotherapeutic agent, it is important to ascertain its activity in an *in vivo* cancer model. However, in order to engage in this type of drug development activity, it is necessary to first understand the physical and stability properties of this potential lead compound.

The U.S. Food and Drug Administration (FDA) provides general guidelines pertaining to drug development activities. The results from studies associated with these recommendations are often used as parts of an Investigational New Drug (IND)

application. Most importantly, the guidelines stress that the chemical quality and stability of the lead compound needs to be well documented. With regard to compound stability and the stability of intended formulations, the FDA suggests the following studies would be helpful. The first studies pertain to the drug substance's physical and chemical characteristics, including the inherent stability of the raw drug material. A program for assessing stability might include storage at ambient temperature under stressed conditions. Stress-testing conditions ordinarily include both increased and decreased temperatures, increased humidity and exposure to various wavelengths of electromagnetic radiation (eg. ultraviolet and visible ranges), as well as high oxygen atmosphere [6]. Additionally, the FDA recognizes the need to undertake stability testing based on the specific product's processes for manufacturing and eventual use. It is critical that the stability tests can assure and support the FDA's requirements for subsequent toxicological studies that are mandated before any clinical studies can be undertaken [7]. Because the chemical substance will be dissolved in various solutions for studies to be performed during drug development, the second major area of concern to the FDA pertains to the drug's stability within these types of preliminary formulations. It follows that the effects of storage and the process of using these formulations such as freezing and thawing steps also need to be studied.

The International Conference of Harmonization (ICH) and World Health Organization (WHO) also provide guidance for IND applications. Their recommendations tend to be more specific. For example, ICH recommends that new drug products undergo long-term stability studies at 25 ± 2 °C for 12 months, an intermediate test at 30 ± 2 °C for 6 months and an accelerated test at 40 ± 2 °C for 6 months. They

recommend that the frequency of long-term tests is performed every 3 months. If the drug products are to be stored in a refrigerator, they suggest a long-term test at 5 ± 3 °C for 12 months. Likewise, if the drug products are to be stored in a freezer, they suggest a long-term test at - 20 ± 5 °C for 12 months [8].

Considering the FDA's flexible guidelines and the specific ICH recommendations, as well as our own need for similar data, we designed a series of tests most relevant for us and the future development of MOMIPP should it continue to be of interest as a drug development candidate. Oxygen and light are normal environmental exposures which cannot be easily avoided during preparation of samples for testing or for eventual use in the clinic. Likewise, freezing samples at low temperature is a common method of convenient storage. But all frozen samples need to be thawed before use and this process can create stability issues. Finally, during high-performance liquid chromatography (HPLC) analysis, the HPLC autosamplers usually will be maintained at 4 °C, such that the effect of these conditions on sample integrity needs to be studied. Thus, the following types of tests seemed most relevant for our needs: (i) 6-month stability test; (ii) air and light exposure tests; (iii) freeze-thaw stability tests; and (iv) 1-week HPLC autosampler stability tests. All tests are to be performed in DMSO or chloroform:methanol (2:1) at -80 °C, -20 °C, 4 °C and at room temperature (25 °C) since these are thought to represent the most appropriate solvents for MOMIPP and the more common temperatures that its formulations may encounter.

In addition to these drug substances and preliminary formulation assays, we felt it would be useful to devise a bioanalytical method that could be used in the future to detect the stability of MOMIPP within biological matrices where the compound would also be

exposed to potential drug metabolism concerns. Because the concentrations usually encountered during these types of biological studies are very low, we felt that a more sensitive LC-MS/MS assay method would be needed. The latter is further discussed in chapter two.

This thesis will describe the results from conducting these types of stability studies on MOMPIP and its preliminary formulations. It will begin by describing the practical assay that was developed to measure MOMIPP under the various drug development conditions, plus the related assay that was specifically developed for use in the anticipated bioanalytical drug metabolism studies that will be conducted in the future. Each type of study will then be delineated and its results discussed in detail. A general summary for all results will be provided at the end.

Chapter 2

Assay Methods

In order to conduct stability tests, one or more assay methods need to be devised in order to measure the analyte, in our case MOMIPP, within the different test matrices. The assay methods used for small molecules such as MOMIPP typically involve HPLC or liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

2.1 HPLC method background

The United States Pharmacopeia (USP) supplies assay methods for existing drug products. Sometimes these can be useful for devising assays for new, promising drug candidates [9]. However, a survey of methods in the USP pertaining to small drug-like molecules did not show any HPLC assays for a novel compound like MOMIPP with its unique structure and physicochemical properties (see Figure 1-1). Background about HPLC is provided below. It serves as a basis from which a new HPLC method can be devised for a new compound's assay.

Modern HPLC techniques represent the culmination of the development of liquid chromatography across many years. Classical liquid chromatography is performed using

a glass column loosely packed with an absorbent that serves to retain analytes on the column. A liquid is allowed to flow through the column and it serves to dissolve and move the analytes with it. This liquid is called the eluent and it originates from a reservoir placed on top of the column. Since the absorbent will retain analytes differently when they have different chemical structures, they will separate as the eluent passes through the column. Eventually, the analytes are eluted from the column by the solvent which has been driven by the flow of gravity during the entire separation. Alternatively, today's HPLC consists of a solvent reservoir, high-pressure pump, an auto-injection system, a re-usable and highly efficient column that is tightly packed with an absorbent so as to dramatically increase the overall separation power, a convenient detector and a computerized data processing system. These components are shown in Figure 2-1 below in a manner that depicts the flow path for the eluting solvent which is called the 'mobile phase'.

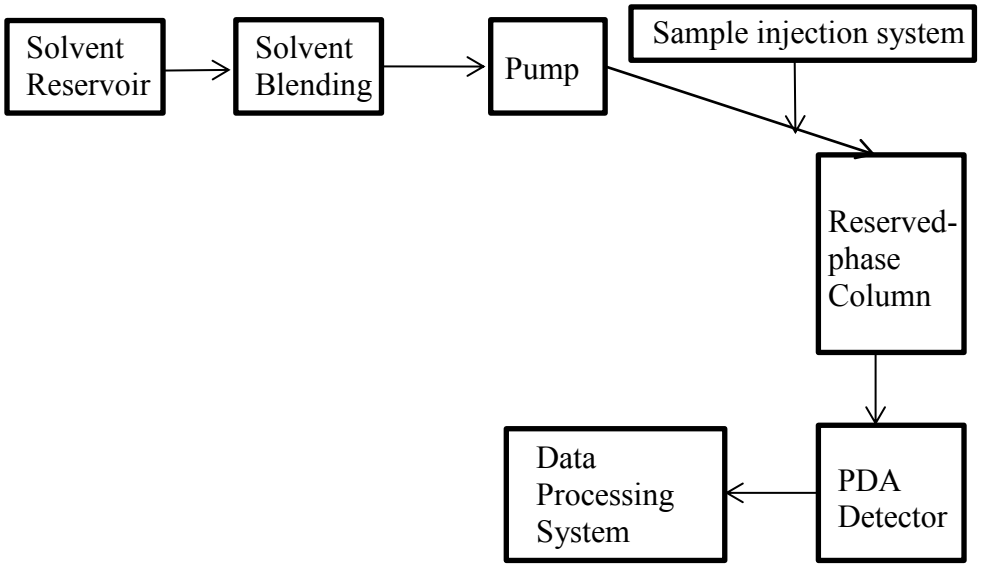


Figure 2-1. Schematic of an HPLC system.

The mobile phase is stored in a solvent reservoir and pumped into the column at high pressure. The pump also provides a solvent blending system which can mix different solvents. The pump keeps the solvent under pressure and maintains the system at a constant flow.

The auto sample injection system is very friendly and convenient. It decreases the variation among parallel injections compared to when they are done manually.

The column is the key component of an HPLC system. Its absorbents are tightly packed so as to increase the 'resolution' or separating properties of the overall system. The tightly packed columns require high pressure to be placed on the mobile phase in order to have the latter move through the column. There are many choices for various types of columns/absorbents. Reversed-phase columns tend to be efficient and reproducible, and are available in a wide range of column choices. Usually, a column's absorbent solid phase consists of silica or alumina. Reversed-phase solid parts are made of bonded stationary particle which contain imbedded silica that is additionally attached to alkyl or aromatic ligands. For example, the column we selected for devising an assay method applicable to MOMIPP was a Nova-Pak®C18 analytical column (reversed-phase). This column is filled with a C18 alkyl bonded silica stationary phase, i.e. the stationary phase is modified with long-chain C18 alkyl groups. The attached groups serve to decrease the ability of hydrophilic substances or compounds having polar groups to adhered too tightly to the column. Because very polar molecules can thus interact more strongly with the polar mobile phase, these compounds are less retained and leave the column first. Alternatively, less polar compounds prefer the nonpolar stationary phase and leave the column last. Hence the name 'reverse' phase when compared to untreated

silica absorbents which tend to retain polar compounds the most and thereby cause their eventual elution to be last.

Ultraviolet absorption (UV) and mass spectrometry (MS) are common detectors for the components of drug-like analytes. The aromatic rings and conjugated ketone within MOMIPP are good UV chromaphores. MOMIPP has strong UV absorbance at 276.3 nm and 455.6 nm. Thus, it became practical to deploy a UV-related photodiode array (PDA) detector to observe the presence of MOMIPP in our intended assay method. The PDA detector consists of a light source, aperture, grating and photodiode array. Figure 2-2 shows the schematic of a PDA system.

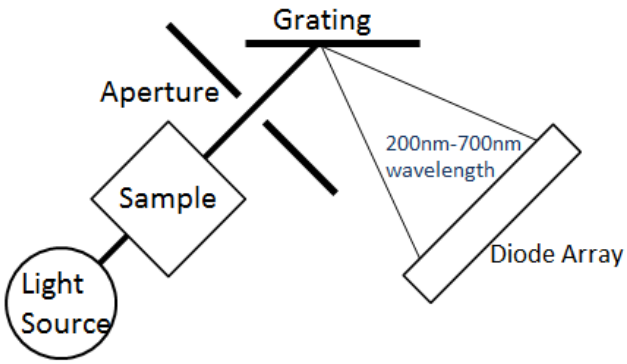


Figure 2-2. Schematic of a PDA system. The light source includes various UV wavelengths.

The light source emits from a deuterium lamp and passes through a beam splitter on route to the flow cell. The light source goes through an aperture, which controls the wavelength, bandwidth and its intensity. It is then dispersed into bands of wavelength by a grating. Finally, the light reaches the photodiode array for detection. Each photodiode acts as a capacitor. The amount of light striking the photodiode will relate to the magnitude at specific wavelengths. The magnitude is recorded in absorbance units (AUs). A typical recording is shown in Figure 2-3 where actual data for MOMIPP is provided.

Note that MOMIPP's absorbance occurs upon its elution from the column and passage across the detector after 5.3 minutes of retention on the reversed-phase column. The latter is called an analyte's 'retention time'.

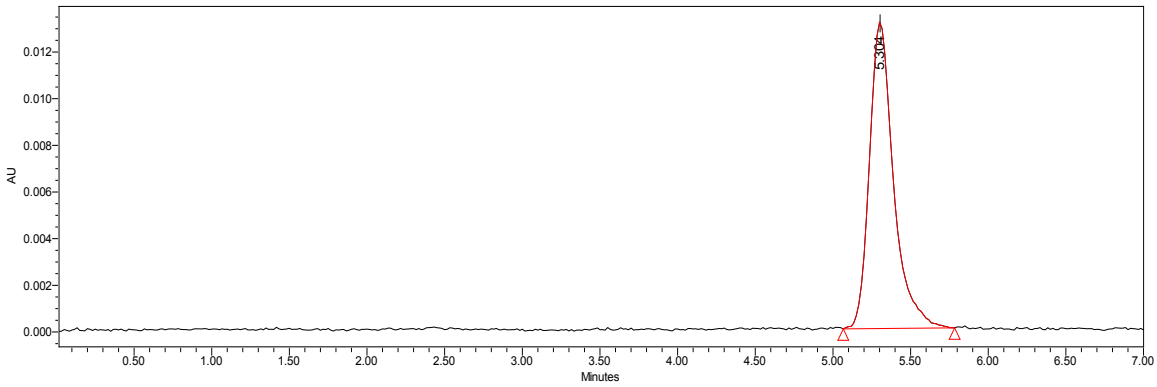


Figure 2-3. A typical HPLC chromatogram for MOMIPP at 278 nm using a UV PDA detector. (Retention time is 5.304 min; Peak Area is 142039 µV*sec)

In our HPLC system, data processing is completed by Empower™ (Build 1154) software from Waters Corporation (Milford, MA, USA). This software can simultaneously record the chromatographic event prompted by the detector, calculate the peak area under the observed absorbance spike or 'peak', and record the retention time. This type of data is then used to further quantify the stability assessments within a given assay.

In our lab we were able to develop a suitable HPLC assay method by revising a former protocol which had been initiated by Dr. Jeff Sarver. The general approach to revise or develop a new HPLC method is to first consider the structure of the molecule. For polar compounds or structures like MOMIPP which have two distinct polar groups, a C18 reversed-phase column is a popular choice. The next step involves a decision about the pH of the mobile phase based on the pKa value of the molecule. MOMIPP's pKa

value was estimated using Marvin™ Software from ChemAxon LLC, (Cambridge, MA, USA). The pKa of pyridine's basic N on MOMIPP is about 3.2. From this we selected an acid to adjust the pH of the mobile phase, so that it would protonate the pyridine group. A general guide is to choose a buffer's pH that is either 2 units below or above the pKa value. This insures that ionizable groups are either largely protonated or largely non-pronated rather than being in an equilibrium mixture of the two states. The usable pH range for reversed-phase on silica-based packing is pH 2 to 8. However, we also wanted to select the same acid for potential use in both our immediate HPLC method and the LC-MS/MS method envisioned for the future bioanalytical studies. Thus, we preferred to use formic acid (FA) because LC-MS/MS can only use volatile acids in its mobile phase. The useful pH range of FA is 2.8-4.8 [10]. Luckily, the behavior of MOMIPP and its retention time under these conditions was very promising despite FA's pH being very close to MOMIPP's predicted pKa value. In addition, we further adjusted the buffer's pH to be exactly 3.00 ± 0.01. Careful adherence to this pH using a high-quality pH meter resulted in limited interday variability in the retention time of MOMIPP. Likewise, the viscosity and absorbance properties of the mobile phase were also considered. Acetonitrile (ACN), methanol (MeOH) and water are very common mobile phase solvents for reversed-phase chromatography. MOMIPP has a strong absorbance at 276.3 nm, which allows us to use an ultraviolet (UV) detector as part of our overall HPLC instrumentation (Figures 2-1 and 2-2). The cut-off wavelength of ACN is 210 nm and MeOH is 205 nm [11]. Thus, both values solvents will not interfere with MOMIPP's absorbance. The next step involves finding the appropriate percentages of the different mobile phase components by using a gradient setting to test the movement of the analyte through the column. In the end, we

also added a small percentage of triethylamine (TEA) to reduce "tailing" of MOMIPP's distinct HPLC peak. Once all of these variables are optimized, they need to be re-checked for intra-day and inter-day variation both of which need to be minimized and accounted for statistically during a method's validation. Importantly, the latter are a key part of the final method's validation in order to assure that everything is very consistently maintained according to the guidelines recommended by the FDA.

2.2 LC-MS/MS method background

An LC-MS/MS is an HPLC system with a tandem mass spectrometry detector. Usually, the method deployed in the HPLC part is reversed-phase chromatography. Just like UV detection, the analyte components will be detected in the order of their elution. After leaving the column, all components will be ionized, then the sensitive MS detector will scan the molecules and show a full, high-resolution spectrum which can account for all parent ions (LC-MS) or fragment ions (LC-MS/MS) having different masses. It is a convenient way to detect specific molecules without fully separating them on the column. The spectrum can discern the presence of a specific compound by selecting its distinctive mass to charge ratio, called "$\frac{[M+H^+]}{z}$", z represents charge.

One aspect of this technique that can be problematic is the need to insure that the mobile phase has a low boiling point. This is why only volatile buffers and acids can be used when the mobile phase needs to have its pH adjusted. Tandem mass spectrometry (MS/MS) can be very useful for detecting the fragment ions of the molecules. These are called 'daughter ions', abbreviated "D^+". Their spectra can be useful to verify a molecule's suspected structure.

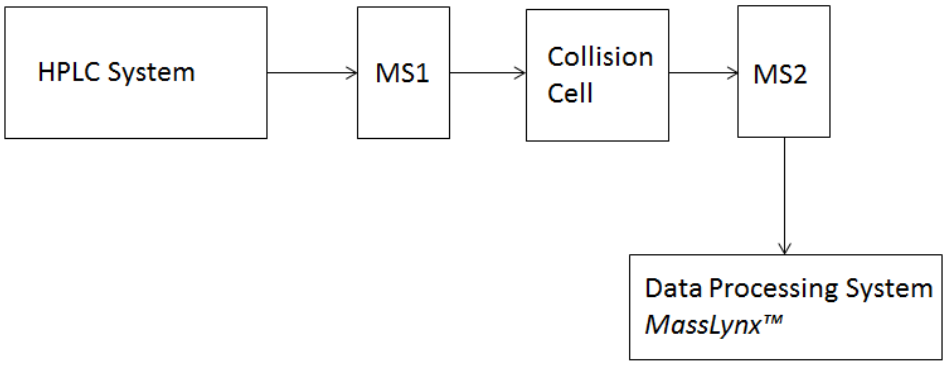


Figure 2-4. Schematic of an LC-MS/MS system.

Figure 2-4 shows the key components of an LC-MS/MS system. The LC part is an HPLC system, usually using a reversed-phase column similar to the former systems that have already been discussed. The quadrupole tandem mass spectrometer serves as the detector. It consists of 2 mass analyzers (MS1 and MS2) and a collision cell between the two parts. For LC-MS analysis, the MS1 is only used for the parent (precursor) analyte via its ion detection. This method is called 'Selected Ion Recording' (SIR). During this procedure, a strong electrical charge emerges from a nebulizer, which produces an aerosol of charged droplets. The droplets interact with the sample compounds and produce parent ions. Then the mass analyzer sorts them by mass-to-charge (m/z) ratio. Both MS components are needed for LC-MS/MS daughter ion detection. This method is called 'Multiple Reaction Monitoring' (MRM). During this procedure, argon is used to allow production of fragments from the precursor within the collision cell which is under a vacuum. Then the MS2 can detect each fragment by the same principle as above. Finally, the system is connected to a data processing system, which can record the chromatogram, mass spectrogram, and also help to quantitate calculations. Figure 2-5 shows the key components for the ionization part of a mass spectrometer, including nebulizer, cone, extractor, etc. Nitrogen as nebulizing gas, helps to evaporate, desolvate

and ionize the solvent and sample. Perpendicularly introduced nebulizing gas flows around the outside of the capillary, also called the sample cone. The mobile phase and sample emerging from the tip is dispersed into an aerosol of highly charged droplets. Transfer optics are used to transfer and focus the ions into the mass spectrometer.

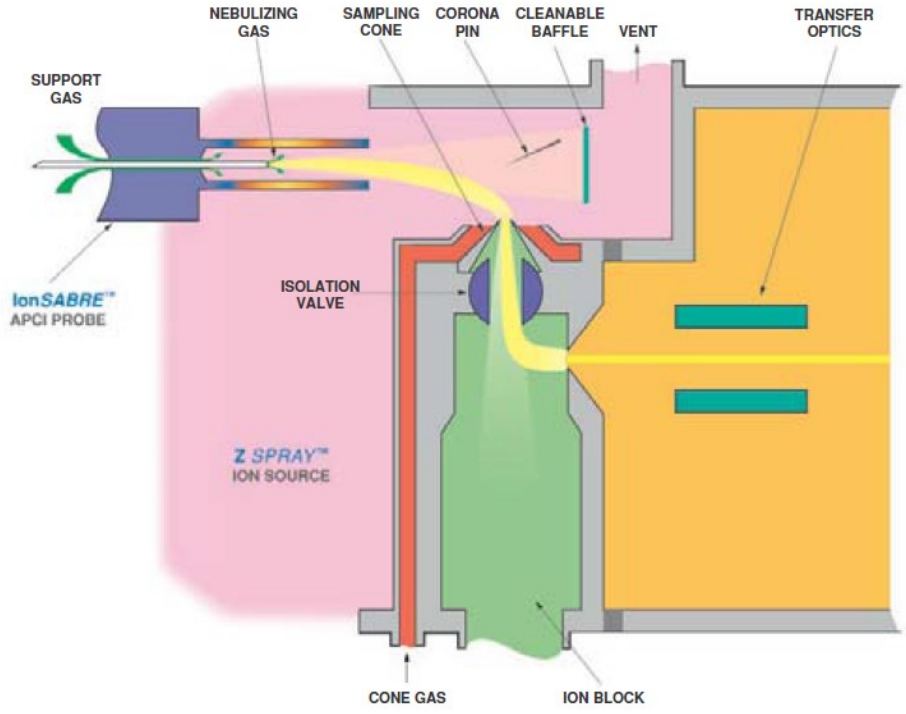


Figure 2-5. Schematic of the key ionization part of a mass spectrometer [12].

An LC-MS/MS method was initially developed by Dr. Jeff Sarver. Preliminary studies suggested that the method works well to detect both MOMIPP and its metabolites produced in cultures of different cell lines and biological media. Because the MS detector is extremely sensitive, it can usually detect analytes at much lower concentrations than HPLC UV detectors. Furthermore, the addition of a second MS to detect daughter ions becomes very specific for each given analyte, and this usually allows the analytes to be discernible in very complex media. These two features become very important to study

drug molecules in biological systems wherein analytes tend to be present at low concentration and the media are highly complex due to the presence of biomolecules.

The specific details for the final HPLC and LC-MS/MS methods that were devised for MOMIPP are provided below. Exceptions to these general descriptions that were needed for some of the distinct tests are described within each test in Chapter 3 and include: specific drug concentrations; differing storage and sample preparation protocols.

2.3 HPLC method devised for MOMIPP

2.3.1 Analytical instrumentation

An Alliance® HPLC (model Waters 2659) equipped with a quaternary pump, an inline membrane degasser, an autosampler, and a column oven from Waters Corporation (Milford, MA, USA) were used. Substances were detected using a Waters® 2996 Photodiode Array (PDA) Detector. Empower software from Waters was used for data acquisition and processing. All weighings were done on an XS205 analytical balance from Mettler Toledo (Columbus, OH, USA). Buffer pH was adjusted and measured using an XL15 pH meter purchased from Thermo Fisher Scientific Inc. (Wayne, MI, USA).

2.3.2 Reagents

HPLC grade acetonitrile (ACN), formic acid (FA), and triethylamine (TEA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC water was purified using a Barnstead International (Dubuque, IA, USA) water purification unit.

2.3.3 HPLC conditions

The HPLC method used a flow rate of 1000 µl/min with an injection volume of 10 µl and the total run time was around 7-10 minutes. The samples were run through a Nova-Pak®C18 analytical column (reversed-phase, 3.9×150 mm, 4µm) purchased from Waters Corporation (Milford, MA, USA). The samples were analyzed for total amount of MOMIPP based upon the area under the curve during MOMIPP's detection (see Figure 2-3). The mobile phase was 30% acetonitrile and 70% buffer. The buffer was made by HPLC water, 0.1% FA and 10 µM/L TEA. Final adjustment to pH 3.00 ± 0.01 was accomplished using 1 mM and 5 mM hydrochloric acid (HCl) and 1 mM and 5 mM sodium hydroxide (NaOH). Fresh buffer was prepared every 24 hrs. The column and samples were kept at 35 ± 5 °C and 5 ± 5 °C, respectively.

2.3.4 PDA detector conditions

Absorption was monitored from 200-700 nm, using 3D data collection to quantify MOMIPP levels and check for breakdown products. Detection at 278 nm was used to quantify the amount of unchanged MOMIPP present. Figure 2-6 shows a typical broad scan with 3D data recorded for MOMIPP. Figure 2-3 shows the same sample's HPLC chromatography at 278 nm. Figure 2-7 shows a spectrogram of MOMIPP from 200 nm to 700 nm. As expected it shows two strong absorptions at about 280 nm and about 430 nm.

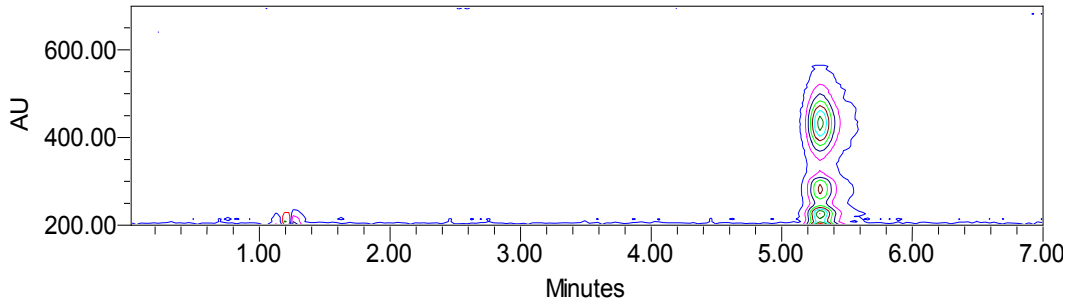


Figure 2-6. A typical broad 3D HPLC chromatogram for MOMIPP from 200 nm to 700 nm using a UV PDA detector.

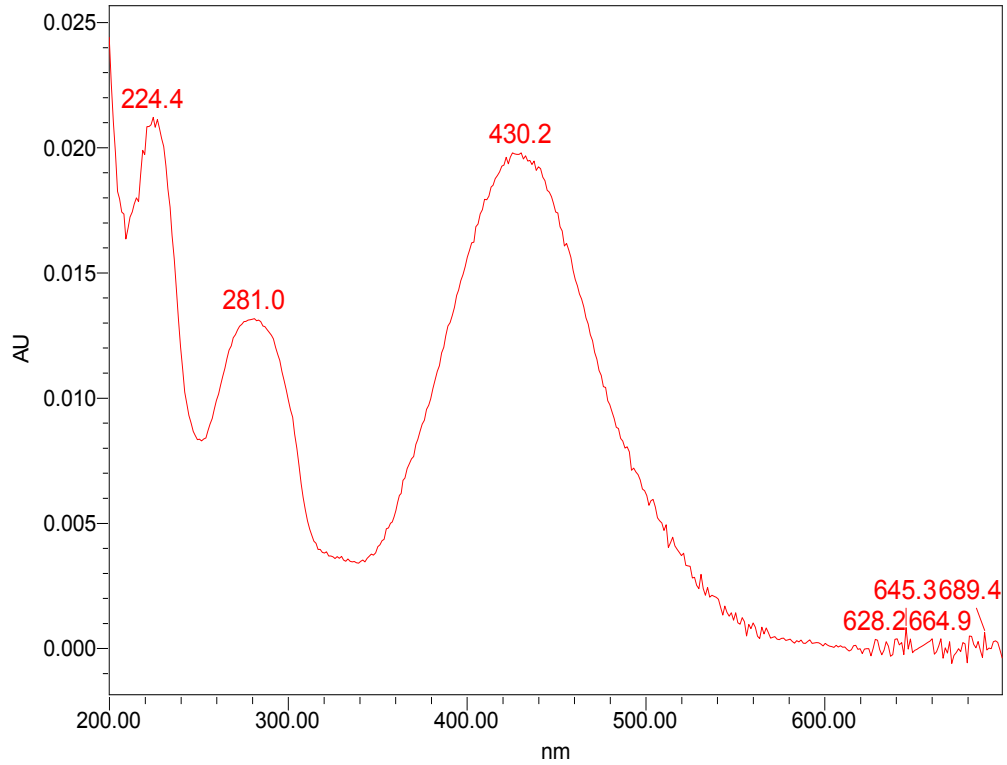


Figure 2-7. A typical spectrogram for MOMIPP from 200 nm to 700 nm.

Figure 2-8 shows a concentration response curve for MOMIPP when measured specifically at 278 nm. It is clear that the concentration response relationship is linear across a range of 1 µM to 20 µM MOMIPP. The calculated coefficient of linearity is 0.9999. Likewise, a recommendation from the FDA indicates that the lower limit of quantification, called LLOQ, should not vary between injections by more than ± 20%. All

of our tests were in accord with this specification, actually being much less than this value.

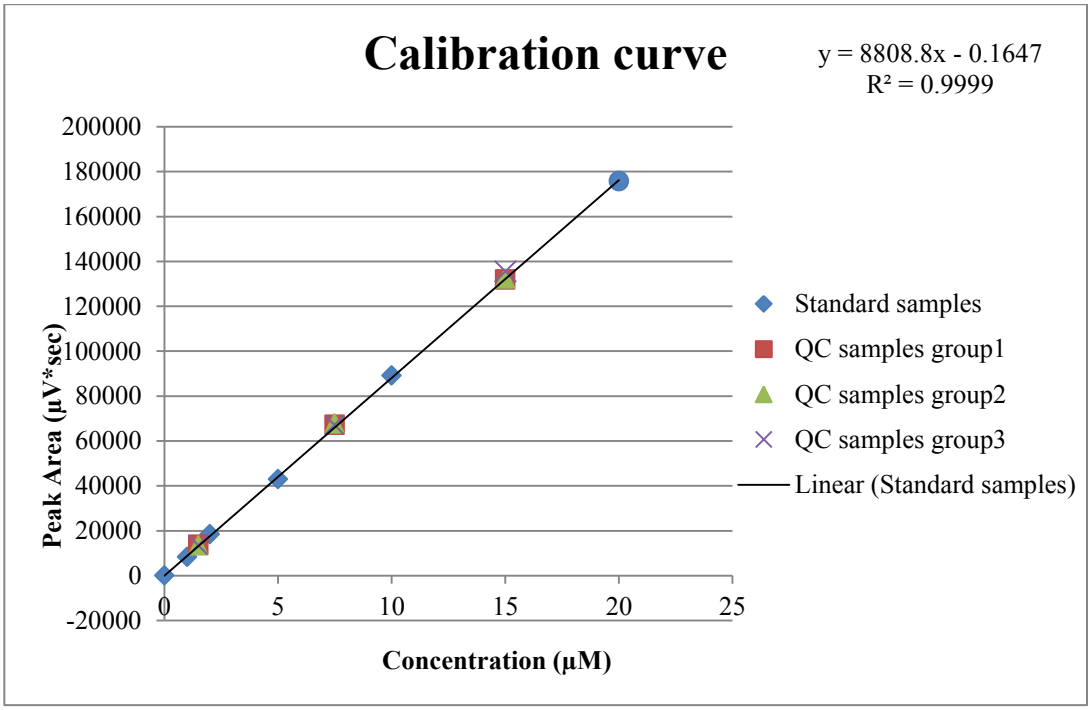


Figure 2-8. A typical calibration curve for 6-month MOMIPP stability tests (in $CHCl_3$:MeOH (2:1))

2.3.5 HPLC method validation

We established the assay selectivity by demonstrating the single peak's chromatogram for MOMIPP when studied from different matrices. Accuracy and precision were demonstrated by the concentration-response studies presented earlier. The linear range extended from 1 µM to 20 µM and had an excellent correlation coefficient (R^2=0.9999). Likewise, the mean value and the coefficient of variation (CV) should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. Recovery criteria pertain to each type of test and they are further shown in the test results section. Intra-day and Inter-day variation studies are shown below. Calibration

standard curves are again applicable to each type of specific test. These are shown in the subsequent tests and results section.

The FDA also provides a guideline for validation of an analytical method to be used during drug development. The items that they specify include: (1) selectivity; (2) accuracy, precision and recovery, including intra-day and inter-day variation; (3) calibration standard curve, including LLOQ and (4) stability, including demonstration of feasibility for intended uses [13].

Three-day tests were performed in order to collect data n intra-day and inter-day variations. Figure 2-9 and Table 2-1 show the calibration curve of the average value of the 3-day standard and QC samples. This curve retains linearity and QC samples match the trendline appropriately.

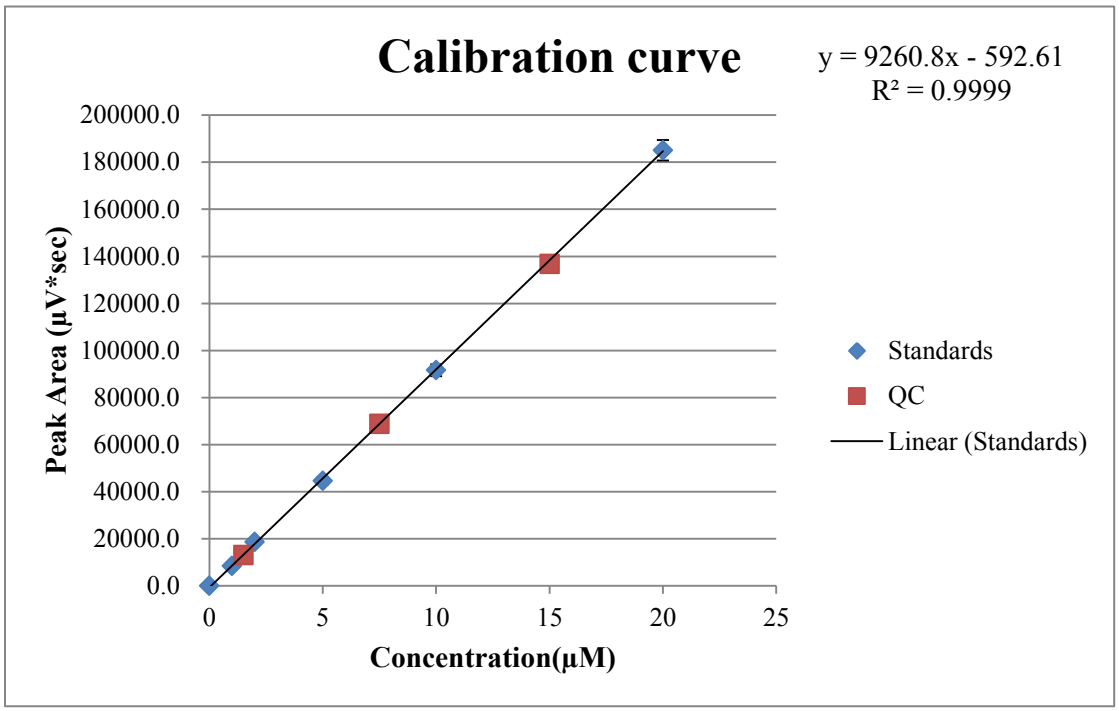


Figure 2-9. Calibration curve showing the average value of continuous 3-day standards and QC samples peak area values.

Table 2.1. Results from 3-day standards and QC samples' peak areas (unit: μV*sec).

Standards:	Average	SD
0	0	0
1	8449.89	1183.38
2	18640.00	733.65
5	44618.33	1488.77
10	91604.44	2565.39
20	185041.67	4310.74

QC:	Average	SD
1.5	13090.33	495.23
7.5	68710.11	1136.34
15	136801.22	1356.90

Each group of QC samples was calculated from each standard calibration curve. The value calculated from the calibration curve is called the "true value." There are 3 groups of standard samples and QC samples for each day. Table 2-2 shows the average of true value from the 3-day test. Comparing of the low-end concentrations where a LLOQ value of 20% is regarded as acceptable, our value of about 8% variation can be considered as exceptionally good. The same holds for the mid-level and high-level concentrations where our validation is less than 5%.

Table 2.2. Average QC values calculated from each standards sample's calibration curve.

Unit:μM	Theory (μM)	Mean	SD	SEM	CV%	Error%
QC1.5	1.5	1.504	0.125	0.072	8.281	0.269
QC7.5	7.5	7.497	0.116	0.067	1.549	-0.035
QC15	15	14.841	0.492	0.284	3.313	-1.069

2.4 LC-MS/MS method devised for MOMIPP

2.4.1 Analytical instrumentation

An Alliance® LC (model Waters 2795) equipped with a quaternary pump, a degasser, an autosampler, and a column oven from Waters Corporation (Milford, MA, USA) were used. The detector was a mass spectrometry MICROMASS® Quatro *Micro*™ API from Waters Corporation. MassLynx™ V4.1 software from Waters Corporation (Milford, MA, USA) was used for data acquisition and processing. Quattro Micro V4.1 software from Waters Corporation was used to set up MS, and Inlet Method V4.1 software from Waters Corporation was used to set up LC.

2.4.2 Reagents

HPLC grade acetonitrile (ACN) and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC water was purified using a Barnstead International (Dubuque, IA, USA) water purification unit.

2.4.3 LC conditions

The LC method was used at a flow rate of 300 µl/min with an injection volume of 10 µl and the total run time was around 5 min. The samples were run through an Ascentis® Express C18 analytical column (reversed-phase, 2.1× 75mm, 2.7µm) purchased from Sigma-Aldrich (St. Louis, MO, USA) preceded by a XTerra® MS guard column purchased from Waters Corporation. The mobile phase was 70% water with 0.1% formic acid and 30% ACN. The column and sample temperatures were set at 35 ± 5 °C and 5 ± 5 °C, respectively.

2.4.4 MS conditions

MS detection was used with the atmospheric pressure ionization (API) source in positive ion mode. Nitrogen was used as the desolvation and cone gas, and was set at a flow rate of 650 L/h and 40 L/h, respectively. The source and desolvation gas temperatures were 100 °C and 400 °C, respectively. The API source tip (capillary) voltage was 3.0 kV, cone was 25 V, extractor was 3 V, and the source was 100 °C. The MRM cone was 30 V, collision was 28 V and the ion energy for MS1 was 0.5 V and for MS2 was 1.0 V. The MS was operated in the MRM mode with argon gas used as the collision gas. The argon gas cell pressure was approximately 3.1×10^{-3} mbar. The dwell time for detection of each compound was 200 ms. Full-scan mass spectra for each compound were obtained by direct infusion into the API source at a flow rate of 5 μl/min. Protonated molecular ions (parent-ions) were produced and optimized using the first quadrupole analyzer MS1 (See Figure 2-4). Collision-induced fragmentation was achieved using suitable collision energy to produce product-ions within the second quadruple analyzer MS2 (See Figure 2-4). Figure 2-10 shows a spectrum of the parent ion for MOMIPP.

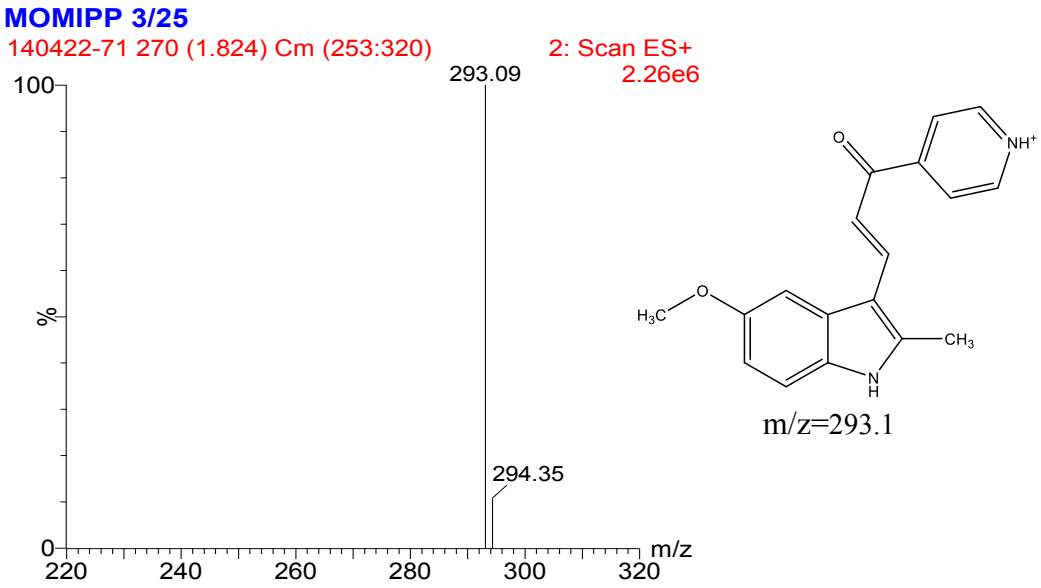


Figure 2-10. A spectrum of MOMIPP's parent ion.

Figure 2-11 to 2-13 shows typical spectra of daughter ions from 3 metabolites of MOMIPP. These two daughter ions' m/z ratios are 174.09 and 122.06, respectively. Their two corresponding structures are also shown below (see Figure 2-14).

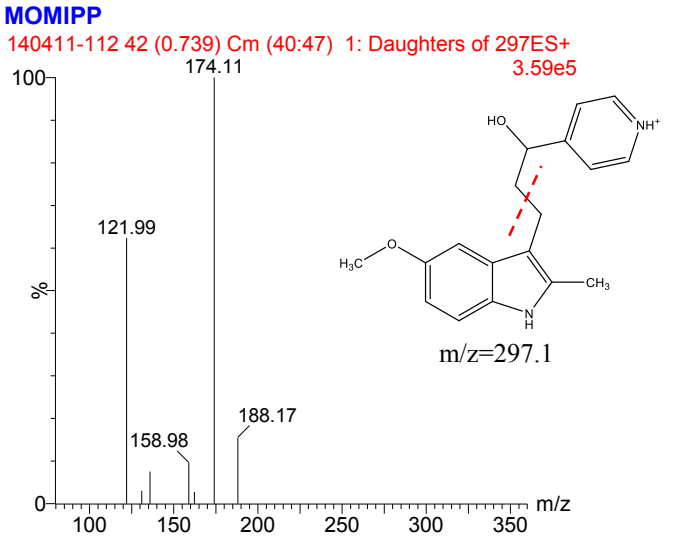


Figure 2-11. A spectrum of daughter ions from a MOMIPP's metabolite, M1.

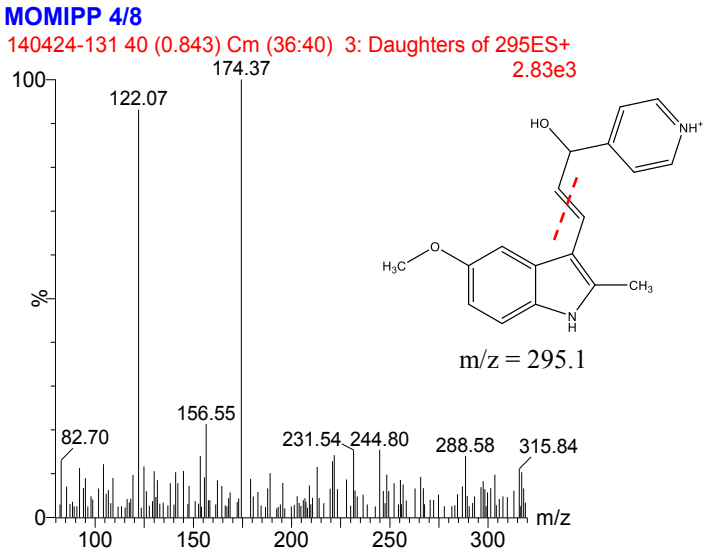


Figure 2-12. A spectrum of daughter ions from a MOMIPP's metabolite, M2A.

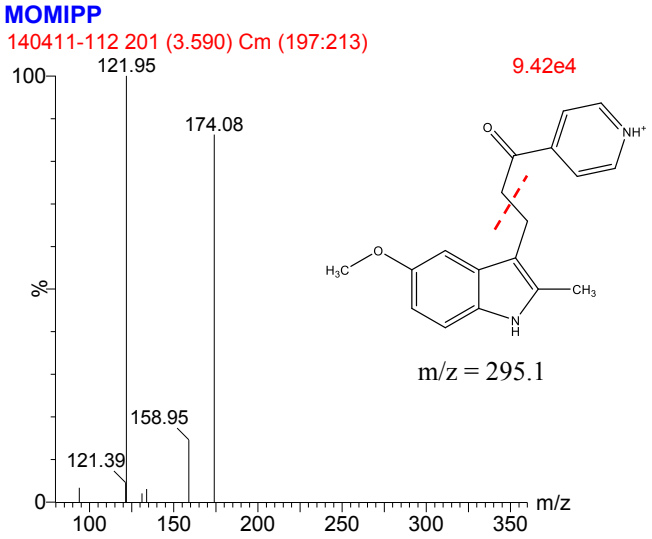
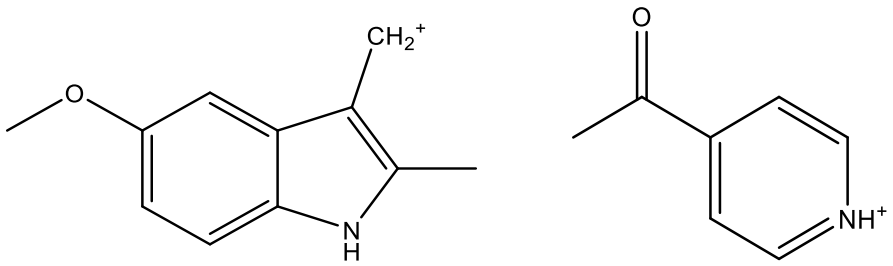


Figure 2-13. A spectrum of daughter ions from a MOMIPP's metabolite, M2B.



174.09 122.06

Figure 2-14: Chemical structures of a metabolite of MOMIPP's two daughter ions and their m/z ratios.

Chapter 3

Stability Tests

In order to quantify MOMIPP within the different tests, a standard concentration calibration curve first needed to be developed. This was accomplished as described below in a uniform manner for all tests. It is also useful to deploy a well-behaved external standard as a quick check of the instrument and column's overall performance at the start and at the end of each set of experimental runs. This general protocol is also described below.

Calibration curves were prepared from solid MOMIPP raw drug compound which was stored at -20 °C. On each test day, 3 separate portions of 5 ± 1 mg MOMIPP were weighed using a 5 decimal the analytical balance (± 0.01mg). Each portion was dissolved in DMSO to a concentration of 15 mM. One calibration curve consists of 5 standard samples and 3 quality control (QC) samples diluted with buffer and ACN to concentrations of 1 µM, 2 µM, 5 µM, 10 µM and 20 µM, and then 1.5 µM, 7.5 µM and 15 µM, respectively. One set of standard samples and the QC samples are derived from the same stock of MOMIPP. Usually, we need to evaporate the DMSO before diluting MOMIPP with ACN and buffer into the different concentrations indicated above. An external standard was also utilized. For this we chose ethylparaben (purchased from

Sigma-Aldrich, St. Louis, MO, USA). Its structure is shown in Figure 3-1. The stock solution of external standard was prepared in ACN at 1.667 mM. Each test day the external standard samples were diluted with mobile phase and buffer to 50 μM. The external standard was then injected once before and once after all test samples on each test day.

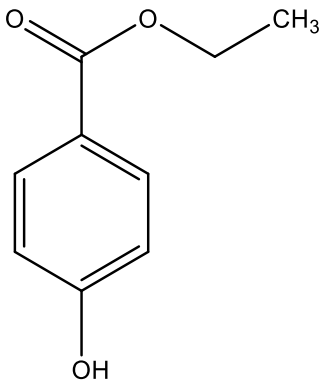


Figure 3-1. Chemical structure of ethyl paraben.

3.1 Stability testing conditions

3.1.1 6-month stability test

We did 7 analyses over a 6-month period starting from Day 0, then Day 7, Day 14, Day 28, Day 56, Day 112 and Day 168. The samples were dissolved in DMSO at 15 mM and stored at -20 °C, 4 °C and room temperate (RT). In addition, samples dissolved in $CHCl_3$:MeOH (2:1) at 1mg/ml were stored at -80 °C, 4 °C and room temperature. Each analysis day utilized 3 stock samples from each of the 6 conditions. The standard concentration-response curve used for these comparisons is shown in Figure 3-2.

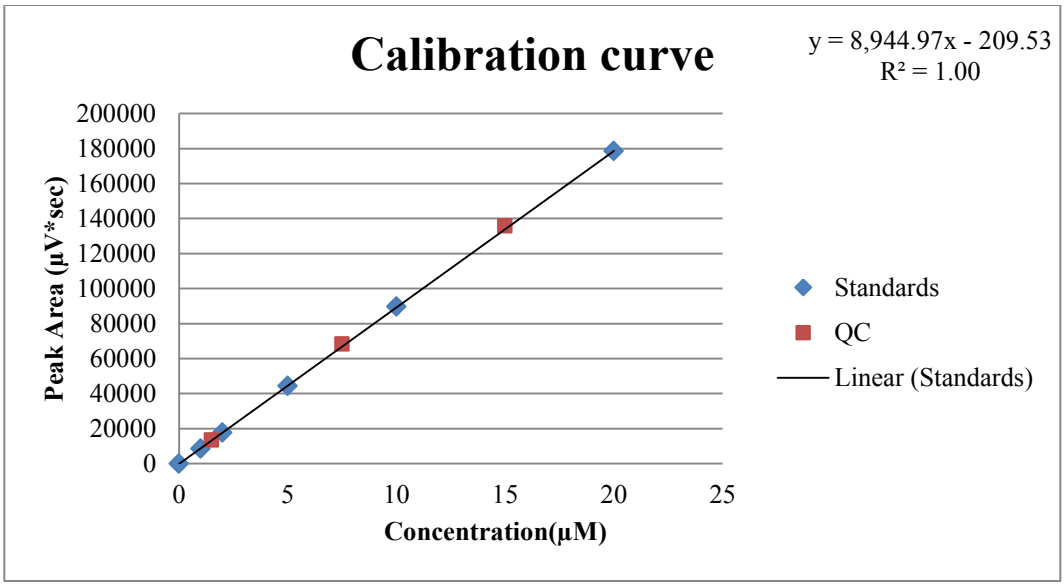


Figure 3-2. Calibration curve showing the average value of the day-180 analysis standards and QC samples' peak area values for 6-month stability tests.

3.1.2 Normal light, ultraviolet (UV) exposure stability test

All samples were dissolved in DMSO at 15 mM in 350 µl glass vials and kept at room temperature. Each test sample had a control-sample which was wrapped with aluminum foil but otherwise maintained under the same conditions (temperature, air, humidity). The normal light source was a fluorescent lamp in the lab. The UV light source was a UV bulb in a biological safety cabinet (series 36209-04U). The UV lamp generates a primary wavelength of light of 254nm. Each analysis day used 3 test samples for each condition. Analysis days were: Day 0 and Day 3. The concentration-response curve used for these studies is shown in Figure 3-3.

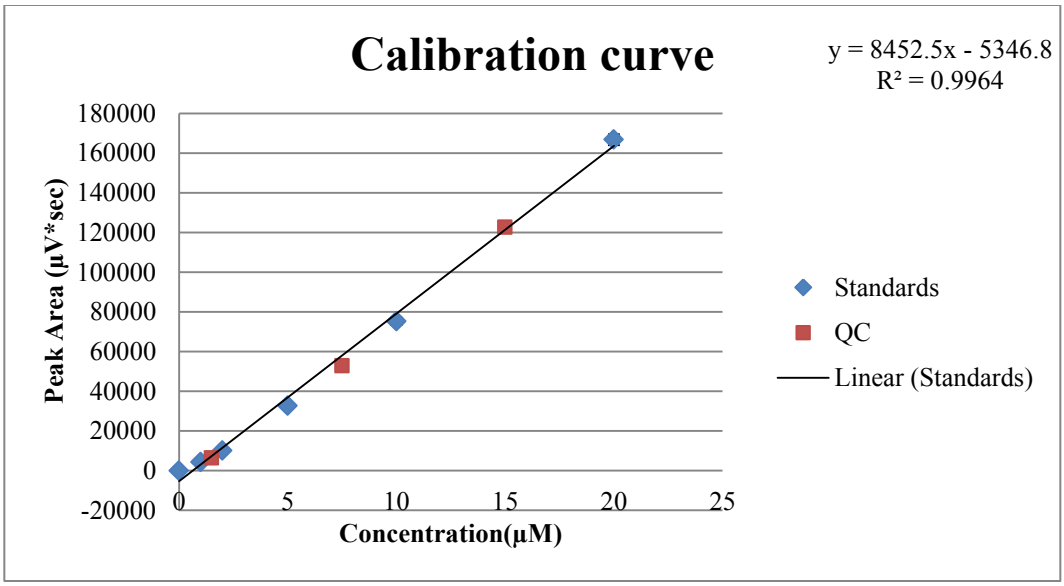


Figure 3-3. Calibration curve showing the average value of the day-3 analysis standards and QC samples' peak area values for light exposure tests.

3.1.3 Oxygen exposure stability test

All samples were dissolved in DMSO at 15 mM in 350 µl glass vials. Each test sample had a control-sample. Test samples were exposed to oxygen by storing in a desiccator that was periodically refreshed by oxygen. Control samples were stored in another desiccator refreshed by nitrogen. Desiccators were refreshed once each day until day 3. Each analysis day used 3 test samples accompanied by their conditions. Analysis days were: Day 0 and Day 3. The concentration-response curve used for these studies is shown in Figure 3-4.

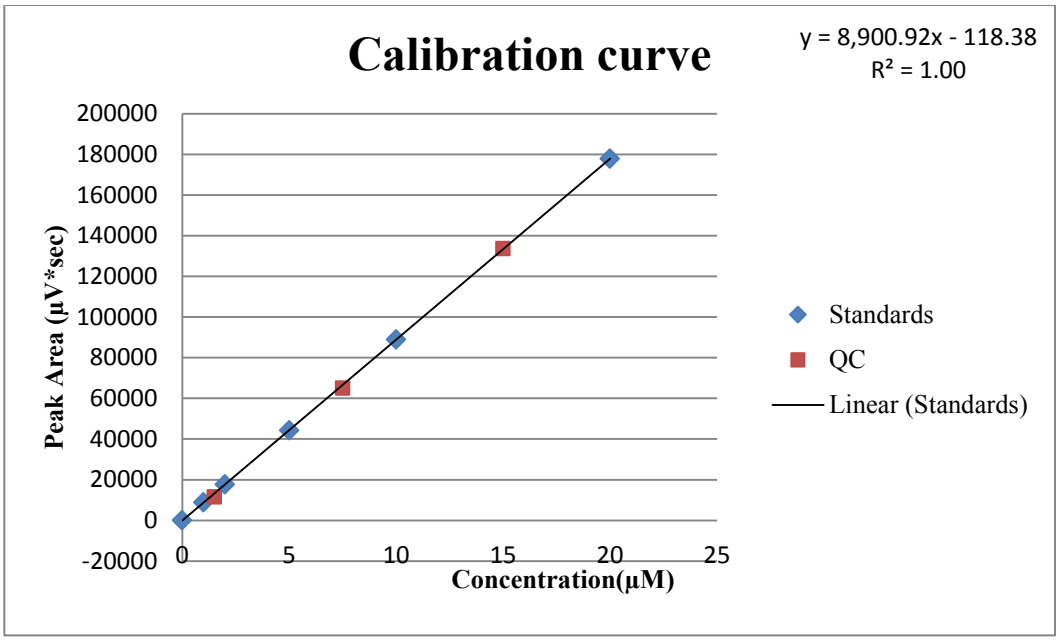


Figure 3-4. Calibration curve showing the average value of the day-3 analysis standards and QC samples' peak area values for oxygen exposure tests.

3.1.4 Freeze-thaw stability test

All samples were dissolved in DMSO at 15 mM in micro-centrifuge tubes. Each test sample had a control sample. They were all stored at -20 °C. Freeze-thaw samples were subjected to freeze-thaw conditions for 6 cycles while the control-sample had only 1 cycle of freeze-thaw, when it was prepared for final analyses. Each analysis day used 3 samples for each condition. The concentration-response curve used for these studies is shown in Figure 3-5.

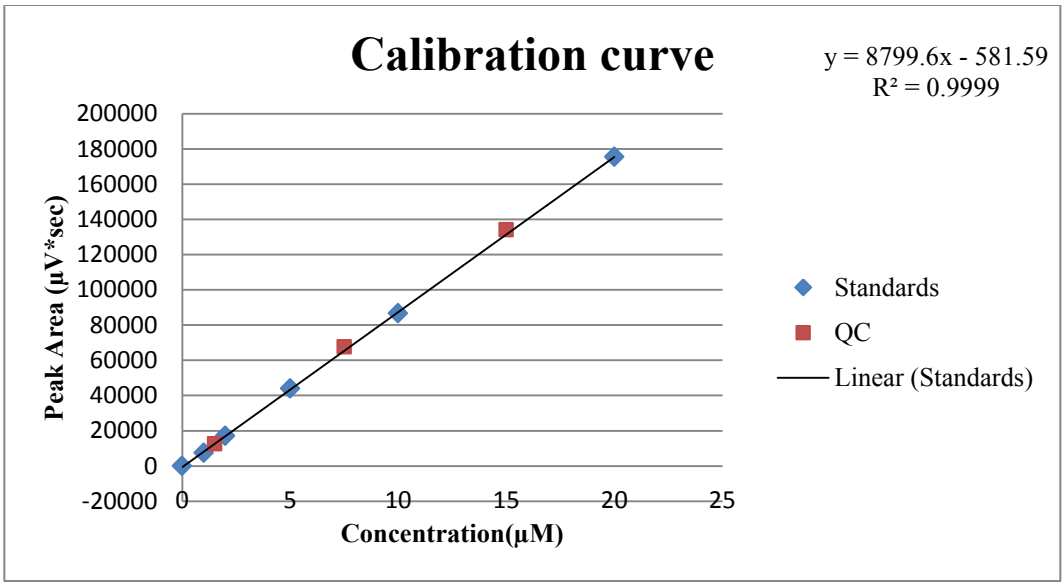


Figure 3-5. Calibration curve showing the average value of the 5-cycle analysis standards and QC samples' peak area values for freeze-thaw tests.

3.1.5 1-week HPLC autosampler stability test

All samples were prepared by the same protocol used to prepare standard and QC samples. The concentrations were 15 µM in mobile phase. Samples were stored at 4 °C and at room temperature. Each analysis day used 3 samples from each condition. Analysis days were: Day 0, Day 1, Day 2, Day 3 and Day 7. The concentration-response curve used for these studies is shown in Figure 3-6.

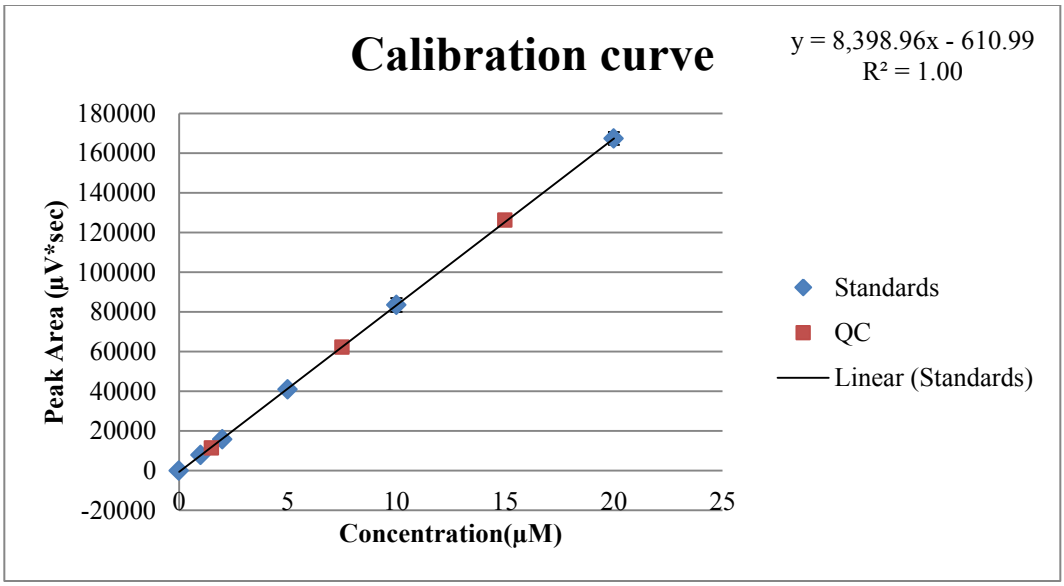


Figure 3-6. Calibration curve showing the average value of the 7-day analysis standards and QC samples' peak area values for HPLC autosampler tests.

3.2 Results from Stability testing

3.2.1 6-month stability test

(1) In DMSO Solution.

The starting concentration of MOMIPP was 15 mM. Statistical analyses were performed by one-way ANOVA, with $P<0.05$ used as the criteria for significant differences. Table 3.1 provides all of the data for all three studies. Figures 3-7 to 3-9 show a bar-graph representation of the results for each of the three conditions. There are no significant differences among each analyses day-to-day results for each of the three temperatures that were studied. However, there are differences observed between Day 0 and Day 180 overall. The room temperature condition showed the largest fall in MOMIPP concentration but even this change remains rather small with a 6% decrease.

It should be noted that the possibility for solvent evaporation can be a problem during such studies, particularly for the case of the samples being stored at room temperature. Solvent evaporation would lead to an erroneous increase in an analyte's concentration. This possibility was monitored by initially marking the volume on the outside of each vial and carefully observing for any decrease from that level. This did not pose problems for the case of DMSO which has a high boiling point.

Our results suggest that it is safe to store solutions of MOMIPP in DMSO for up to 6 months at either -20 °C or 4 °C with less than 5% deterioration of the sample. Alternatively, there could be slightly more than 5% loss of sample integrity when stored at room temperature.

Table 3.1. Results from stability testing in DMSO under different temperatures.

Day/Temp	-20 ºC	4 ºC	RT
Day 0	100.00 ± 2.83	100.00 ± 2.83	100.00 ± 2.83
Day 7	101.48 ± 0.63	99.05 ± 0.64	100.80 ± 1.14
Day 14	97.64 ± 3.04	98.80 ± 1.13	99.67 ± 2.11
Day 34	96.45 ± 4.01	97.67 ± 4.23	98.82 ± 3.07
Day 56	96.98 ± 2.98	98.56 ± 4.14	98.77 ± 3.74
Day 180	96.40 ± 1.91	95.91 ± 1.12	93.67 ± 2.55

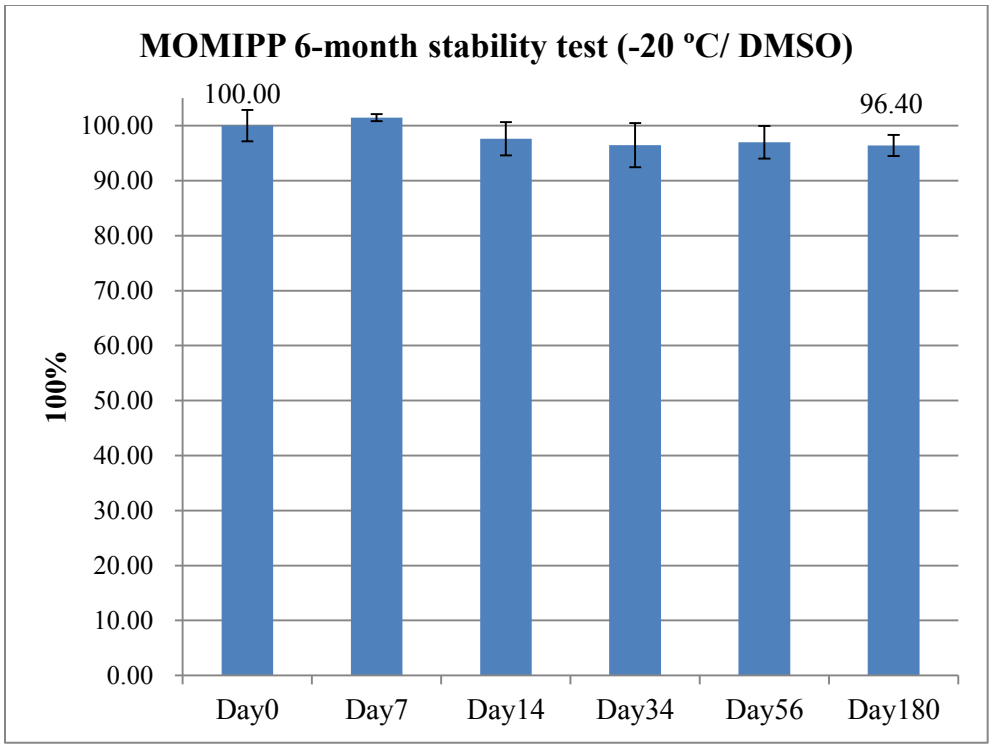


Figure 3-7. MOMIPP 6-month stability test at -20 ºC in DMSO.

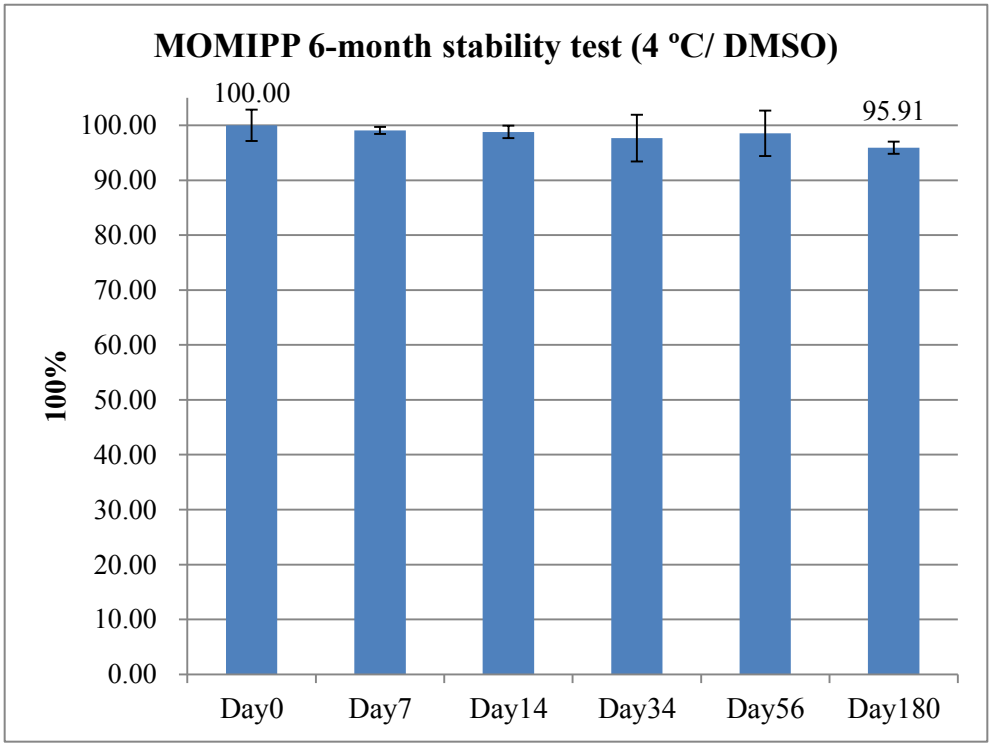


Figure 3-8. MOMIPP 6-month stability test at 4 ºC in DMSO.

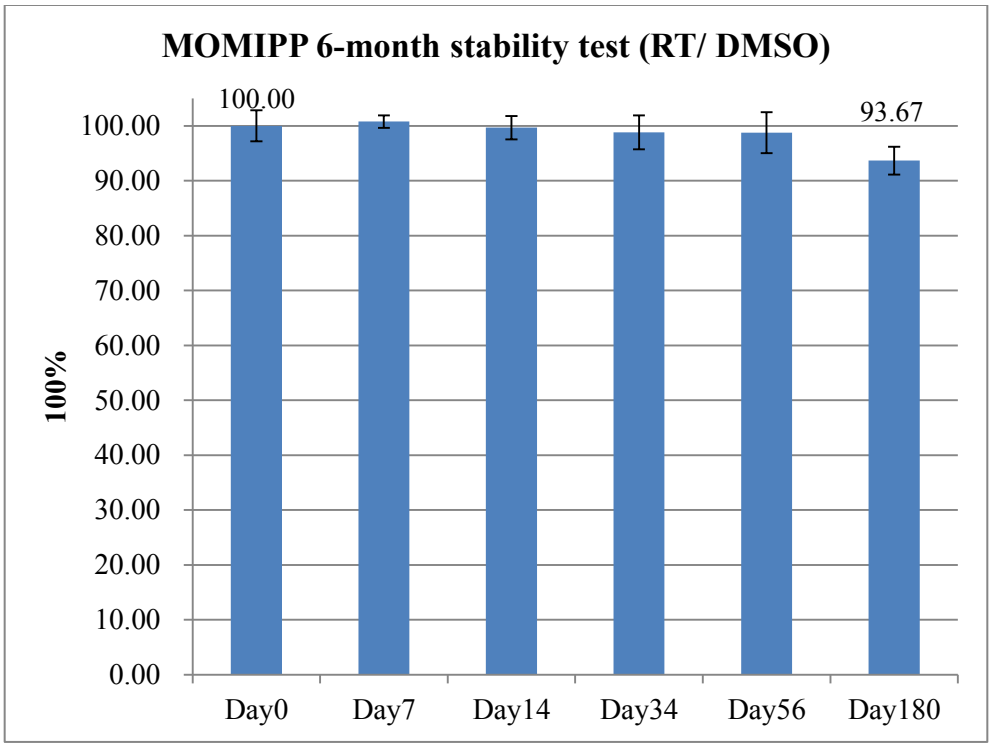


Figure 3-9. MOMIPP 6-month stability test at RT in DMSO.

(2) In $CHCl_3$:MeOH (2:1) Solution.

The starting concentration of MOMIPP was 1mg/ml (3.42 mM). Statistical analyses were performed by one-way ANOVA, with $P<0.05$ used as the criteria for significant differences. Table 3.2 provides all of the results from the three different temperature conditions. Figures 3-10 to 3-12 convey the results for each temperature condition in bar graph form. Table 3.3 provides a comparison of the Day 180 analysis results to that of Day 0 for all tests.

Storage at -80 ºC (Figure 3-10) clearly indicates that there is no change in the integrity of the solution. Alternatively, at 4 ºC (Figure 3-11) there appears to be an increase in concentration just above 5%. However, this difference is not statistically significant (Tables 3.2 and 3.3).

At room temperature (Figure 3-12) this type of increase in concentration becomes more than 10% and it is statistically significant from the Day 0 concentration (Tables 3-2 and 3-3). While not a deterioration in MOMIPP, this solution integrity has become compromised and storage under these conditions is not recommended. The reason for this is clearly due to solvent evaporation which has indeed become problematic in line with the more volatile nature of these components when compared to that of DMSO. Another note of caution when deploying formulations of MOMIPP in $CHCl_3$:MeOH solution is that precipitation can occur upon storage at these lower temperature and so it becomes important to vigorously remix and slightly warm their vials before withdrawal of an aliquot.

Table 3.2. Results from stability testing in $CHCl_3$:MeOH under different temperatures.

Day/Temp	-80 ºC	4 ºC	RT
Day0	100.00 ± 8.54	100.00 ± 8.54	100.00 ± 8.54
Day22	102.20 ± 3.36	103.58 ±3.33	112.13 ± 2.85
Day146	105.37 ± 2.91	107.61 ± 2.57	111.49 ± 1.79
Day180	100.63 ± 4.17	106.41 ± 2.31	116.43 ± 7.07

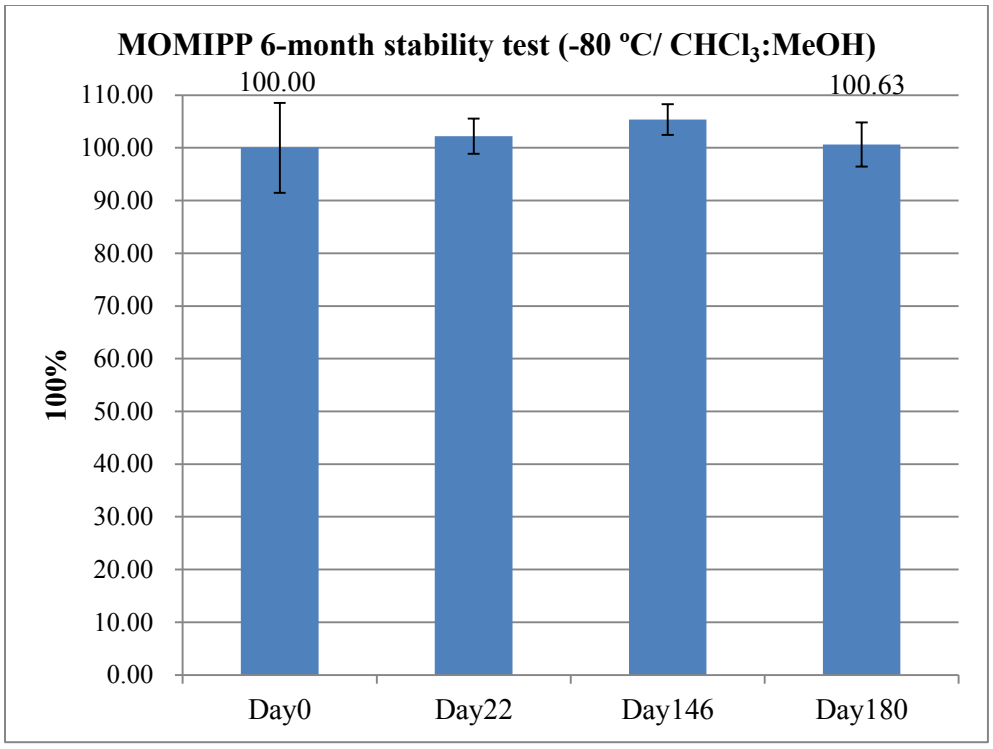


Figure 3-10. MOMIPP 6-month stability test at -80 ºC in $CHCl_3$:MeOH.

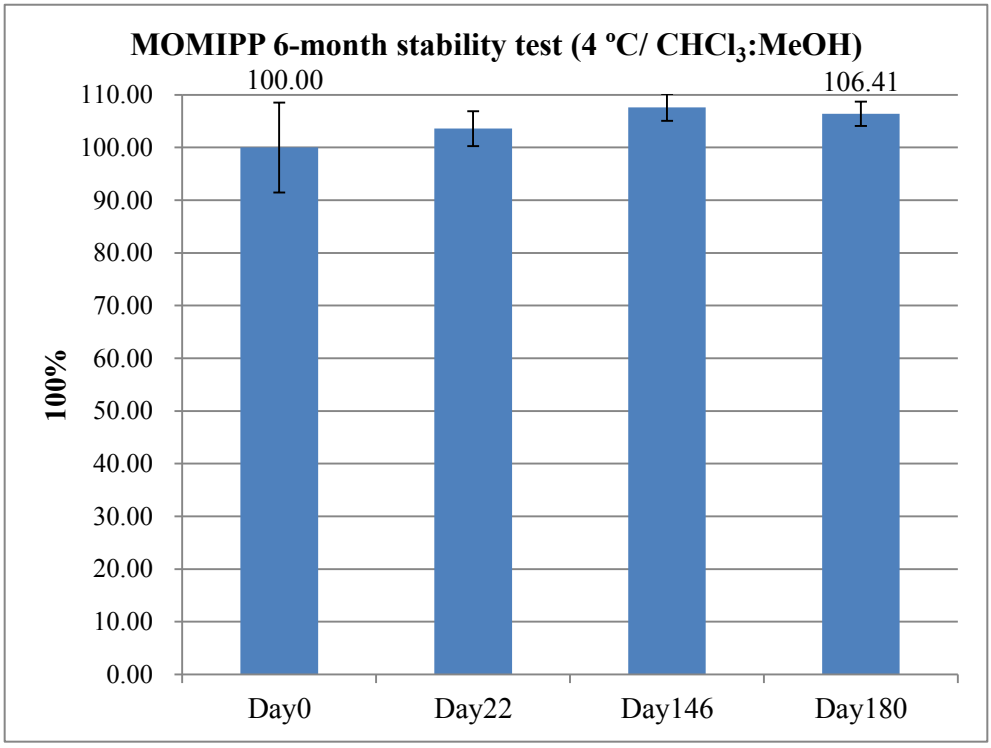


Figure 3-11. MOMIPP 6-month stability test at 4 ºC in $CHCl_3$:MeOH.

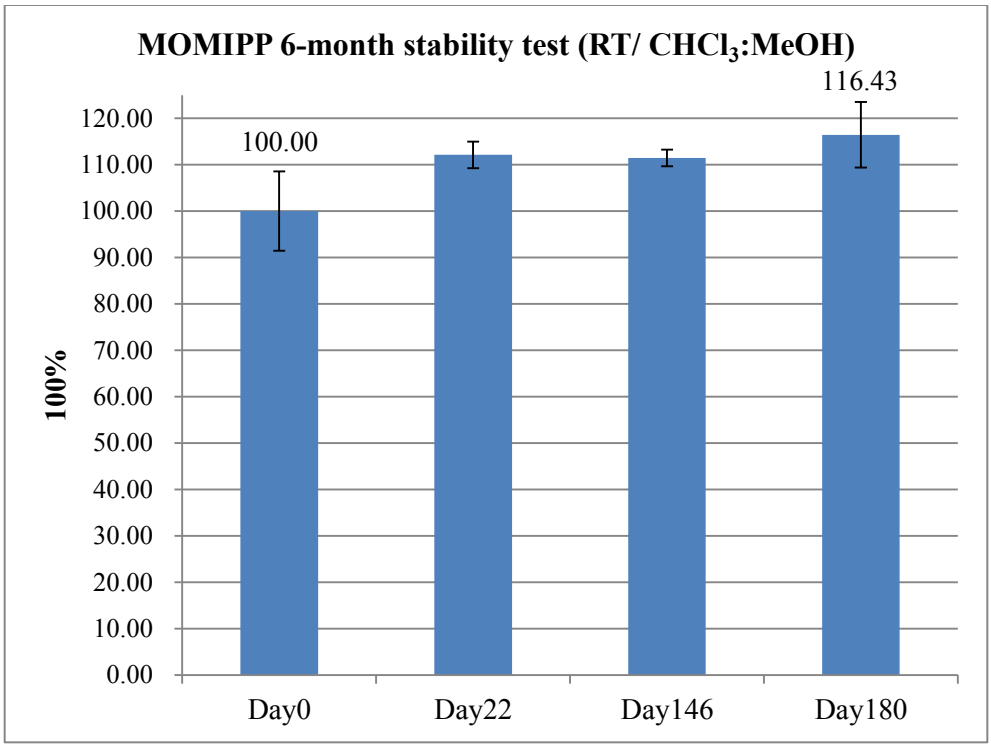


Figure 3-12. MOMIPP 6-month stability test at RT in $CHCl_3$:MeOH.

Table 3.3. Day180 results for MOMIPP 6-month stability test in $CHCl_3$:MeOH (2:1).

unit:μM	Theory	Mean	SD	SEM	CV%	Error%
Control Samples-a	15.000	15.062	0.082	0.047	0.541	-0.412
Control Samples-b	15.000	15.244	0.190	0.110	1.247	-1.600
Control Samples-c	15.000	15.423	0.118	0.068	0.764	-2.741
-80d Samples	14.613	14.609	0.606	0.350	4.147	0.031
4d Samples	14.613	15.448	0.336	0.194	2.172	-5.405
RT Samples	14.613	16.903	1.027	0.593	6.075	-13.546

Each group of samples are consist of 3 test samples, i.e. the Control Samples-a is consists of 3 test samples (a-1, a-2, a-3). Standard deviation (SD) describes the dispersion of each value from the average value. Standard error of mean (SEM) represents the error between the sample value and the population value. (a-1, a-2, a-3 represent "samples", the control samples-a represents "group".) Coefficient of variation (CV) represents precision, this is used to compare among groups with different units. Error% represents accuracy, also describes the difference between theoretical value and true value.

3.2.2 Normal light, ultraviolet (UV) exposure test

(1) Normal light.

The starting concentration of MOMIPP was 15 mM in DMSO. Statistical analyses were performed by a Two-tail t-test, with $P<0.05$ used as the criteria for significant differences. As seen in the Table 3.4, the CV and error values are less than 10%. As shown in the accompanying bar graph (Figure 3-13), the average difference between light exposure samples and controls is far less than 10%. These data show that even 3-days of constant light won't affect MOMIPP's quality. Thus, during our normal operations, between 5 to 30 min light exposures, this will not affect the quality of MOMIPP's DMSO formulation.

Table 3.4. Results from 3-day normal light exposure stability tests.

Unit:μM	Theory	Mean	SD	SEM	CV%	Error%
Control samples	15.076	16.038	0.919	0.531	5.733	-5.995
	15.076	15.904	0.645	0.373	4.057	-5.208
	15.076	16.104	0.851	0.491	5.286	-6.383
Light exposure sample	15.076	15.764	0.657	0.380	4.170	-4.362
	15.076	15.720	1.000	0.578	6.363	-4.099
	15.076	16.242	0.999	0.577	6.152	-7.179

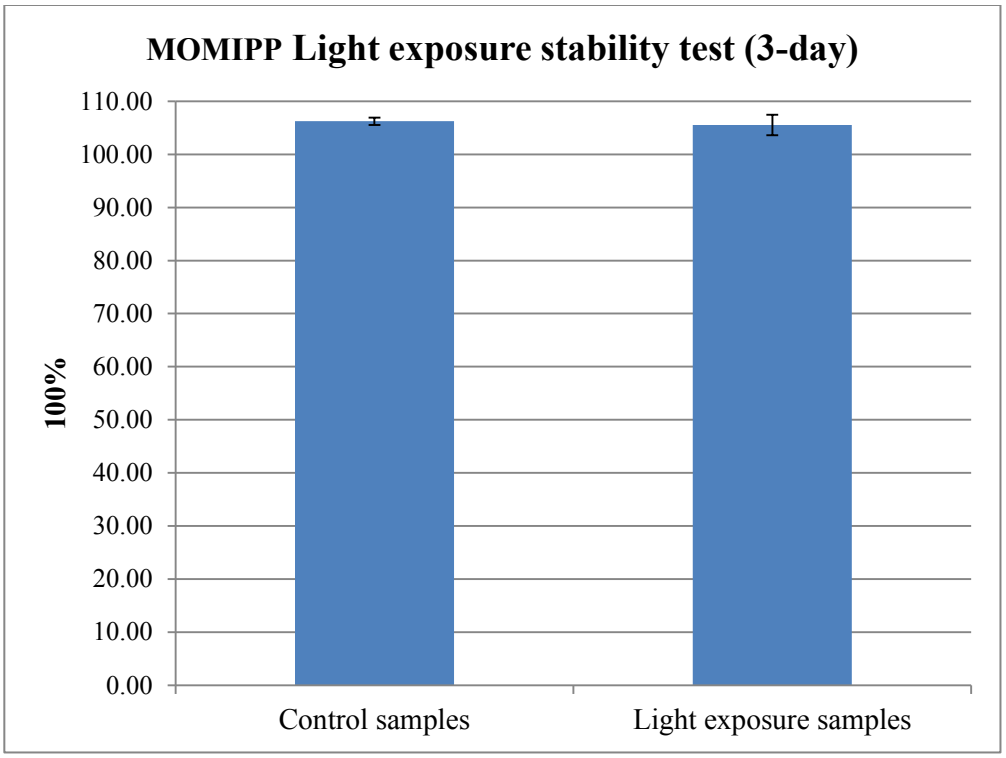


Figure 3-13. MOMIPP 3-day normal light exposure stability test. The value of control samples is 106.23 ± 0.674%; the value of light exposure samples is 105.52 ± 1.92%. Difference between these two is -0.707%.

(2) Ultraviolet (UV) light.

The starting concentration of the MOMIPP was 15 mM in DMSO. Statistical analyses were performed by a Two-tail t-test, with $P<0.05$ used as the criteria for significant differences. The Two-tail t test shows these results to be significantly different. As can be seen in Table 3.5 and Figure 3-14, the CV and error values are far less than 10%, and the average difference between UV exposure samples verses controls is less than 10%. Interestingly, no additional peaks were detected during analyses of the broad HPLC chromatogram. Thus, the suspected decomposition materials in this case are not readily detectable by our existing method. This underscores the value of having a standard concentration-calibration curve available to assess the integrity of a given analyte when

its solutions are exposed to variant conditions. Nevertheless, the bar graph in Figure 3-14 clearly shows that the concentration decreases by 8.34%, and this is a statically significant number. Thus, it is better to keep MOMIPP away from ultraviolet light, e.g. the use of an amber vial is recommended. Likewise, Figure 3-15 shows that there are no extra peaks when focused at 278nm detection while Figure 3-16 shows no extra peaks when the entire spectrum is scanned via 3D collection across 200-700 nm.

Table 3.5. Results from 3-day UV light exposure stability tests.

Unit:μM	Theory	Mean	SD	SEM	CV%	Error%
Control samples	15.076	15.843	0.670	0.387	4.229	-4.839
	15.076	15.913	0.871	0.503	5.474	-5.260
	15.076	16.113	0.827	0.477	5.131	-6.438
UV exposure sample	15.076	14.572	0.182	0.105	1.249	3.461
	15.076	14.753	0.064	0.037	0.435	2.191
	15.076	14.771	0.320	0.184	2.163	2.068

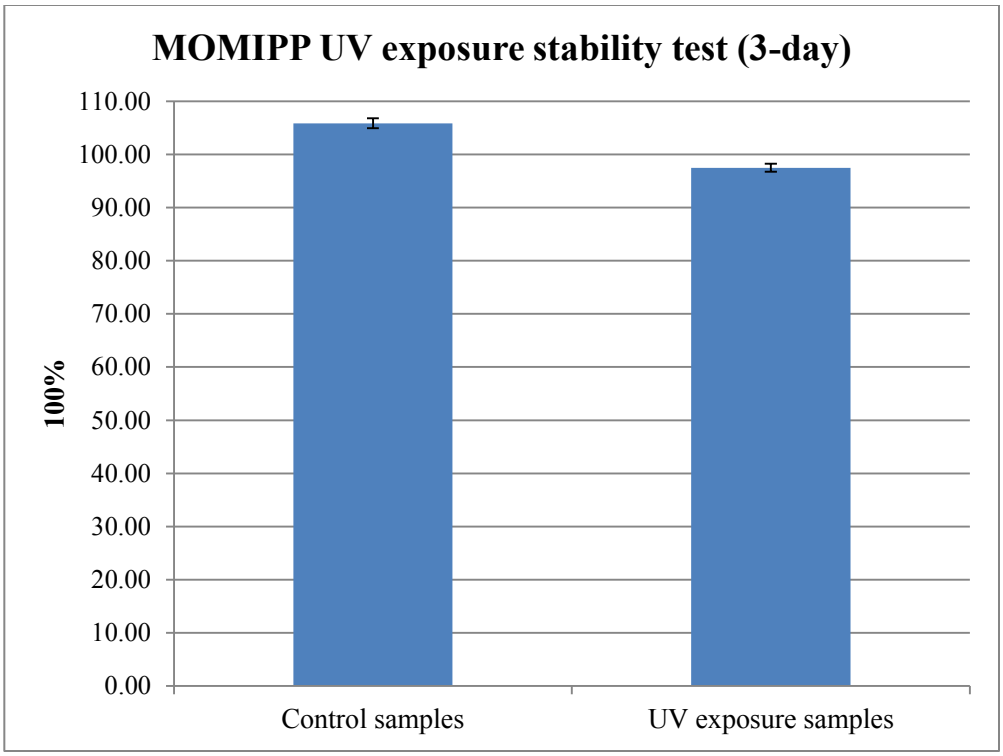


Figure 3-14. MOMIPP 3-day UV light exposure stability test. The value of control samples is 105.84 ± 0.932%; the value of light exposure samples is 97.50 ± 0.73%. Difference between these two is -8.34%.

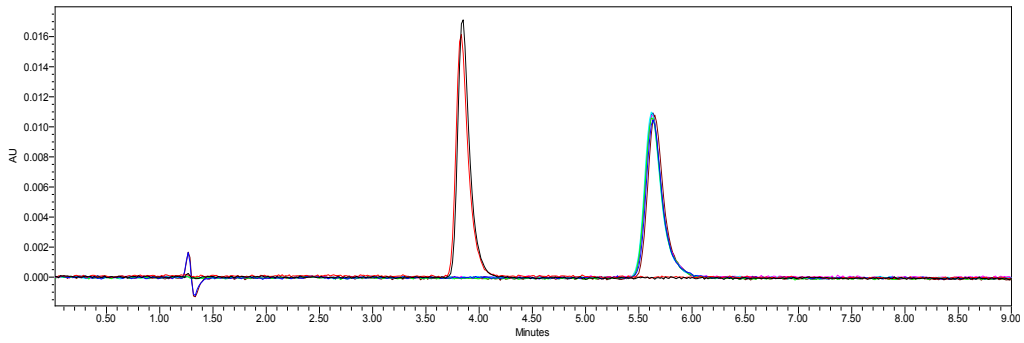


Figure 3-15. Overlap chromatogram of UV light exposure stability test, also including external standard at 278 nm by deploying UV-PDA detector.

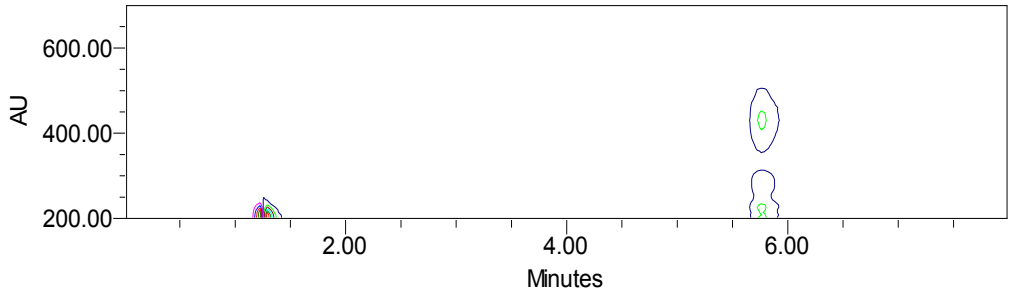


Figure 3-16. 3D chromatogram of UV light exposure stability test data. There are no extra peaks.

3.2.3 Oxygen exposure stability test

The starting concentration of MOMIPP was 15 mM in DMSO. Statistical analyses were performed by a Two-tail t-test, with $P<0.05$ used as the criteria for significant differences. The Two-tail t test shows that the results are not significantly different. As seen in Table 3.6 and Figure 3-17, the CV and error values are less than 10% and the average difference between the oxygen exposure samples and the nitrogen exposure control samples is less than 10%. The bar graphs in Figure 3-17 indicate that the concentration decreases only 1%. We conclude that exposure to air should not be a problem for MOMIPP.

Table 3.6. Results from 3-day oxygen exposure stability tests using nitrogen exposure samples as control.

Unit:μM	Theory	Mean	SD	SEM	CV%	Error%
Nitrogen exposure samples	14.700	14.529	0.108	0.062	0.743	1.174
	14.700	14.589	0.200	0.116	1.373	0.762
	14.700	14.497	0.168	0.097	1.157	1.398
Oxygen exposure samples	14.080	14.222	0.079	0.045	0.553	-1.001
	14.080	14.548	0.164	0.095	1.130	-3.215
	14.080	14.425	0.100	0.057	0.690	-2.394

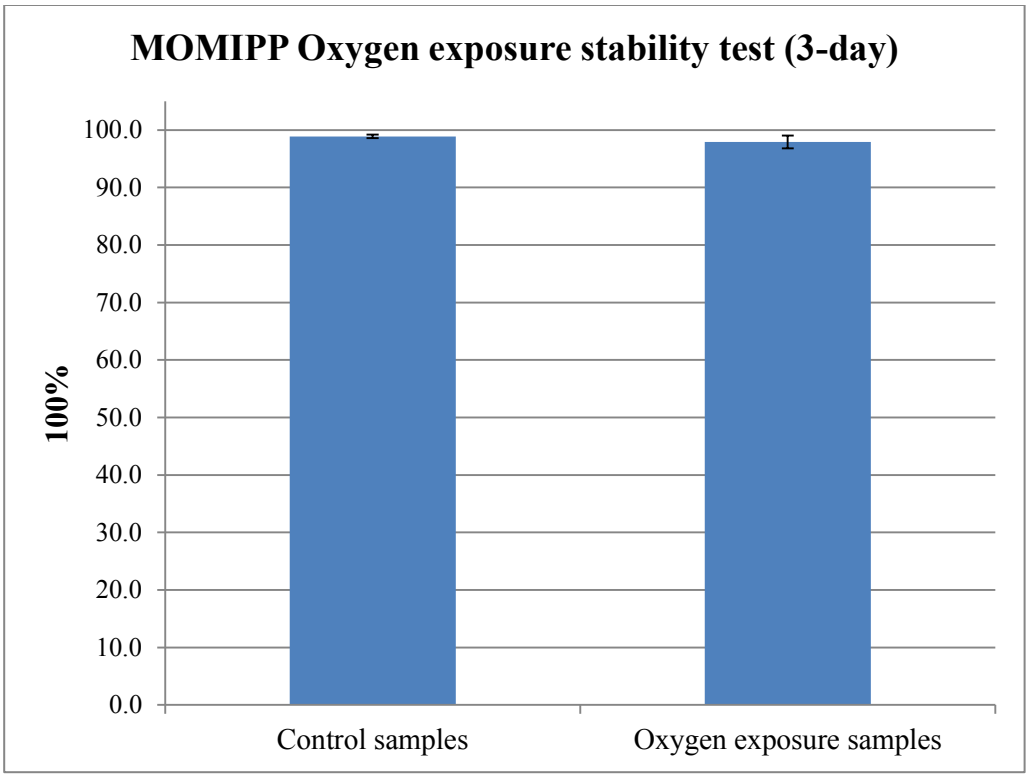


Figure 3-17. MOMIPP 3-day oxygen exposure stability test. The value of control samples is 98.9 ± 0.316%; the value of light exposure samples is 97.9 ± 1.118%. Difference between these two is -1%.

3.2.4 Freeze-thaw stability test

The starting concentration of MOMIPP was 15 mM in DMSO. All solutions were stored at -20 °C. Statistical analyses were performed by a Two-tail t test, with $P<0.05$ used as the criteria for significant differences. The Two-tail t test shows that the two groups' results are significantly different. From Table 3.7, the CV and error value are much less than 10% and Figure 3-18 shows that the average differences between the freeze-thaw samples and the control samples is less than 10%. Thus, while statistically different the changed reasonably small (< 5%) and we regard them as being acceptable after 5 cycles of freeze-thaw protocols. Likewise, Figure 3-19 shows that there are no

extra peaks when focused at 278 nm detection while Figure 3-20 shows no extra peaks when the entire spectrum is scanned via 3D collection across 200-700 nm.

Table 3.7. Results from 5-cycle freeze-thaw stability tests.

Unit:μM	Theory	Mean	SD	SEM	CV%	Error%
Control samples	15.000	15.173	0.178	0.103	1.174	-1.137
	15.000	15.113	0.214	0.123	1.413	-0.747
	15.000	15.457	0.257	0.148	1.660	-2.956
Freeze-thaw samples	15.000	14.914	0.204	0.118	1.368	0.577
	15.000	14.821	0.253	0.146	1.706	1.205
	15.000	14.620	0.282	0.163	1.927	2.599

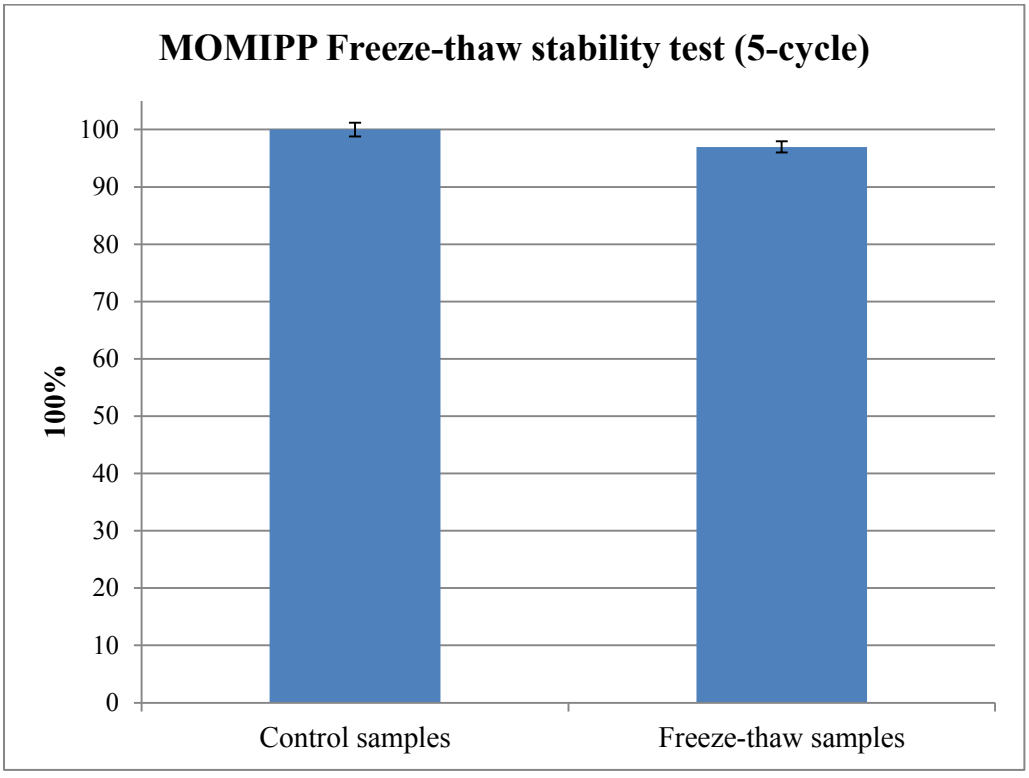


Figure 3-18. MOMIPP freeze-thaw stability test (5-cycle). The value of control samples is 100 ± 1.21%; the value of light exposure samples is 97.0 ± 0.986%. Difference between these two is -3%.

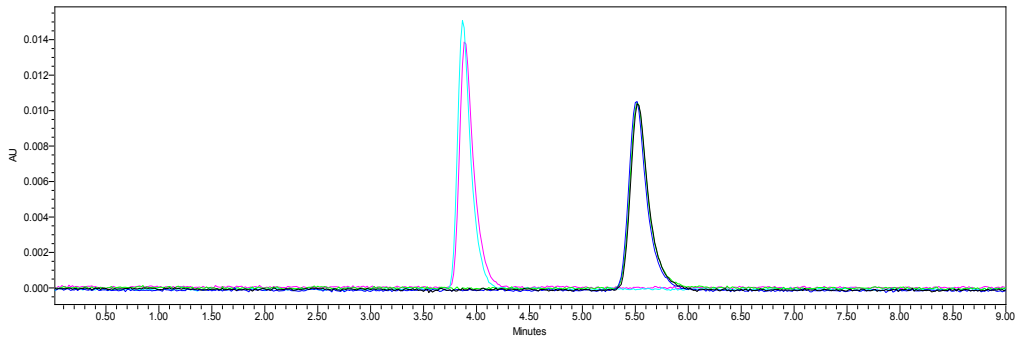


Figure 3-19. Overlap chromatogram of freeze-thaw stability test, also including external standard at 278 nm by deploying UV-PDA detector.

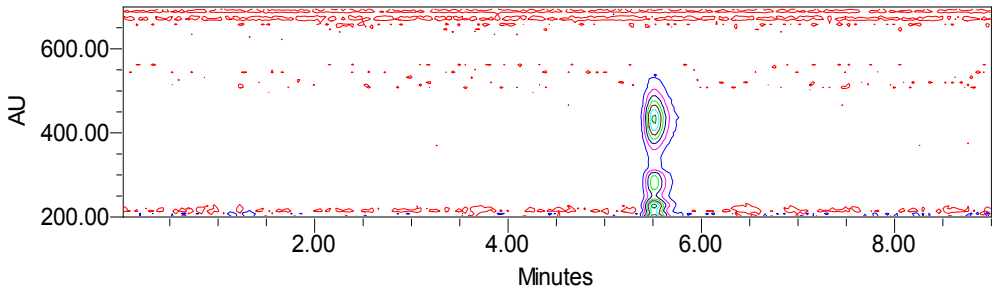


Figure 3-20. 3D chromatogram of freeze-thaw stability test data. The small spots on the graph represent system noise. There are not extra peaks.

3.2.5 1-week HPLC autosampler stability test

The starting concentration of MOMIPP was 15 μM in the mobile phase, this was the same concentration as the other 15 μM control samples. These tests also utilized the same preparation procedure. Statistical analyses were performed by one-way ANOVA, with $P<0.05$ used as the criteria for significant differences. According to Table 3.8, Figures 3-21 and 3-22, significant statistical differences exist from Day 0 when compared to the rest days of the week at room temperature. The table also presents that the data between Day 3 and Day 0, between Day 7 and Day 0 have statistical significant differences at 4 °C.

Evaporation appears to be a major problem that changes the concentration. There are no extra peaks in the chromatographs. Figures 3-21 and 3-22 show the HPLC autosample stability test at 4 °C and RT, respectively. Table 3.9 and Figure 3-23 indicated the concentration which was calculated according to the weight lost. In the calculation, the original weight of the autosamples was obtained from the first day during preparation of the storage samples. Before each analysis day, the autosamples were weighed again. Density (d) was derived by preparing the same concentration solution and calculated from d= mass/ volume. Given this problem with solvent evaporation, it is recommended that samplers placed on the autosampler should be set-up to be analyzed within no longer than 2 days.

Table 3.8. Results from 1-week HPLC autosampler stability testing under different temperatures.

Day	4 °C	RT
Day0	100.00 ± 0.16	100.00 ± 0.16
Day1	103.07 ± 1.32	105.79 ± 0.57
Day2	103.00 ± 0.58	106.91 ± 0.63
Day3	108.06 ± 0.89	111.12 ± 0.90
Day7	120.31 ± 4.82	128.71 ± 2.24

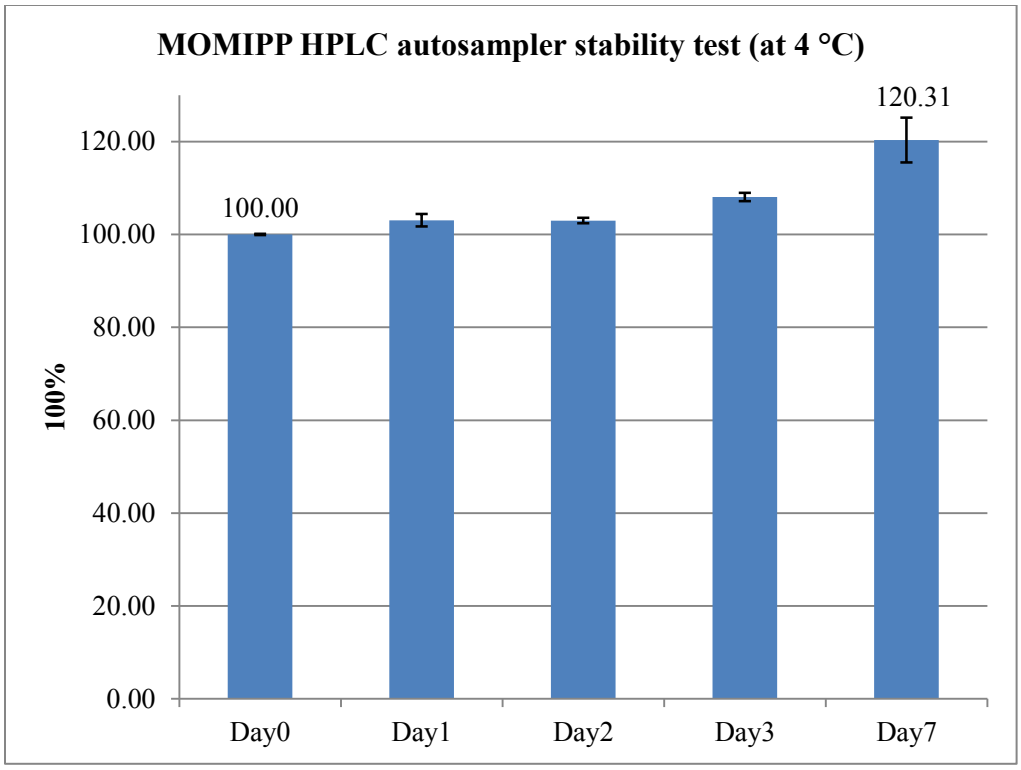


Figure 3-21. MOMIPP HPLC autosample stability test at 4 °C.

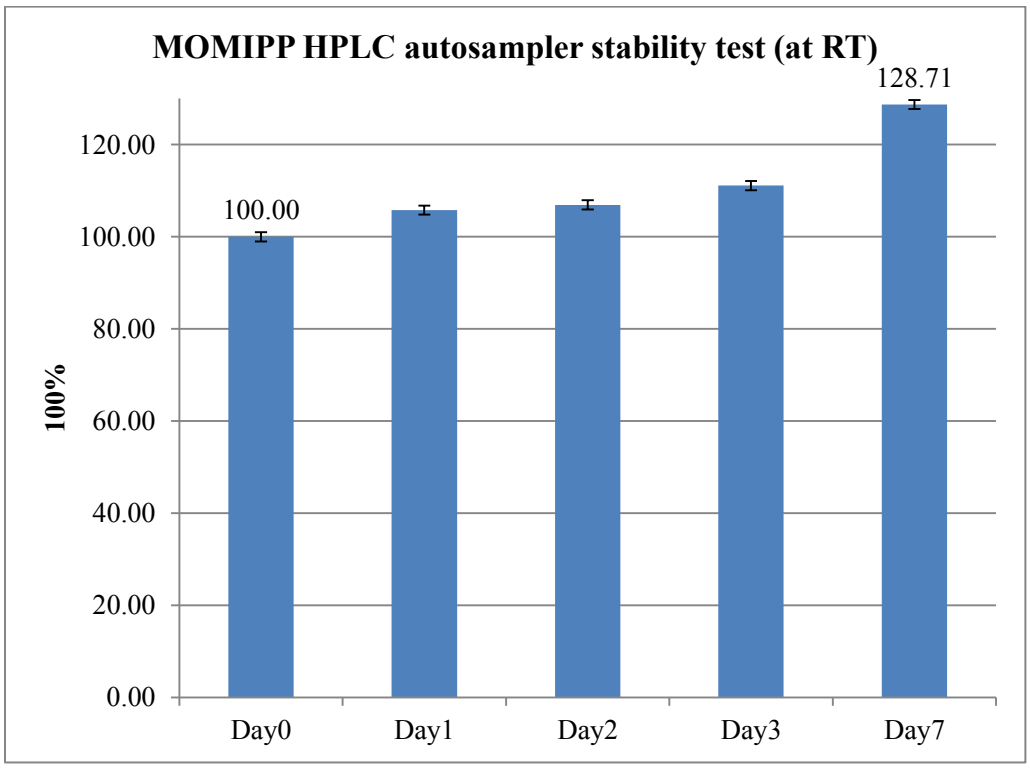


Figure 3-22. MOMIPP HPLC autosampler stability test at RT.

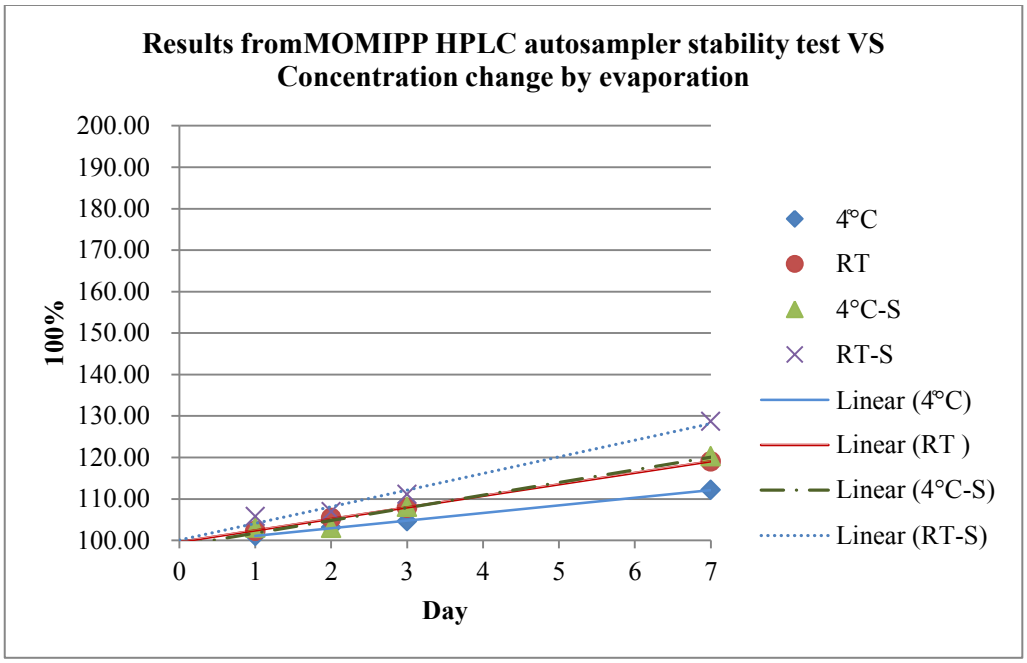


Figure 3-23. Results from HPLC autosampler stability testing versus the concentration change due to evaporation.

Table 3.9. Results from HPLC autosampler stability testing versus concentration change due to evaporation.

	c% by evaporation		Results from stability tests	
	4 °C	RT	4 °C	RT
Day 1	101.02	102.29	103.07	105.79
Day 2	103.11	105.37	103.00	106.91
Day 3	104.59	107.84	108.06	111.12
Day 7	112.17	119.07	120.31	128.71

Chapter 4

Cell Metabolism Studies

Preliminary metabolism studies were conducted so as to provide an initial assessment of the practical utility of our LC-MS/MS assay described in Chapter 2. These studies were conducted directly on an *in vitro* cell culture system being exposed to MOMIPP drug treatments. The cell culture media from these treatments was used for analysis. This required a sample preparation step prior to actual assay by LC-MS/MS.

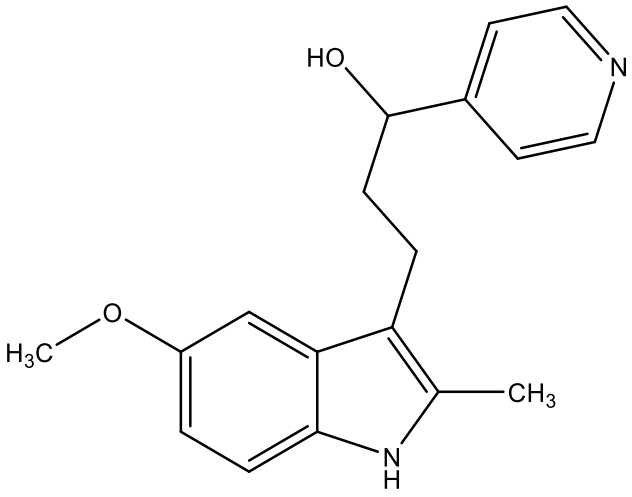
The protocol developed for the sample preparations is described below and our preliminary results follow.

4.1 LC-MS/MS samples preparation and storage conditions

The cell culture media aliquots were stored at -80 ºC. Aliquots were thawed at RT over a period of 10 min. They were then extracted by placing 500 µl media in 1000 µl ethyl acetate for 30 minutes. A sample was obtained by taking 800 µl of the upper-layer (ethyl acetate and evaporated by a vacuum centrifuge. The resulting residue was resuspended in 200 µl of mobile phase which was then centrifuged before transferring it to the LC-MS/MS autsampler.

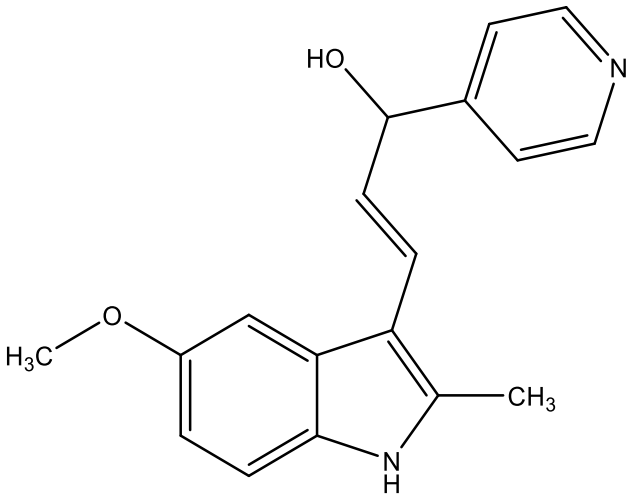
4.2 Results of metabolism studies

The observed metabolites of MOMIPP are called M1, M2A and M2B. The structures are shown below in Figures 4-1, 4-2 and 4-3. The MS-detectable mass to charge species for these metabolites are: M1, $[M+H]^+ = 297.16$; M2A, $[M+H]^+ = 295.14$; M2B, $[M+H]^+ = 295.14$. Figure 4-4 depicts the chromatogram for all of these metabolites within an LC-MS assay. Figure 4-5 delineates the order of metabolites formation of MOMIPP.



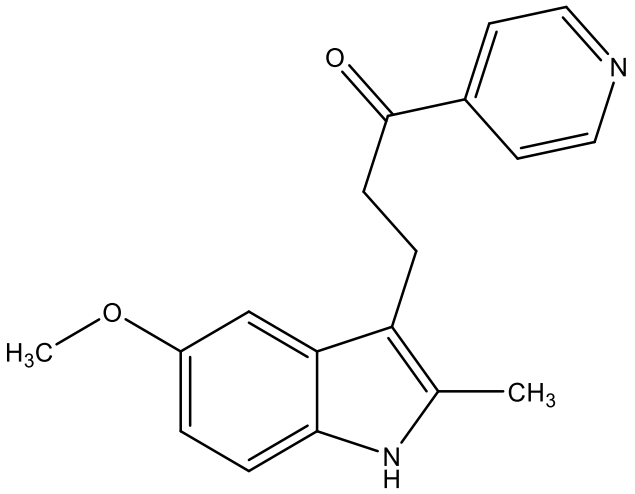
M1

Figure 4-1. Chemical structure of MOMIPP's metabolite M1.



M2A

Figure 4-2. Chemical structure of MOMIPP's metabolite M2A.



M2B

Figure 4-3. Chemical structure of MOMIPP's metabolite M2B.

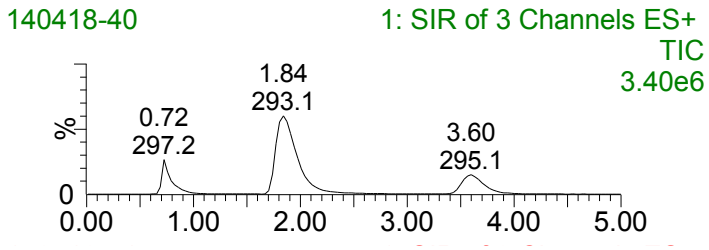


Figure 4-4. A chromatogram with all metabolites and MOMIPP. MOMIPP's retention time (minutes) is 1.84, M1's retention time is 0.72, m/z is 297.2; M2A's retention time is also 0.72, m/z is 295.1; M2B's retention time is 3.60, m/z is 295.1; MOMIPP's retention time is 1.84, m/z is 293.1.There is only a small amount of M2A formed.

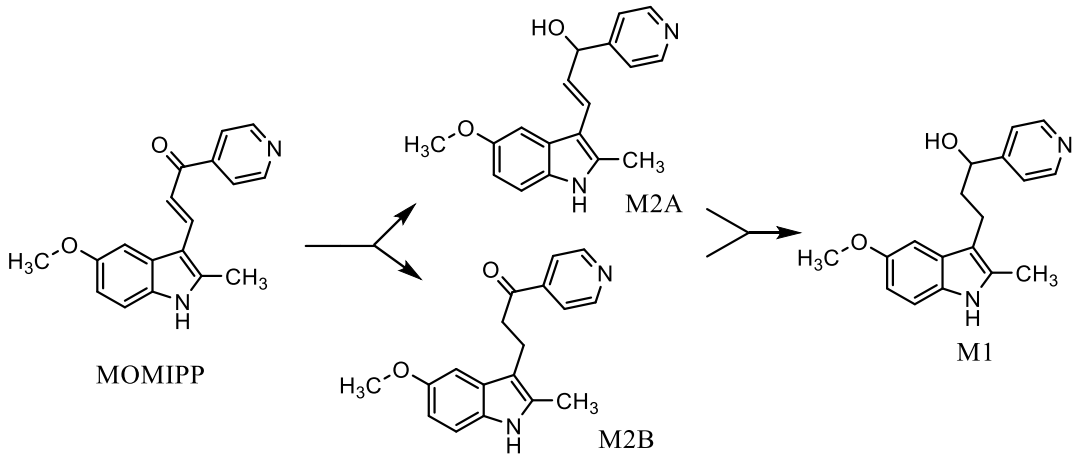


Figure 4-5. The order of forming the metabolites of MOMIPP. MOMIPP incubated in glioblastoma cells follows a similar metabolism pattern. M2A and M2B are formed first. Then, these two metabolites were partially converted into M1.

4.2.1 MOMIPP metabolites from U251 cells

The starting concentration of MOMIPP in media is 10 μM. Figure 4-6 represents results from U251 media which were collected at different time points: 0-hr, 4-hr, 24-hr, 48-hr and 72-hr. Figure 4-7 shows a clear relationship among the metabolites of MOMIPP by expanding the scale of the y-axis. The initial sharp rise tends to decrease after 24 hrs for M2B. M1 continues to be gradually formed from M2A and M2B. There is only a small amount of M2A formed, about 2% of the total M2 metabolites. Figure 4-8

shows a series of LC chromatograms which include control samples of media. The two chromatograms of MOMIPP without incubation and MOMIPP incubated without cells are similar. Thus, media itself cannot deteriorate MOMIPP. In addition, the chromatogram shows that U251 cells without MOMIPP do not contain interfering peaks that need to be accounted for. There is no other effect from background solvent. The chromatograms for 1 day, 2 days and 3 days with U251 cells show the concentration change of metabolites by time. Most metabolites form after 24 hrs.

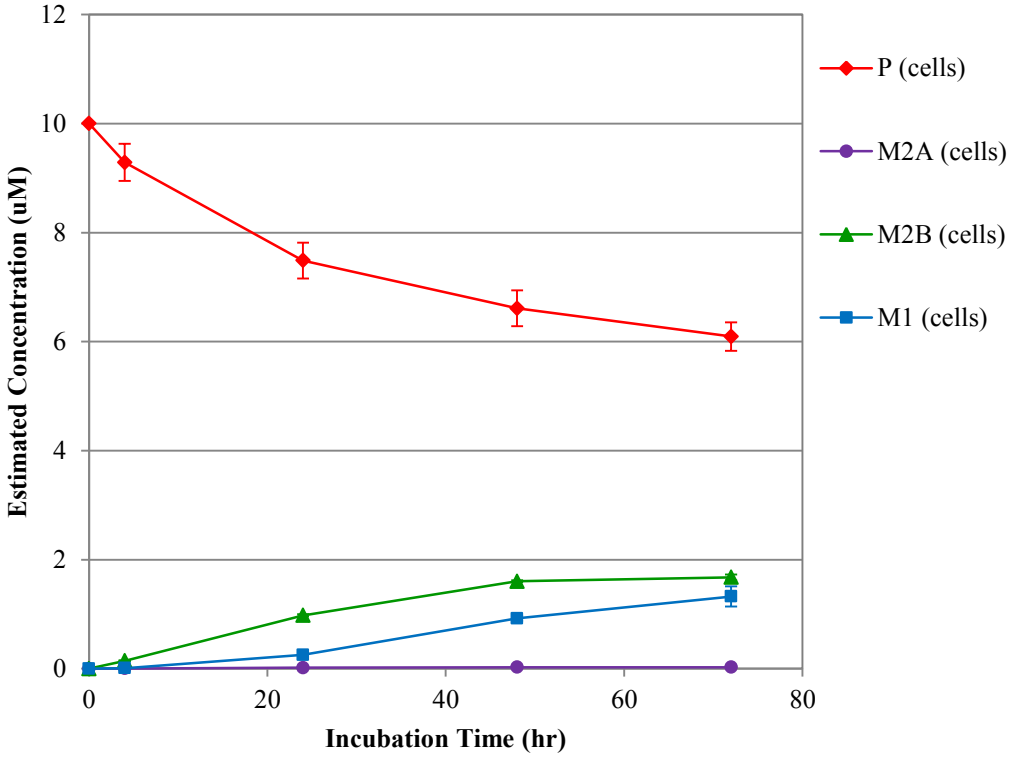


Figure 4-6. The concentration change of MOMIPP and its metabolites incubated in U251 cells in 72 hrs.

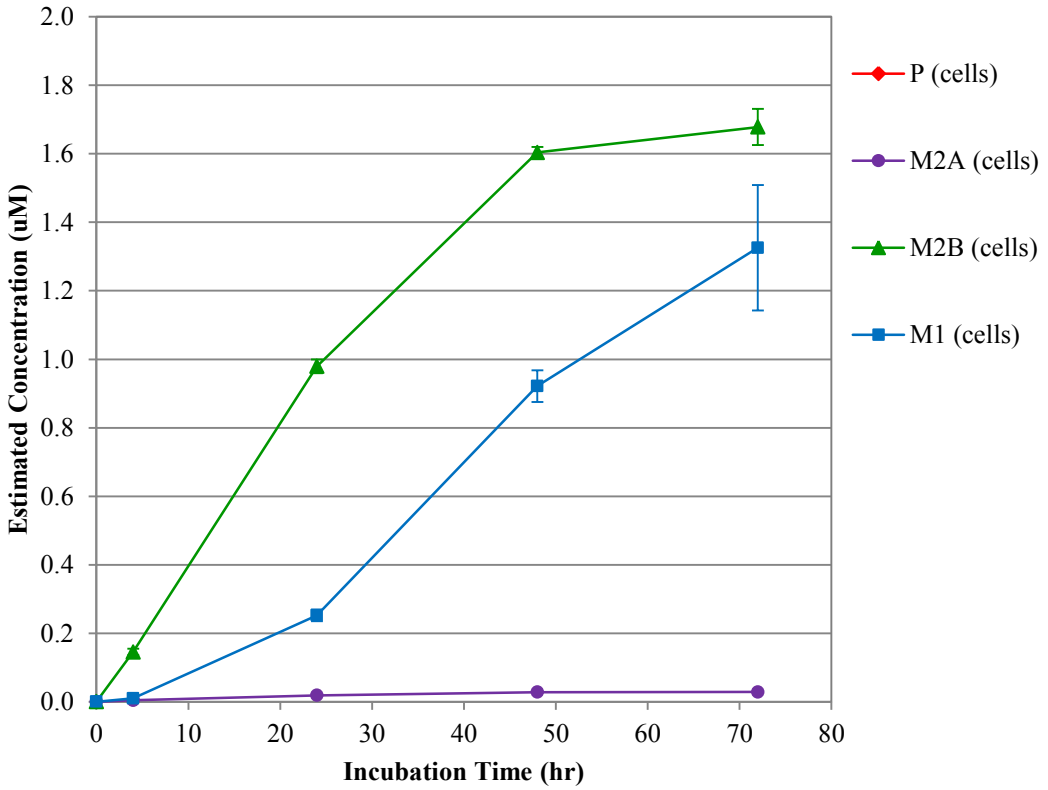
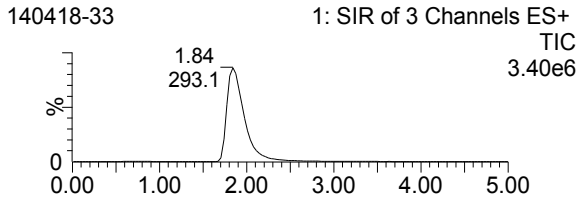
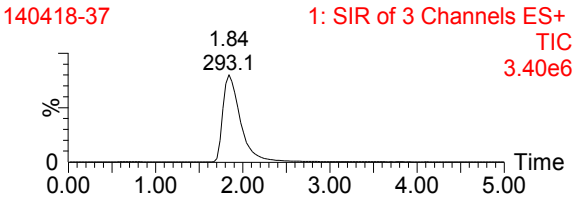


Figure 4-7. The concentration change of MOMIPP's metabolites incubated in U251 cells in 72 hrs using an enlarged scale on the y-axis.

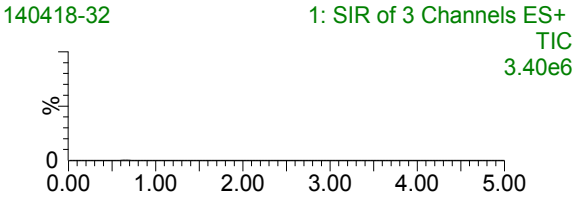
No incubation



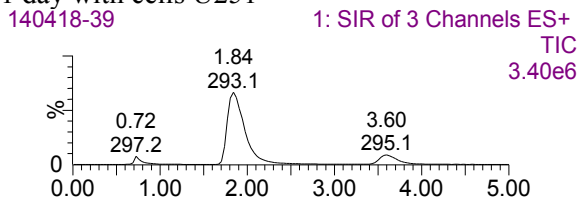
3 days without cells U251



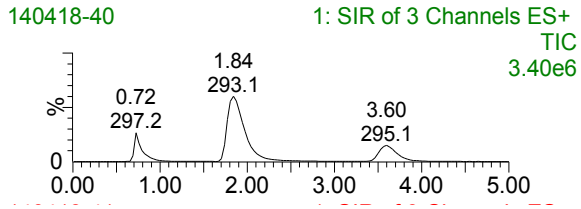
3 days with cells U251 No MOMIPP



1 day with cells U251



2 days with cells U251



3 days with cells U251

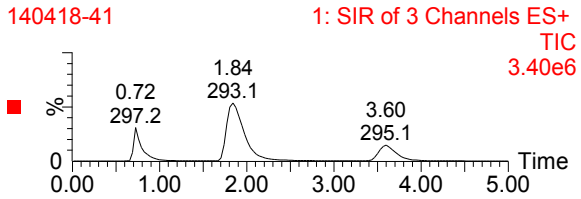


Figure 4-8. The concentration change of MOMIPP and its metabolites incubated in U251 cells in LC chromatograms, with control samples' chromatograms.

4.2.2 MOMIPP metabolites from GL261 cells

The starting concentration of MOMIPP in media is 10 μM. Figure 4-9 presents the results from GL261 cell media which were collected at different time points: 0-hr, 4-hr, 24-hr, 48-hr and 72-hr. Figure 4-10 shows a clear relationship between metabolites by having an expanded y-axis. MOMIPP incubated in GL261 metabolizes faster than in U251 cells. The concentration of MOMIPP decreases nearly to 0 after the 2nd day. M2B formed quickly during the1st day and then this subsided. The figures show a clear trend that M1 was formed from M2A and M2B especially after 24 hrs where there is only a small amount of MOMIPP left, but M1 was formed faster during the first 24 hrs. In addition, there is only a small amount of M2A formed. Figure 4-11 shows a series of LC chromatograms that include control samples of media. The two chromatograms of MOMIPP without incubation and MOMIPP incubated without cells are similar. As before media does not deteriorate MOMIPP. In addition, the chromatogram again shows that U251 cells without MOMIPP do not have interfering peaks. There is no other effect from background solvent. The chromatograms for 1 day, 2 days and 3 days with U251 cells show the concentration change of metabolites with time. MOMIPP was almost gone after the 1st day, the M2B metabolite's peak rises and falls during the1st day. At the same time, the M1 and M2A metabolite peaks grow larger after each day.

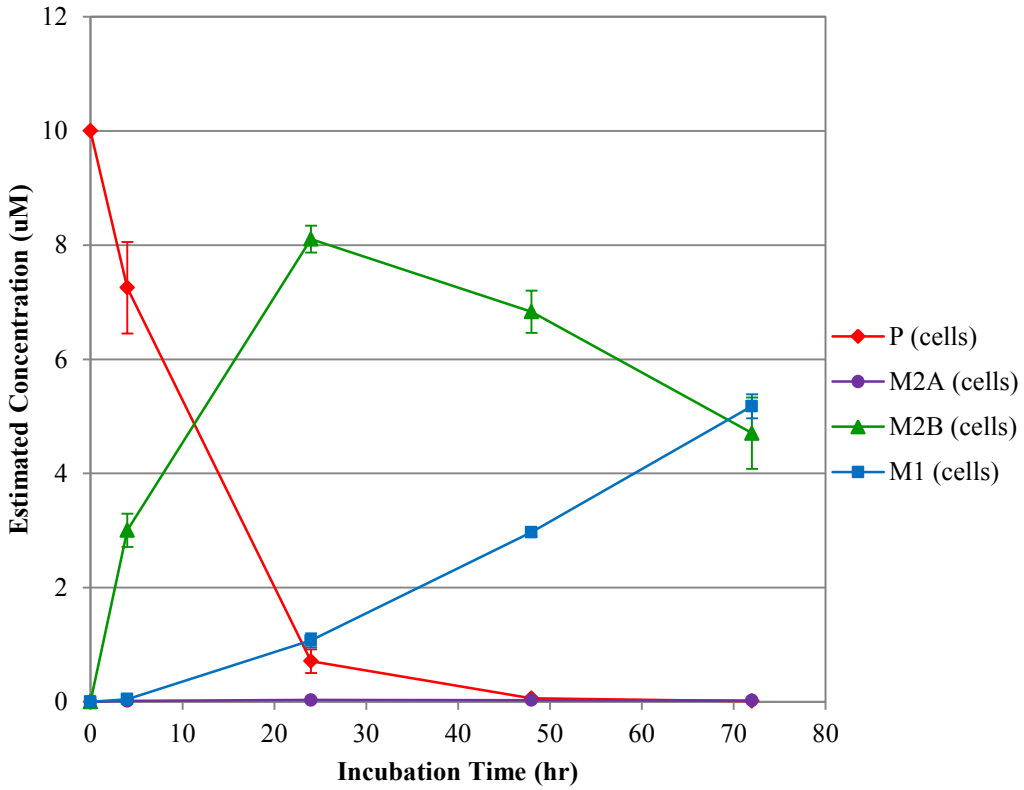


Figure 4-9. The concentration change of MOMIPP and its metabolites incubated in GL261 cells in 72 hrs.

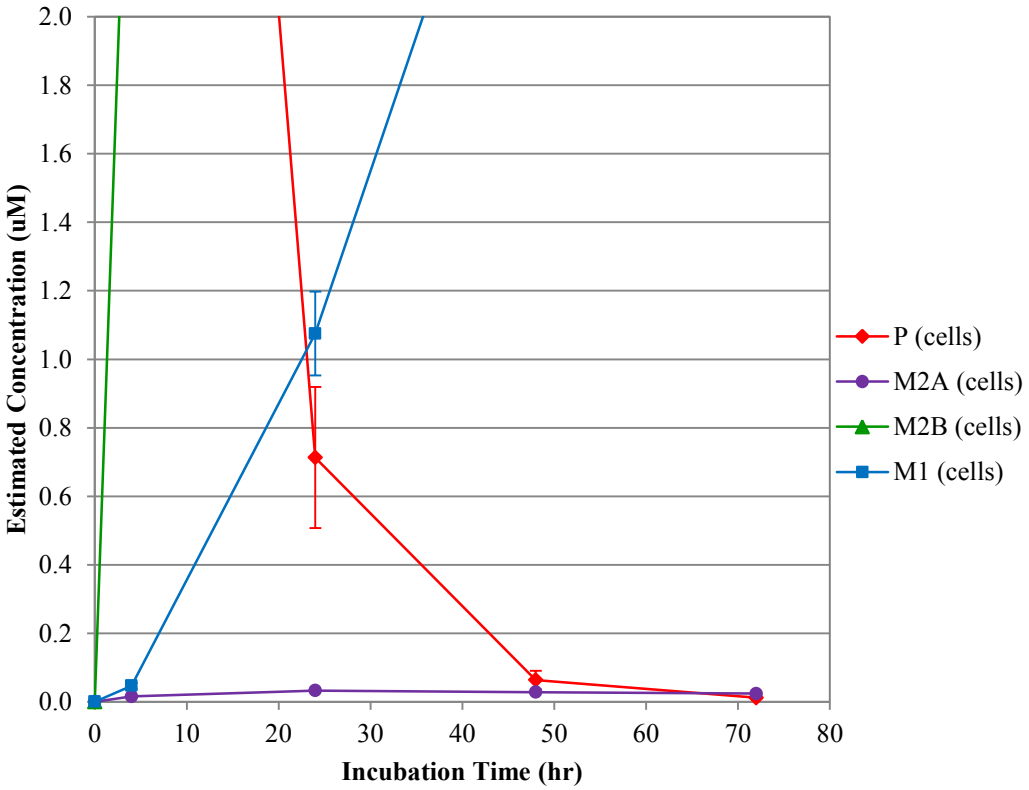
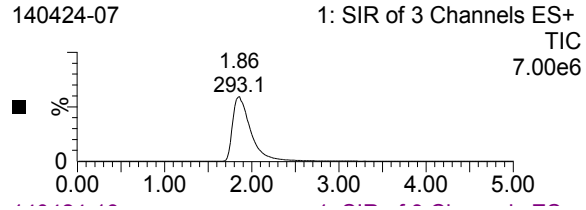
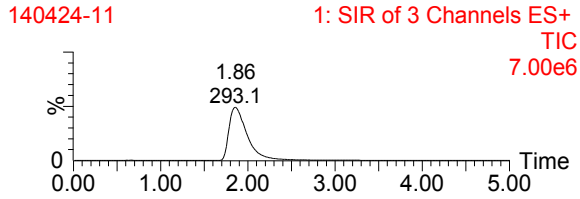


Figure 4-10. The concentration change of MOMIPP and its metabolites incubated in GL261 cells in 72 hrs using an enlarged scale on the y-axis.

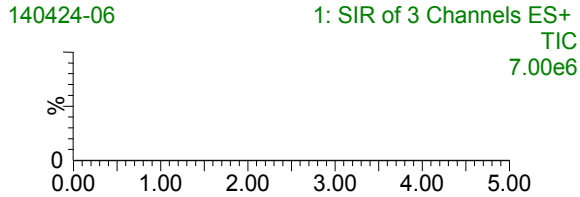
No incubation



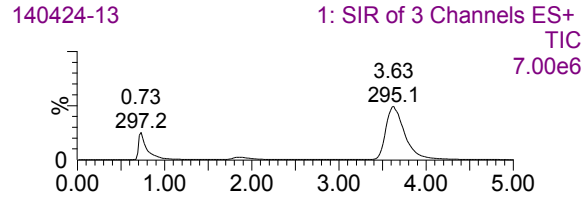
3 days without cells GL261



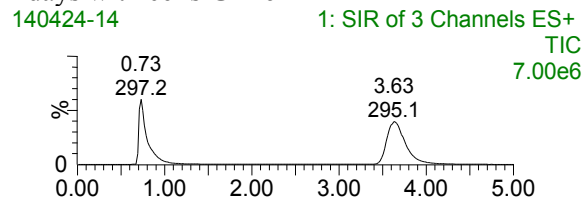
3 days with cells GL261 No MOMIPP



1 day with cells GL261



2 days with cells GL261



3 days with cells GL261

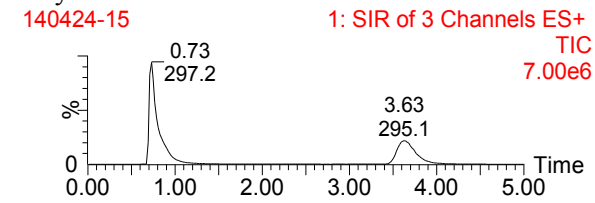


Figure 4-11. The concentration change of MOMIPP and its metabolites incubated in GL261 cells in LC chromatograms, with control samples' chromatograms.

4.3 Conclusion

The metabolites from MOMIPP incubated with two different cell lines of glioblastoma are similar. The only difference was the rate of their formation. M2A only forms a small amount overall. M1 is formed from M2A and M2B. Importantly, the feasibility of deploying our LC-MS/MS method to study MOMIPP's metabolism has been clearly demonstrated.

Chapter 5

Overall Conclusions and Future Studies

Based on the results of the stability tests for MOMIPP, it is clear that this chemical structure is stable for at least 6 months in DMSO. Low temperature is recommended as the better storage condition in terms of temperatures. $CHCl_3$:MeOH (2:1) is a volatile solvent compared to DMSO, and so it can be subject to evaporation during storage. However, because it can be quickly evaporated during sample preparation steps, it is still a useful solvent. To avoid unwanted evaporation, it is convenient to store $CHCl_3$:MeOH solutions of MOMIPP in well-sealed vials.

As common exposure factors in the lab, normal light and oxygen will not affect MOMIPP's stability. However, UV light is a damaging factor and it will affect MOMIPP's concentration to a small degree. In that regard we did not identify extra peaks or degradation compound information from these tests. Similarly, from freeze-thaw tests, it is not clear about the information pertaining to degradation compounds, because while our data showed a decrease in concentration, there were no extra peaks detected. Using a solution for more than 5 cycles of freeze-thaw procedures is not recommended. For analysis of samples using an HPLC autosampler, it is recommended that they should be

stored at 4 °C for no longer than 2 days before injection. Otherwise, the results could become compromised by evaporation of the solvent.

In future studies, it will be interesting to determine the reason that the concentration of the freeze-thaw (5 cycles) samples and the UV exposure samples' decrease. This can be attempted by increasing the overall quantities deployed and then utilizing the decomposed mixture for separation of their components by preparative column chromatograph (Chapter 2). Once separated, the component structures can be ascertained by MS and NMR analysis.

To summarize the metabolism of MOMIPP, it is clear that incubation with glioblastoma cell lines U251 and GL261 have similar results. From these two cell lines, the metabolism pattern is similar except for the rate of their formation. In the future, it will be interesting to learn about the metabolism pattern of MOMIPP incubated with other cell lines. Likewise, it will be especially interesting to understand the metabolism pattern after *in-vitro* testing in rodents.

Finally, because MOMIPP's chemical structure contains a central scaffold that is common to all of the analogs of interest for our methuosis program; these results should be generally applicable even to other compounds having similar structures should they be selected for development. While specific tests will need to be repeated in every of such case, these new tests should now be able to be completed with very little additional development work, and the overall framework for their design and execution can be essentially the same.

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