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Quantitative Analysis of Bleomycin in Rat Plasma by LC-MS/MS

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QUANTITATIVE ANALYSIS OF BLEOMYCIN IN RAT PLASMA BY LC-MS/MS

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QUANTITATIVE ANALYSIS OF BLEOMYCIN IN RATS PLASMA

WITH LC-MS/MS

HUAWEN LI

ABSTRACT

Bleomycin is the most commonly used compound in its group of antineoplastic drugs. It works on tumor cells by single and double stranded DNA cleavage after its activation, in which it blocks tumor cells' DNA replication or transcription activities to inhibit tumor cells' growth. Bleomycin sulfate (Blenoxane) is the most popular preparation used in clinical research, and contains Bleomycin fractions of A_2 and B_2, which causes difficulties in quantitative analysis. This work uses the metal chelating property of Bleomycin as an advantage to simplify and improve sensitivity of existing quantitative methods.

Copper was spiked in excess to plasma samples, followed by liquid-liquid extraction. Samples were then subjected to analysis by high-performance liquid chromatography electrospray ionization tandem mass spectrometry using a quadrupole trap mass analyzer. Samples spiked with copper showed improved selectivity over samples without excess copper, thereby making use of standard mass spectrometers a possibility in the clinic. In comparison with current methods of quantification of Bleomycin in plasma, this method achieved higher percent recoveries of the chemotherapy drug, higher sensitivity of quantification, with lower matrix effects, as well as a more simple preparation method. Linear range in the lower nanogram per milliliter range with a correlation coefficient over 0.99 makes this method promising for improved quantification and monitoring of Bleomycin in plasma.

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CHAPTER I

INTRODUCTION

1.1 Blenoxane

Bleomycin sulfate (Blenoxane; BLM; DB00290; Fig. 1)[3] is a widely used drug in oncology used to treat a variety of cancers, such as Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, ovarian cancer, and cervical cancer among others.[1] BLM is also used as a highly effective cytostatic antibiotic. In its use as an anticancer agent, BLM can be given intravenously by injection into the muscle or under the skin.[1] The advantage of the BLM compared to other chemotherapeutic drugs and other drugs in its class includes use in multi-cancer therapy, fewer side effects, and that it is not damaging to the bone marrow and the immune system.[1, 2, 3] Bleomycin was discovered in 1962, and the most widely used drug is isolated from the fermentation broth of *Streptomyces verticillus*. Current clinically administered preparations of the drug mixture has more complex contents. The most common Bleomycin drug is the Bleomycin sulfate (Blenoxane), which mainly consists of metal (Cu) ions, 55-70% of Bleomycin A_2 [molecular weight (M.W.) of 1414.52], and 25-32% of Bleomycin B_2 (molecular weight of 1424.56).[2,3] The rationalization as to when bleomycin sulfate is used as a treatment in clinical use is with a

deep consideration of its toxicity; therefore the research on its side effect from hydrolysis studies is an interest of some research groups.

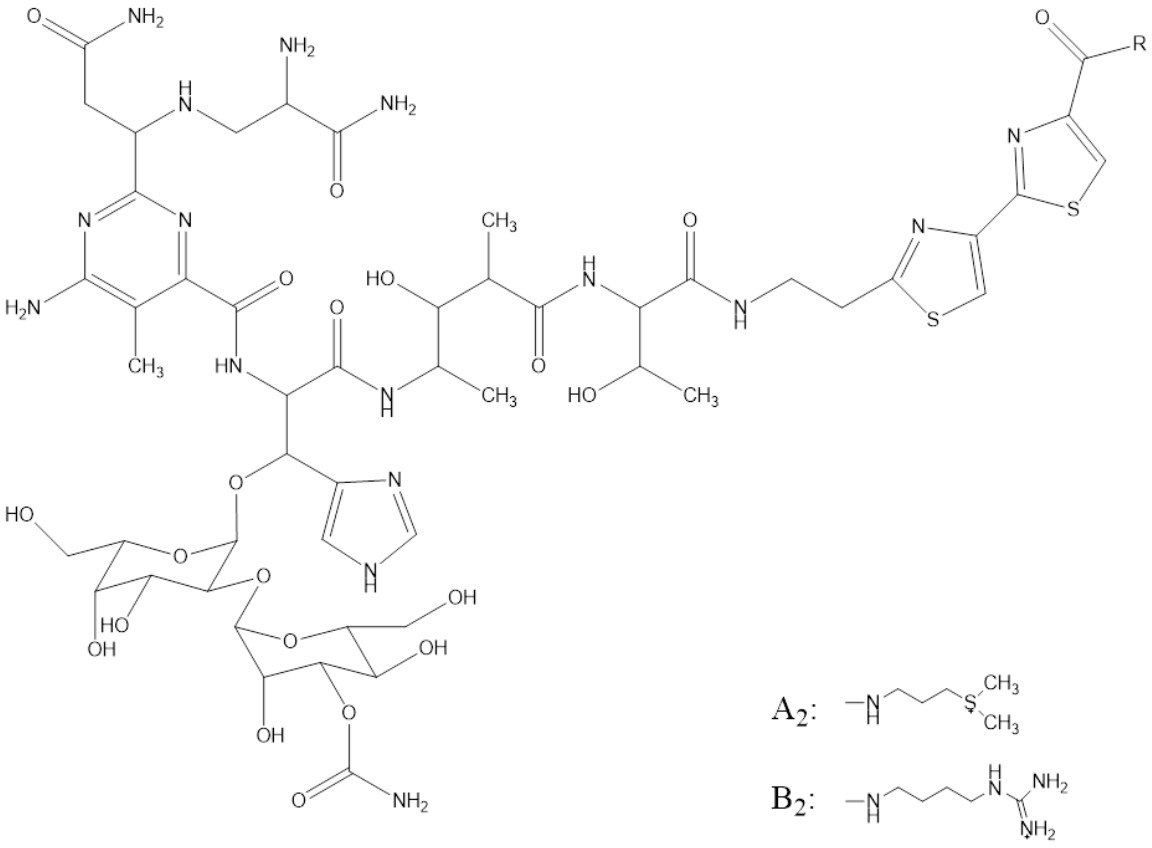

Figure 1. The chemical structure of bleomycin sulfate. IUPAC name: {(3-{[2-(2-{2-[(2S,3R)-2-[(2S,3S,4R)-4-[(2S,3R)-2-({6-amino-2-[(1S)-1-{[(2S)-2-amino-2-carbamoylethyl]amino}-2-carbamoylethyl]-5-methylpyrimidin-4-yl}formamido)-3-[(3-{[4-(carbamoyloxy)-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl)oxy]-3-(1H-imidazol-5-yl)propanamido]-3-hydroxy-2-methylpentanamido]-3-hydroxybutanamido]ethyl}-1,3-thiazol-4-yl)-1,3-thiazol-4-yl]form-amido}propyl)dimethylsulfanium}.[3,4]

1.2 Overview of Research on Bleomycin

It is important to understand the mechanism and metabolism of Bleomycin, not only for its biochemical mechanism of DNA damage knowledge but also to be able to accurately quantify this significant anticancer drug for clinical use. The most commonly

used Bleomycin sulfate preparation contains two main fractions, as well as chelating copper (II) ions. A clearly understood mechanism and metabolic pathway facilitates the generation and development of clinically relevant treatments and diagnostic methodologies, for example the production of agonists and antagonists that reduce harm to patients from its side effects and moreover analytical methods can also be developed to improve quantification, diagnosis, and therapeutic monitoring all more accurately and precisely.

Bleomycin, a glycoprotein, that is comprised of two sugar rings and a polypeptide chain if four amino acids in length (Fig. 2). Five amino groups confer the molecule's high polarity. It is documented that the anticancer properties of Bleomycin must be activated by incorporation with metal ions and free molecular oxygen.[6] Bleomycin can be activated by a metal binding ions and oxygen molecules enabling it to react with double stranded DNA, specifically with deoxyguanosine-phosphate-deoxycytosine (GpC) and deoxyguanosine-phosphate-thymidine (GpT) residues, which then produces a pseudoenzyme and hydroxide free radicals, thereby cleaving both strands of DNA. Bleomycin hydrolase is the only enzyme that is known to metabolize Bleomycin on its α-amino-carboxylamide group. Bleomycin hydrolase is a cytosolic cysteine proteinase enzyme that is widely distributed in normal tissues except for the skin and lungs, in which case, targets of Bleomycin toxicity cause extreme side effects.

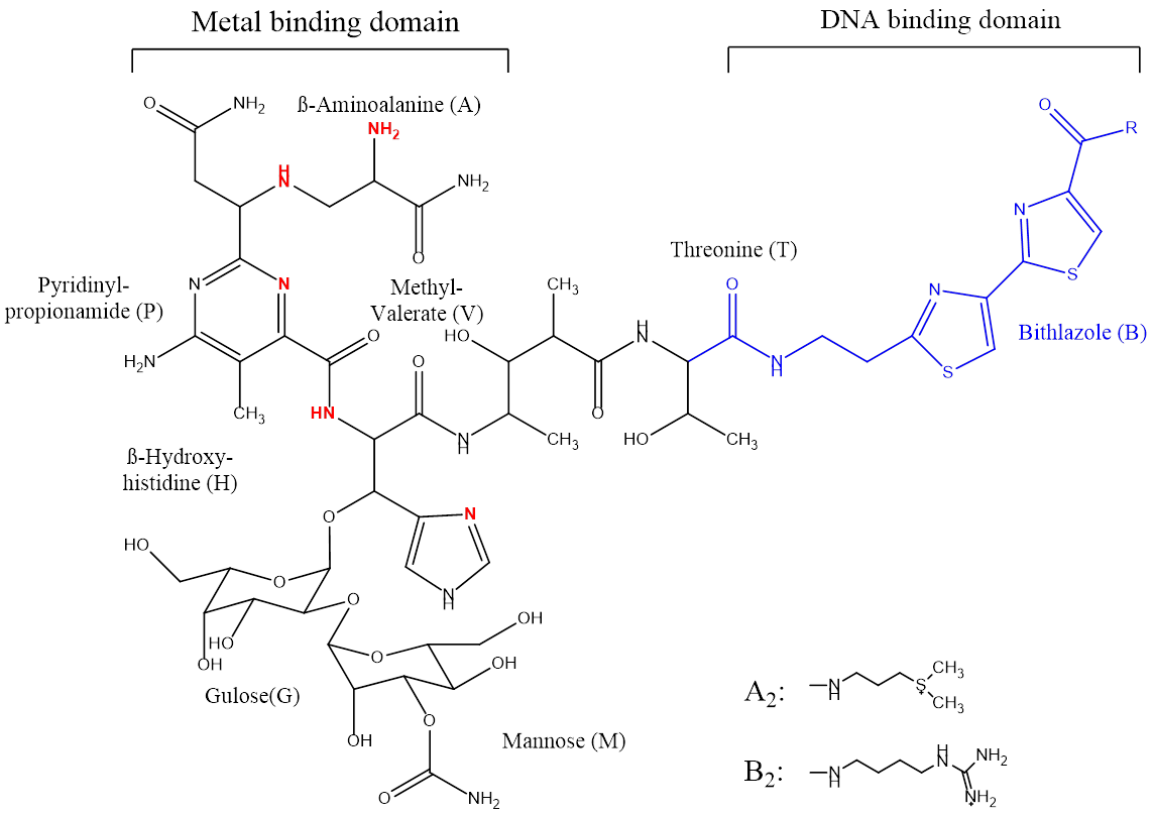

Figure 2. The structural contents of bleomycin sulfate.[5] Bleomycin hydrolase converts Bleomycin to desamido-bleomycin, which presents as its fifth coordinate nitrogen is replaced by a carboxyl group, decreasing the oxygen activating affinity of the Bleomycin-Fe(II) complex resulting in the loss of Bleomycin's activity. In this case, bleomycin's 5'-amine might be the key to control the activity of Bleomycin.

1.3 Mechanism of Action

Bleomycin is a structurally complex compound containing distinct regions that participate in the compound's biological activity. Bleomycin sulfate structure is divided into three domains: Metal-binding domain, two sugar moieties, and DNA binding domain. The metal-binding domain binds a metal ion and an oxygen molecule to activate bleomycin. The sugar moieties facilitate uptake into the cellular membrane for drug delivery. And lastly, the DNA binding domain, a bithiazole, specifically identifies and

binds to deoxyguanosine at the minor groove of both single- and double-stranded DNA, which is the precursor step to cleave DNA strands.

1.3.1 Metal-Binding Domain

In the metal-binding domain, only the deprotonated amide nitrogen binds to the metal biding site.[5] The metal-binding domain can bind a variety of metal ions, however Cu(II) has the highest coordinate affinity and BLM is most often found as a complex with Cu(II). Once the activated bleomycin that binds with Cu(II) is transported into the cells, Cu(II) is converted into Cu(I) by intracellular reductants and then is unbounded from BLM-Cu complex and is converted back to free BLM (Fig. 3).[6] Intracellular Fe(II) binds to BLM on its metal-binding domain at the deprotonated amide nitrogen sites, then the complex is oxidized to BLM-Fe(III)-OOH^-, thereby releasing hydroxide free radicals that cleave DNA. BLM-Fe(III) is then converted to BLM-Fe(II) by intracellular reductants as NADH, ascorbic acid, or 2-hydroxy-1-ethanethiol (Fig. 4).[7,8]

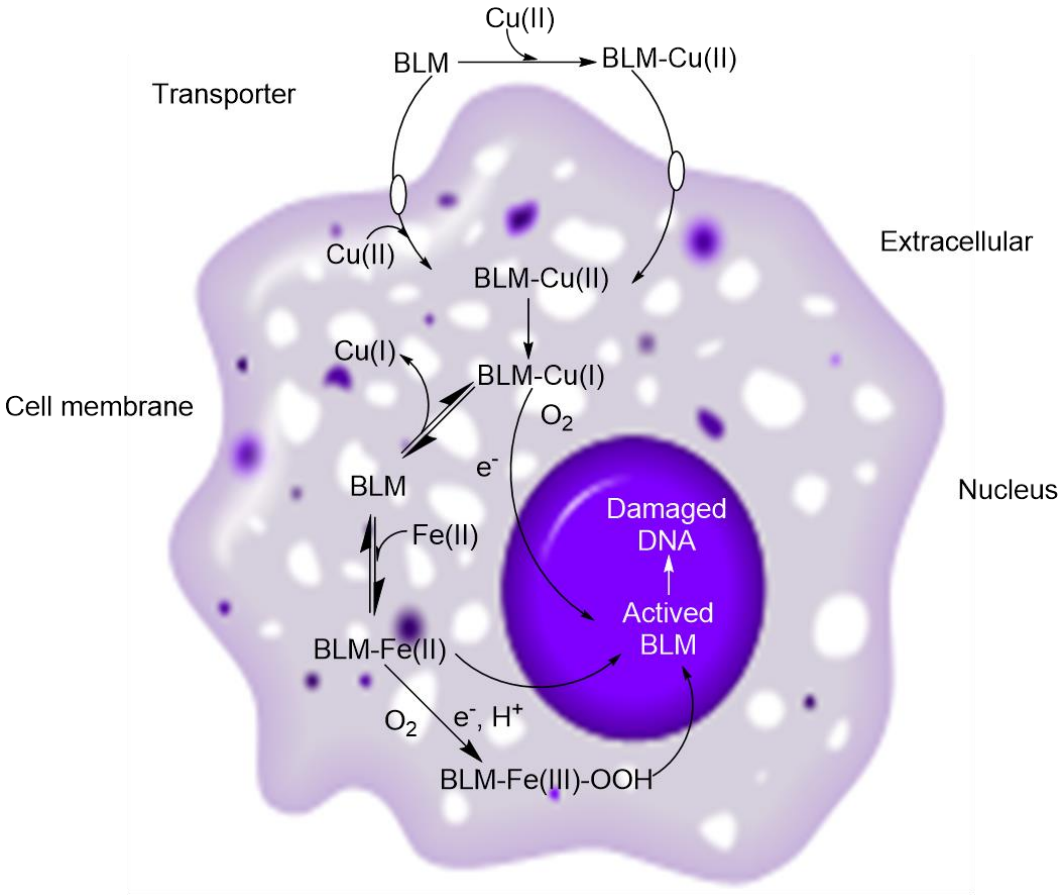

Figure 3. The pathway of bleomycin transportation into the cell and its metal-binding condition. Bleomycin sulfate binds to Cu(II) as an extracellular activation to be transported into the cell. Cu(II) ion is unbound in the cell, and bleomycin then binds to the Fe(II), and then to be oxidized to a BLM-Fe(III)-OOH^- to release hydroxide free radicals to catalyzing DNA cleavage in the cell nucleus.[6,7,8]

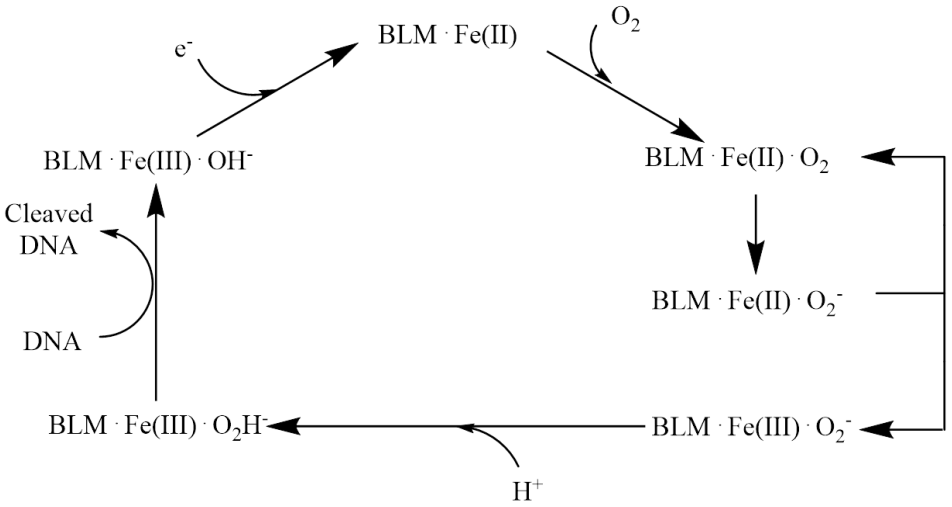

Figure 4. Hypothesis of catalyzing BLM-Fe(II) complex by oxygen.[8]

1.3.2 Sugar

Glucose and mannose form a disaccharide structure that does not directly bind to the DNA. This structure's purpose is to stabilize the BLM-Fe complex, and enhance the drug's uptake into the biological membrane.

1.3.3 DNA Binding Domain

The DNA binding domain is a bithiazole structure, which is the main structure that binds to the DNA. The bithiazole preferentially identifies the Guanine nucleobase of DNA.[8] Bithiazole has properties of aromaticity and coplanarity. Since it identifies Guanine of the DNA, it inserts specifically between both single or double strand deoxyguanosine-phosphate-deoxycytosine and deoxyguanosine-phosphate-thymidine at the minor groove to make an interlayer complex.[9] BLM-Fe-OO extracts a hydrogen atom from the 4' carbon of the guanosine-connecting deoxyribose then reacts with the oxygen free radical to break the chain.[9] The identification of products indicates that there are two pathways that activate BLM-Fe to induce the mechanism of DNA cleavage. When there is excess of oxygen, the 4' carbon hydroperoxide intermediate is produced as base-propenal to break the deoxyribose.[8] In a hypoxic enviornment, the dissociative base can be detected. The bond between 3'-phosphate and 3' carbon of deoxyribose breaks to cleave the chain, and oxygen on the deoxyribose breaks with 4' carbon to produce the dissociated base radical (Fig. 5).[8]

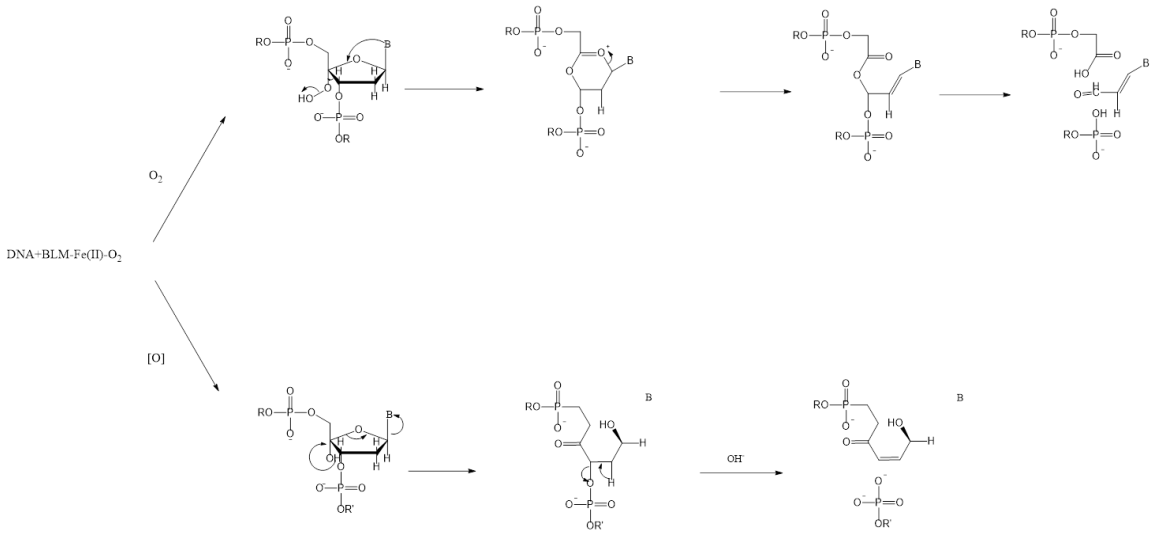

Figure 5. Two pathways that BLM-Fe(II)-O_2 catalyzes DNA cleaverage.[8]

1.4 Metabolism

The metabolism and distribution of BLM hydrolase in an organism's body is the main cause of some adverse side effects including conditions of the lung, fibrosis and rash development. To reduce the side effects, current research groups are investigating the hydrolysis pathway of Bleomycin and toxic effect dosage by employing analytical techniques such as chromatography and spectroscopy with statistical analyses to gain information on target molecule distribution on organs and toxicity.

Bleomycin hydrolase is the only enzyme that inactivates bleomycin. Bleomycin hydrolase is a group of cytosolic cysteine proteinase enzymes that are widely distributed in normal tissue with the exception of the skin and lungs, which causes extremely toxic side effects in these areas which include fibrosis of the lung tissue and rashes.[3] BLM hydrolase metabolizes BLM on its α-amino-carboxylamide to loose and amide to produce product as desamido-bleomycin (desBLM). In desBLM, Fe(II) ion binds to the carboxyl group instead of a nitrogen, thereby decreasing the oxygen activating affinity of this

desBLM-Fe(II) complex, in which case desBLM-Fe(II) complex weakens or loses its ability to release oxygen free radicals to fail on DNA cleavage.

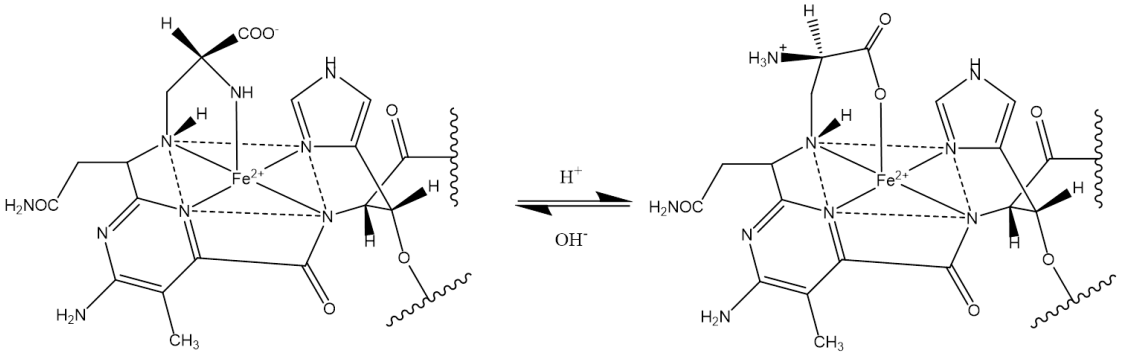

Figure 6. Change of metal-binding site of desamido-bleomycin. BLM hydrolase metabolizes BLM on its α-amino-carboxylamide. Desamido-bleomycin then changes its metal-binding site into the carboxyl group instead of nitrogen, thereby decreasing the oxygen activating affinity of this desBLM-Fe(II) complex.

1.5 Consideration of Bleomycin Quantitative Analysis

Most assays for the quantification of Bleomycin employ techniques with poor sensitivity such as ultraviolet or fluorescent detection or radioimmuno-assays. Current quantification mostly uses liquid chromatography (LC) with fluorescence or ultraviolet (UV) detection. Detector of UV is limited to where saturated organic compound transitions occur. Also, only π-π and p-π conjugated unsaturated esters absorb in the near UV band. The first group to publish a method for the analysis of Bleomycin by HPLC was Shiu and Goehl[10] in 1980 followed by Mahdadi et al.[11] a decade later who published a more sensitive method with a fluorescence detector and ion-paring reverse phase HPLC. These two methods were only validated for the A2 fraction and in addition only achieved a sensitivity of 70 nanograms per milliliter, which is not sufficient for detection in human plasma. In 2012 Mabeta et al.[12] developed a HPLC method for separation and quantification of both A2 and B2 fractions, however this method had several weaknesses,

in that it had a narrow linear range and the actual BLM concentration would exceed their upper limit of quantification.

Mass spectrometry is able to overcome these limitations of other detection methods and is unparalleled in the quantification of small molecule metabolites. Tandem mass spectrometry ensures confidence in quantification and the highest selectivity. The biggest advantage in using MS/MS is no need to consider about separating impurities. The dosage of Blenoxane is about 0.25-0.50 units/kg, and it would reach peak plasma concentration (1-3ug/mL) in 30-60 minutes. The terminal half-life is approximately 3 hours, and about 65% of administered intravenous dosage is excreted in urine within 24 hours. MRM channel has enough sensitivity (pg/mL) to quantify such low level of concentration.

Although several research groups have developed LC-MS methods to determine the concentration of BLM in plasma samples, these methods have low selectivity and would present a problem in the presence of analytes of similar structure. More successful methods such as Galba et al.[13] and Kosjeck et al.[14] rely on high resolution mass analyzers (Q-TOF; quadrupole-time-of-flight) to elucidate quantitative peaks in a complex spectra of many isotopic patterns. Such instruments are not readily available nor widespread in the clinical setting. The methods mentioned also require time consuming sample preparation steps or uncommon chromatography conditions, making the methods impractical. Since there does not exist a simple and accessible method for the quantification of Bleomycin in plasma, development of such method is in need as the ability to quantify the chemotherapeutic drug in plasma is important in determining the

therapeutic window of the drug as well as toxicity in addition to monitoring the efficacy of therapy.

1.6 Aims and Objectives

The objective of this project is to design a method to quantify BLM-Cu complex in plasma, overcoming its low concentration and multiple formations. The purpose of analyzing activated BLM is for its clinical utility and it is thus important to convert all BLM into BLM- Cu^{2+} complex. As same molecular weight with desBLM and BLM, the targets on BLM-Cu^{2+} complex would also eliminate the interference of desBLM to the final quantitation of BLM.

Specifically the methods developed aims to reduce the interference and instability of BLM and increase the sensitivity by transferring all non-metal binding BLM into metal binding BLM. The second aim is to utilize widely-available LC-MS instrumentation that is translatable to the clinical setting, therefore the method was developed using LC-ESI-triple-quadruple mass spectrometer with appropriate conditions.

1.7 Method Development

This method was developed to utilize a quick and efficient extraction technique, liquid-liquid extraction, in order to reduce effect matrix, and improve sensitivity of LC-MS/MS detection, which realizes high sensitivity, high selectivity, and reduced loss of analyte in the process of the analysis. The cost of mass spectrometer is high; however, it satisfies the highest sensitivity and selectivity of the analysis with its MRM channel. The solvent buffer and mobile phases does not contain complex contents, which is easily prepared and remained stable. To extract analytes from the plasma samples, only simplest

protein precipitation by using high concentration of methanol would satisfy high recovery of the analysis.

1.7.1 Liquid-Liquid Extraction

BLM is highly polar and easily dissolved in water and methanol, and there is no effect with the proteins in plasma. In this method, 70% and higher concentration of methanol is prepared for protein precipitation. After ice-dry evaporation and concentration with 20% methanol, the working solutions would remain ＞95% of the original spiked BLM. In comparison with solid-phase extraction, liquid-liquid extraction saves solid-phase column and complicated extraction procedures.

1.7.2 Mass Spectrometry

Mass spectrometry provides qualitative and quantitative information about the atomic and molecular composition of inorganic and organic materials. The mass spectrometer produces charged particles that consist of the parent ion and ionic fragments of the original molecules. The mass analyzer then sorts these ions according to their mass/charge ratio. The mass spectrum is a record of the relative numbers of different kinds of ions and is characteristic of every compound, including isomers. In tandem quadrupole MS, collision induced dissociation in the second quadrupole cell, Q2, and selection of fragments of parent ion in the ion trap is advantageous in the quantitative analysis of compound in solvent.

Electrospray ionization (ESI) is a soft ionization technique that produces ions using an electrospray in which a high voltage is applied to a liquid to create an aerosol. As use of MALDI, ESI is especially useful to produce ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionized. A solution of

macromolecules is sprayed in the form of fine droplets from a glass capillary under the influence of a strong electrical field. The droplets pick up charge as they exit the capillary, and the evaporation of the solvent leaves highly charged molecules. Since droplets “break down” to form spray and evaporate, the charge increases many times until the gas-phase molecules or fragments finally enter the analyzer. The ESI is mostly used to analyze thermo-instable polar macromolecules, and the sample molecules hardly disintegrate during the ionization. Slower flow rate causes more fragments via ionization, furthermore contributing to the high degree of sensitivity. However, ESI limits the molecular structures, which easily cause thermo-dissociation. The chemical noise presents clearly since the molecular mass is low. It is also not suitable for large M.W. molecules which larger than 1000Da, but this can be fixable by changing flow rate. The molecular weight limit is too small for Bleomycin which averages as ~1414Da. However this is overcome by the ability of ESI to form multiple charges. At +2 charges channel, that has satisfied the molecular weight limit and the drug can be detected in the spectrum although the spectrum does not show the singly charged compound.

1.7.3 High-Performance Liquid Chromatography (HPLC)

HPLC is widely used to separate mixed compounds based on their polarities. HPLC consists of liquid mobile phases and a stationary phase that can be reused. It uses high pressure to ensure the mobile phase quickly passes through the stationary phase packed in the column. It has a quicker flow rate and more rapid analysis than traditional liquid chromatography. It is highly effective and highly sensitive to analyze low concentration of analytes (0.01 ng).

The mostly commonly used stationary phase in HPLC is reverse phase columns, which strongly retain polar compounds on the column and eluted with reversed polarity of mobile phases to realize high selectivity. In combination with tandem quadrupole MS, the LC-MS/MS system delivers the highest selectivity of separating compounds from complex biological matrices and with little to no interference from compounds of the same molecular weight as the target analytes. In this method, the column specially used is a SYNERGI^{TM} HYDRO-RP Polar Endcapped C18 Column (Fig. 7), which specializes in the application for extreme retention of non-polar and extremely polar alkyl compounds. The mobile phase A is 20mM of ammonium formate and 0.1% of formic acid and mobile phase B is 0.1% of formic acid in 100% of methanol. The elution appears to be polar with time gradients of 20-60% of mobile phase B.

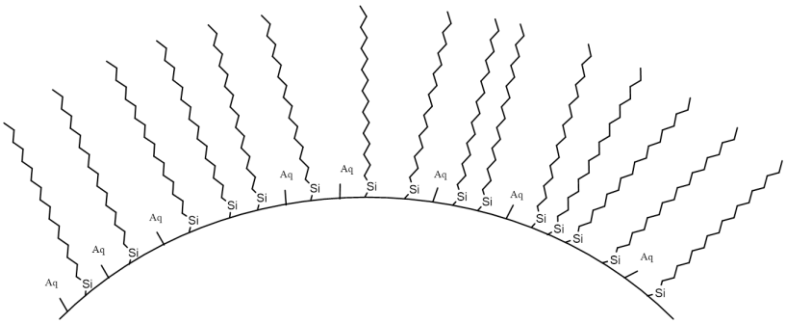

Figure 7. Structure of SYNERGI^{TM} HYDRO-RP Polar Endcapped C18 Column. On the inter-surface of the column, there are C18 and polar site, which presents good retention on both polar and non-polar compounds.[15]

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and Solutions

Blenoxane was purchased from Alexis Biochemicals, in which contains ~67% of BLM A_2 and ~29% of BLM B_2. The internal standard Vancomycin hydrochloride (VCM) was purchased from Sigma Aldrich (St. Louis, MO). Methanol (≥99.9%) and ammonium formate ((≥99%) were purchased from Sigma Aldrich (St. Louis, MO). Water is purified using the Millipore Milli-Q system (Milford, MA). Formic acid was purchased from Fluka.

Mobile phase A is prepared by dissolving ~0.63g of ammonium formate and 1mL of concentrated formic acid into 1L of DI-water, which is prepared as 20mM of ammonium formate and 0.1% formic acid. Mobile phase B is prepared as 0.1% formic acid by dissolving 1mL of formic acid into 1L of 100% methanol.

2.2 Sample Preparation

Two groups of rat plasma were generously provided by a research group at Cleveland Clinic Lerner Research Institute. In 2012, the lab in Cleveland Clinic Lerner Research Institute commenced a project that injecting BLM into the lungs and bronchia of rats, and records the condition of lung fibrosis by timekeeping. Eight rats at an age of

eight weeks old were injected with 25mg of BLM into lungs and bronchia while the other six were kept as controls. After 14 or 28 days, plasma samples were collected and 0.5mL/L EDTA was treated. All plasma samples had been kept at -80℃ until obtained at April 2016. The obtained 50mg of BLM standards were dissolved in DI-Water and stored in -80 degree in Celsius, which caused a problem of sample degradation for subsequent stability tests. BLM is a highly polar compound, which has high solubility in water and methanol, and neither would ensure that it would retain its stability stored in solvent.

2.3 Calibration Curve Standard Preparation

BLM is very toxic and is delivered as a powder, so it is not suggested to prepare the solutions by weighing. The BLM analyte standard consisted of 50 mg of powder, and was directly dissolved in 1mL of DI-water. At the temperature of -80℃ for storage, water is more stable than that in methanol. As high concentration of methanol would need to precipitate proteins in plasma, further stock solutions would be prepared in 100% methanol. From the 1mL of 50mg/mL stock solutions, 50μL was vortexed and mixed with 49.95mL of 100% methanol to prepare a concentration of 50μg/mL secondary stock solution. From the secondary stock solution, 500μL was vortexed and mixed with 49.5mL of 100% methanol to prepare a concentration of 500ng/mL stock solution. 20mL from the 500ng/mL stock solution was vortexed and mixed with 30mL of 100% methanol to prepare a concentration of 200ng/mL stock solution, and from which 5mL was vortexed and mixed with 45mL of 100% methanol to prepare a concentration of 20ng/mL stock solution. 500ng/mL, 200ng/mL, and 20ng/mL stock solutions were stored at -20℃ until use.

0.5 mg of the internal standard VCM was vortexed and mixed with 1mL of 100% methanol to prepare the 0.5mg/mL main stock VCM solution, and from which 0.5mL was vortexed and mixed with 49.5mL of methanol to prepare the 5μg/mL secondary solution. All these stocks were stored at -80℃. 2mL of the 5μg/mL stock was vortexed and mixed with 48mL of methanol to prepare 200ng/mL of VCM stock, and which was stored at -20℃ until use.

As checked common bio-clinical treated plasma, there usually is 1.5-1.8mg/mL of EDTA treated, which is transferred as 4mM. The provided plasma samples were treated by 0.5M of EDTA. However, only to consider of the chelation between Cu^{2+} and EDTA (if there is any) by 1:1 ratio, 4mM of EDTA was prepared by dissolving 75.0mg of EDTA (molecular weight of 372.24g/mL) in 50mL of DI-water.

2mM of $CuSO_4 \cdot 5H_2O$ (molecular weight of 249.677) was prepared by dissolving 25mg of solid $CuSO_4 \cdot 5H_2O$ in 50mL of DI-water.

To test selection and sensitivity of the method, the calibration standards were prepared as the final concentration of BLM from 1ng/mL, 5ng/mL, 10ng/mL, 20ng/mL, 50ng/mL, 100ng/mL, and 200ng/mL. In the test period, plasma was replaced by DI-water to save the cost of plasma. To ensure the accuracy of concentration prepared, three BLM stock solutions were used to prepare corresponding standards. Final concentration of VCM was 40ng/mL. The solvent was prepared as 70% methanol. The calibration curve standards were prepared as shown in Table I.

BLM Stock conc. (ng/mL)		Blank Plasma or DI-water (μL)	2mM $CuSO_4$ (μL)	100% Methanol (μL)	VCM (200ng/mL) (μL)	BLM (μL)
	Double Blank	32	16	112		
	Blank	32	16	80	32	
20	1ng/mL	32	16	72	32	8
	5ng/mL	32	16	40	32	40
	10ng/mL	32	16	0	32	80
200	20ng/mL	32	16	64	32	16
	50ng/mL	32	16	40	32	40
	100ng/mL	32	16	0	32	80
500	200ng/mL	32	16	16	32	64
20	LQC (2.5ng/mL)	32	16	60	32	20
200	MQC (20ng/mL)	32	16	64	32	16
500	LQC (160ng/mL)	32	16	28.8	32	51.2

Table I. Calibration Curve Standards Preparations. Concentration of 70% methanol was used to precipitate proteins from plasma. In the future research and method optimization, each standard would be ice-dried and dissolved in 20% methanol for instrumentation injection and detection.

2.4 Protein Precipitation

All plasma samples were stored at -80℃ until use. After the working standards were prepared, they were vortexed for 1 minute, and then centrifuged at 14,000 rpm for 15 minutes. Transferred 140μL of supernatant into vials with inserters and sent to the HPLC auto sampler for injection.

In the future research of optimization, each unit of supernatant was ice-dried and dissolved in 40μL of 20% methanol. Under this condition, the contents of standards preparation were changed. Prepared standards with concentrations of 1ng/mL, 20ng/mL, 50ng/mL, 100ng/mL, and 500ng/mL in 100% methanol. The final methanol

concentration was changed to 80% to precipitate plasma proteins. The optimized standards were prepared as shown in Table II.

	Blank Plasma or DI-water (μL)	2mM $CuSO_4$ (μL)	100% Methanol (μL)	VCM (200ng/mL) (μL)	BLM (μL)
1ng/mL	32	16	160	32	2
20ng/mL	32	16	160	32	2
50ng/mL	32	16	160	32	2
200ng/mL	32	16	160	32	2
500ng/mL	32	16	160	32	2

Table II. Optimized Standards Contents. This preparation plan was to optimize the protein precipitation and tried to higher the sensitivity of the method. However, in case of BLM stability reduce, it would not be used to make a calibration curve.

2.5 HPLC-MS/MS Instrumentation

The instruments used were a HPLC and tandem quadrupole mass spectrometer system. The mass spectrometer was 5500 QTRAP triple quadrupole, tandem mass spectrometer (AB Sciex, Toronto, Canada). The high-performance liquid chromatography (HPLC, Shimadzu, Columbia, MD, USA) was composed of two LC-30 AD pumps, DUG-20A3R inline degasser, a SIL-30 AC autosampler, a CBM-20A controller and a CTO-10AVP column oven (Shimdazu, Tokyo, Japan).

2.6 HPLC-MS/MS Optimization Parameters

High-performance liquid chromatographic separation was carried using a Synergi 4μ Hydro-RP 80A (50×2.00mm, 4 micron). The parameters of HPLC and MRM channel are shown in the Table III, IV, and V below.

Shimadzu LC System Equilibration Time	10.00 min
Shimadzu LC System Injection Volume	5.00 µL
Pump	
Pumping Mode	Binary Flow
Total Flow Rate	0.2 mL/min
Pump B Concentration	5%
Oven	
Temperature	40℃
Time Program	
0.01 min	Pump B Concentration 20%
2.00 min	Pump B Concentration 50%
9.50 min	Pump B Concentration 60%
9.51 min	Pump B Concentration 2%
10.00 min	Stop

Table III. Shimadzu LC System Parameters.

	Q1 m/z	Q3 m/z	Collision Energy Start	Collision Energy Stop
BLM A_2	739.0	707.7	40	40
BLM B_2	744.3	551.7	45	45
VCM	725.9	100.2	47	47

Table IV. MRM Fragmentation Transition in Positive Ion Mode.

Ion Source	Turbo Spray
Curtain Gas (CUR)	40.0
Collision Gas (CAD)	High
IonSpray Voltage (IS)	4500.0
Temperature (TEM)	650.0
Ion Source Gas 1 (GS1)	31.0
Ion Source Gas 2 (GS2)	31.0
Declustering Potential (DP)	60.0
Entrance Potential (EP)	10.0
Collision Cell Exit Potential (CXP)	13.0

Table V. Optimized Mass Spectrometry Parameters.

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Method Application

Method validation according to FDA guidelines was not completed for this method. However, based on the completed method optimization, this method can quantify at least 0.2ng/mL of analyte concentration in plasma and standard calibrations curves with a correlation coefficient above 0.99 indicate the method indeed satisfies the requirements. The sensitivity is improved by solvent buffer optimization to increase injection volume and a concentration process to satisfy lower concentration analysis. As the BLM-Cu complex is more active in the mechanism of action, an appropriate amount of $CuSO_4$ is added to enhance the components of BLM-Cu. One research group published an HPLC/MS/MS method to quantify Vancomycin, and in which mentioned to consider about using BLM as internal standards.[16] This study used Vancomycin as the internal standard for BLM quantification successfully. The structure of Vancomycin is a glycoprotein, in which there are two sugar residues just like BLM. Also there lots of aromatic rings just like BLM. So the structures are very similar, which makes a good choice to make as an internal standard.

3.2 Mass Spectrometry Results

3.2.1 Mass Spectrometry Q1 Infusion – BLM and VCM

In the Blenoxane that was obtained from Alexis Biochemicals, the main compositions are BLM A_2 (67%) and BLM B_2 (29%). The other compositions are shown in Table VI below.

Bleomycin A_2	67%
Bleomycin B_2	29%
Demethylbleomycin A_2	0.3%
Bleomycin B_4	0.03%
Other Related Substances	3.5%
Copper	109ppm
Loss on Drying	2.5%
Product Description	Isolated from *Streptomyces verticillus*

Table VI. Compositions in Blenoxane.

The primary Q1 scan is shown in Figure 8 below.

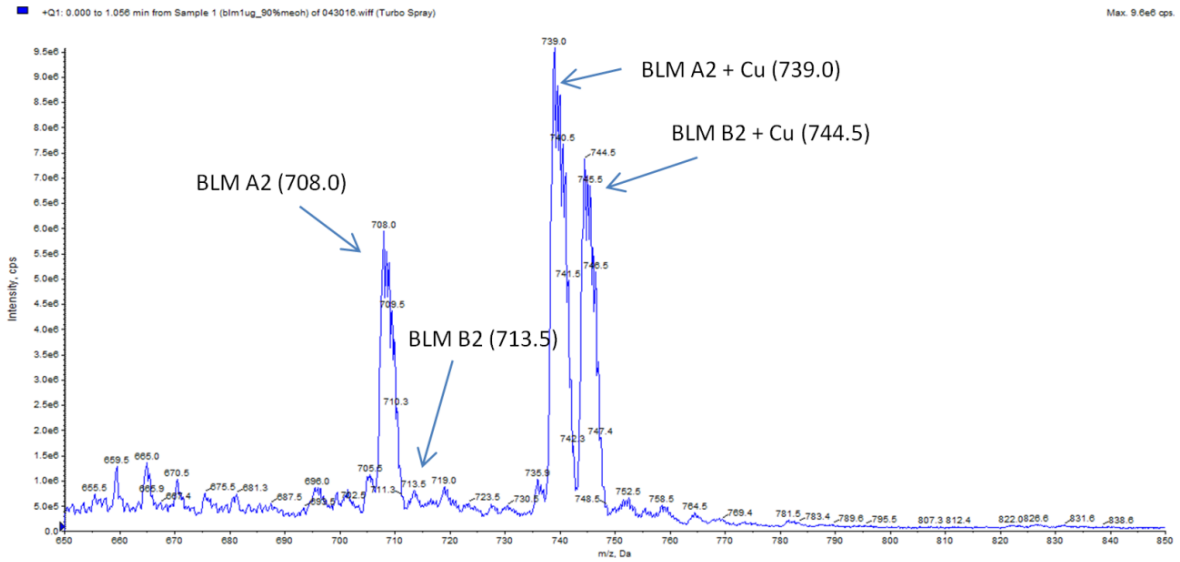

Figure 8. 1µg/mL BLM in 90% Methanol Q1 Scan. The B2 fraction, which is the more relevant biological form, in the spectra chelated with spiked excess of Cu(II) reduces the signal interference as it is almost completely converted to the copper-complex.

The molecular weight of BLM A_2 is 1414.52 and BLM B_2 is 1424.56. To chelate with Cu^{2+}, the corresponding molecular weights change to 1478.066 and 1489.206. The

m/z limit of the Qtrap instrument is 1250, therefore, the BLM A_2 and B_2 are present as their m/z of bivalence.

	m/z
BLM A_2	708.0
BLM B_2	713.5
BLM A_2 – Cu^{2+}	739.0
BLM B_2 – Cu^{2+}	744.5

Table VII. m/z of Each BLM Fractions in Spectrum with Q1 Channel.

According to the spectrum, there is BLM A_2 without chelating with Cu^{2+}, which explains that BLM B_2 has stronger chelation with Cu^{2+}. In the future optimization, excess of Cu^{2+} from $CuSO_4$ would be added to the standard preparation to transfer all BLM to BLM-Cu as much as possible.

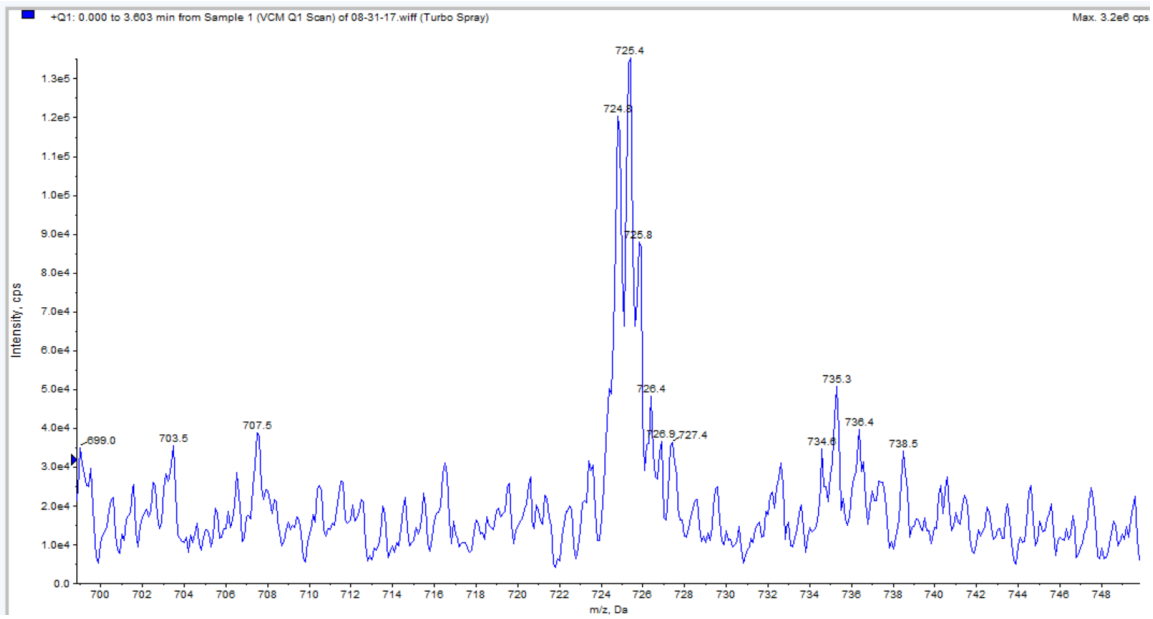

Figure 9. 500ng/mL VCM (725.4) in 70% Methanol Q1 Scan.

3.2.2 Mass Spectrometrum Q1 Infusion – BLM and Cu Chelation

According to the spectrum, BLM A_2 has mostly transferred to BLM A_2 – Cu chelation. However, to consider if there is reversible reaction on this chelation, a pair of comparative Q1 spectrums with time differentiation is shown in Figure 11 and Figure 12.

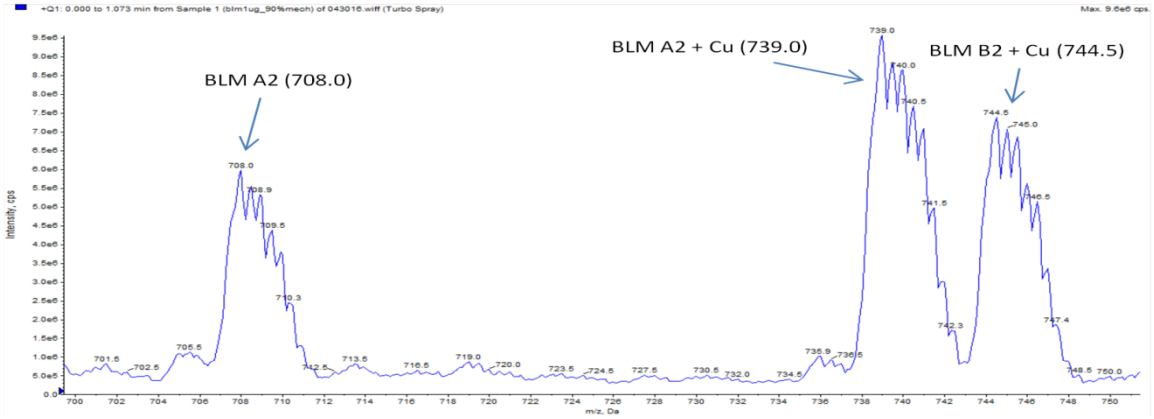

Figure 10. BLM Q1 Scan without adding Excess of Cu^{2+}.

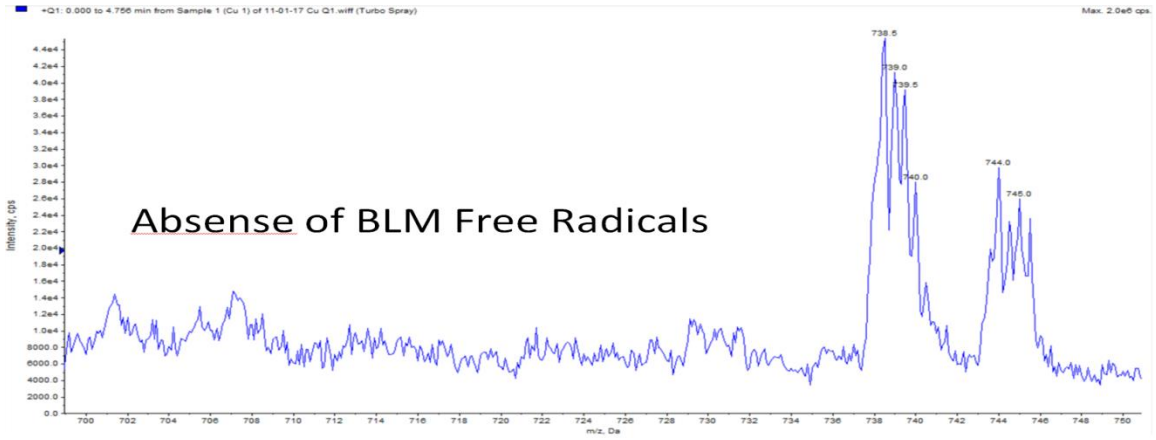

Figure 11. Excess of Cu^{2+} and BLM with Molecular Ratio of 50:1 at 3 minutes after Preparation.

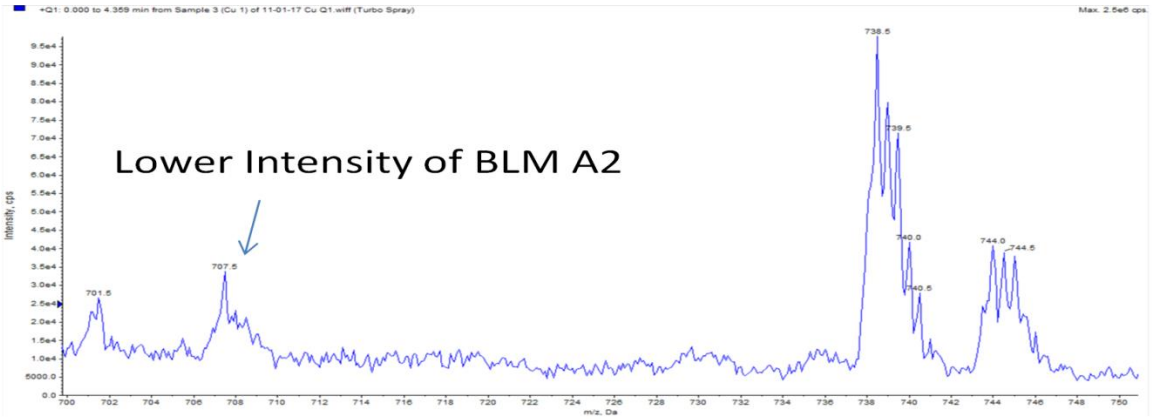

Figure 12. Excess of Cu^{2+} and BLM with Molecular Ratio of 50:1 at 15 minutes after Preparation.

In Figure 11 and Figure 12, it illustrates that the chelation between BLM and Cu has reversible possibility. At 3 minutes after the sample preparation, almost all BLM had been transferred into BLM – Cu complex. In the future study, the reaction condition is able to study, in which the temperature and pH value are changeable in primary.

3.2.3 Mass Spectrometry Q1 Infusion – EDTA Interference

As all plasma samples are treated with EDTA, and EDTA might chelate metal ions to interfere the result of analysis, it is necessary to research if EDTA functions on chelation with Cu^{2+} in this method.

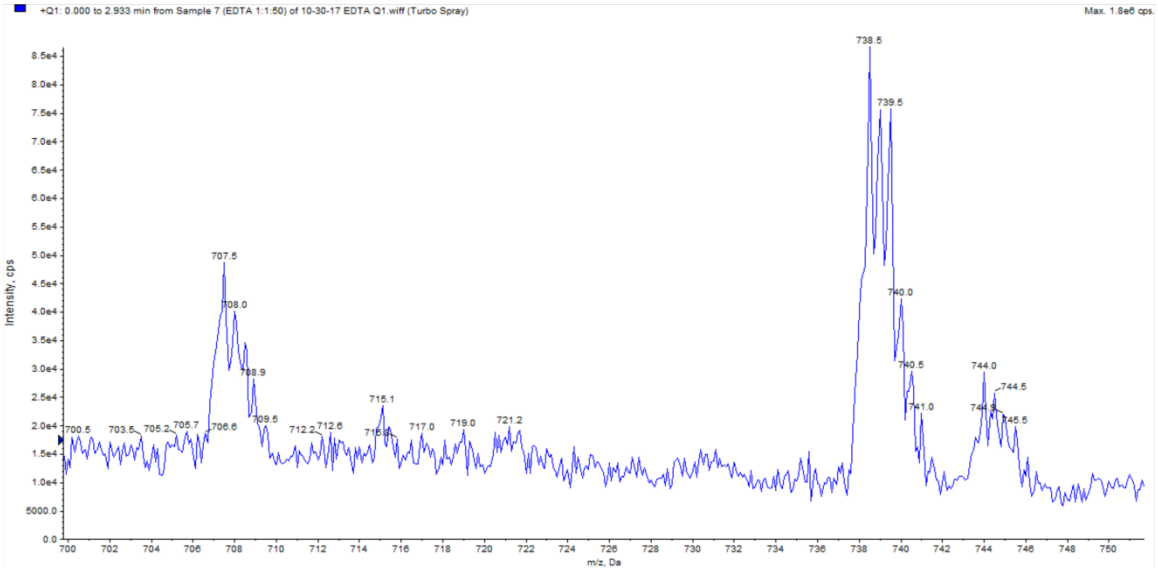

Figure 13. EDTA and BLM with Molecular Ratio of 50:1.

According to the spectrums in Figure 10 and Figure 13, EDTA is rarely interfering the result. However, as BLM B_2 is more active to chelate Cu than A_2, the signal of BLM B_2 – Cu is less than expected presence. There is no BLM B_2 present in the spectrum, so that it is hard to explain whether EDTA is interfering B_2.

3.2.4 Mass Spectrometry Q2 Fragmentation – BLM and VCM

To identify the highest signals of fragments for BLM A_2 and B_2, two different collision energies were used for each of those fractions. The chosen fragment needs to be stably present to different collision energy, which indicates its stability and reliability.

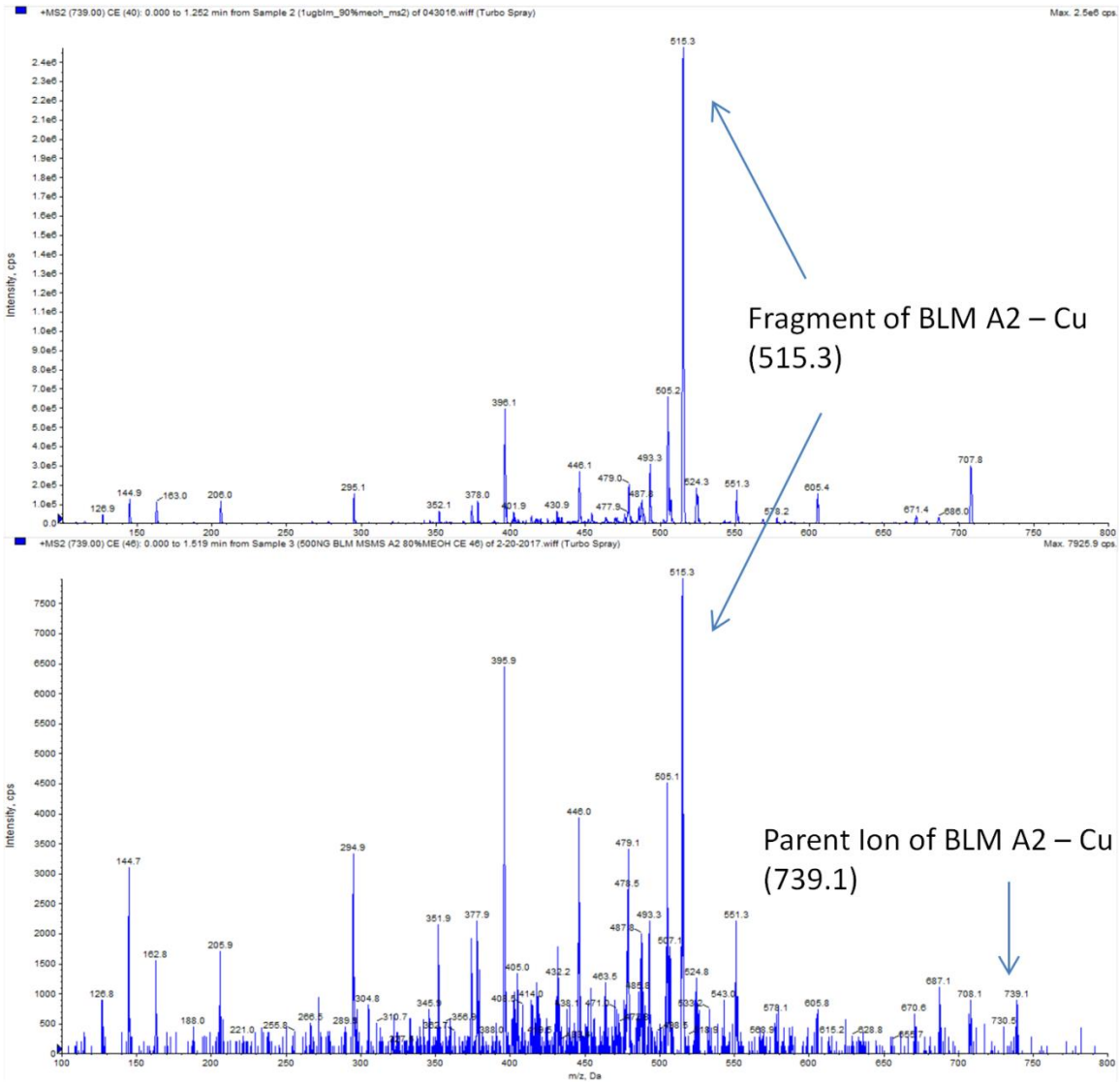

Figure 14. Fragmentation of BLM A_2 with Collision Energy of 40 and 46.

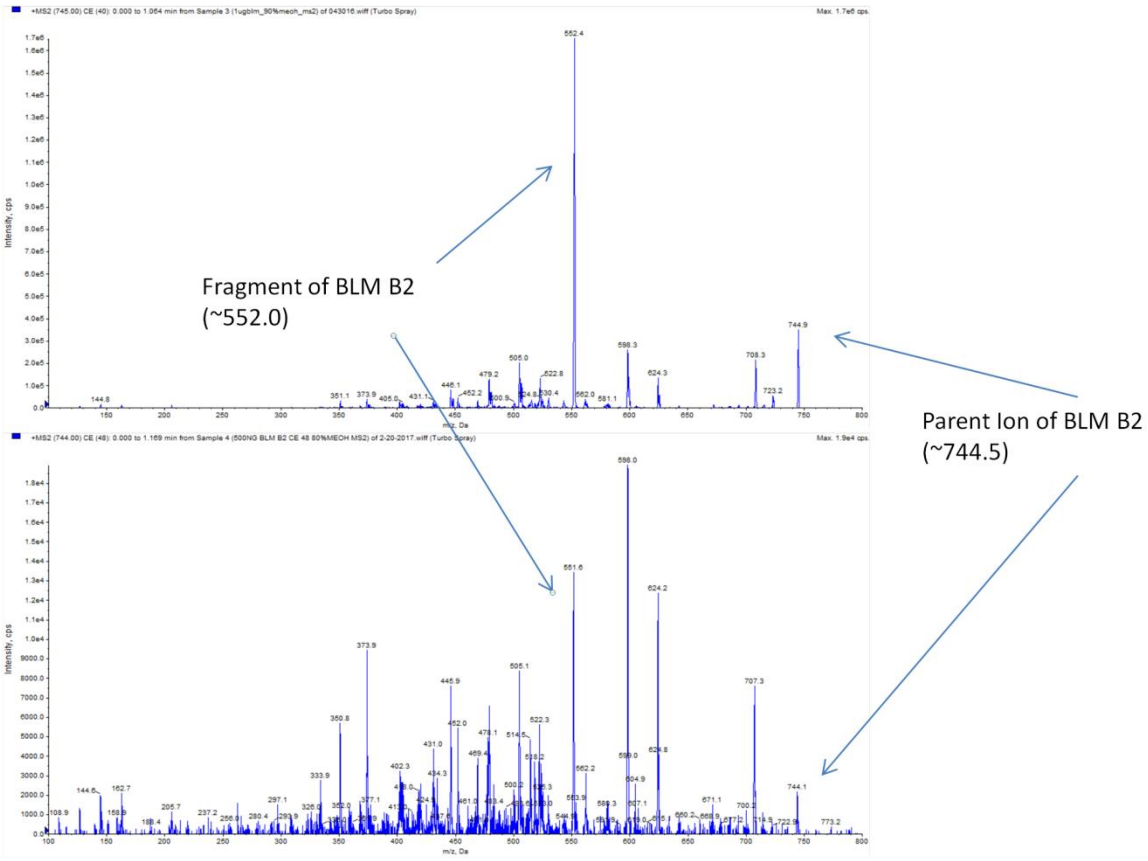

Figure 15. Fragmentation of BLM B_2 with Collision Energy of 40 and 48.

According to Figure 14 and Figure 15, the MRM transition of BLM A_2 was m/z 739.0 → 515.3, and BLM B_2 was m/z 745.0 → 552.0. However, in a following LC-MRM test, there was a stronger signal of transition of BLM A_2 was m/z 739.0 → 708.0.

3.3 HPLC-MS/MS Results

3.3.1 Selection of HPLC Column

As described before, BLM and VCM are polar compounds. Therefore, the best column for the HPLC analysis is a reverse phase column, and the mostly common choice would be a C18 column. However, according to a HPLC-MS/MS test (Figure 16), C18 present peaks with obvious tailing issue, and the retentions were around 1 min, which would easily be indicated as an interference of solvent peak or impurities peak. In this

method, the final choice was a Synergi 4µ Hydro-RP 80A (50×2.00mm, 4 micron) column, from which the spectrum present good shapes of the peaks and retentions were all after 2 minutes (Figure 17).

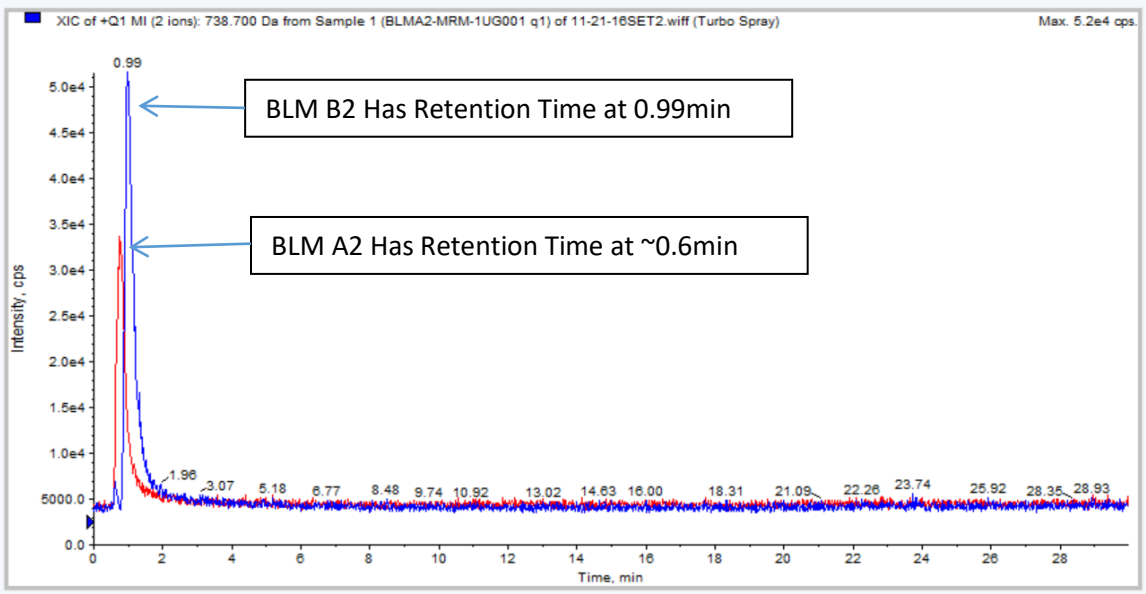

Figure 16. HPLC Chromatogram of BLM A_2 and B_2 Using a C18 Column.

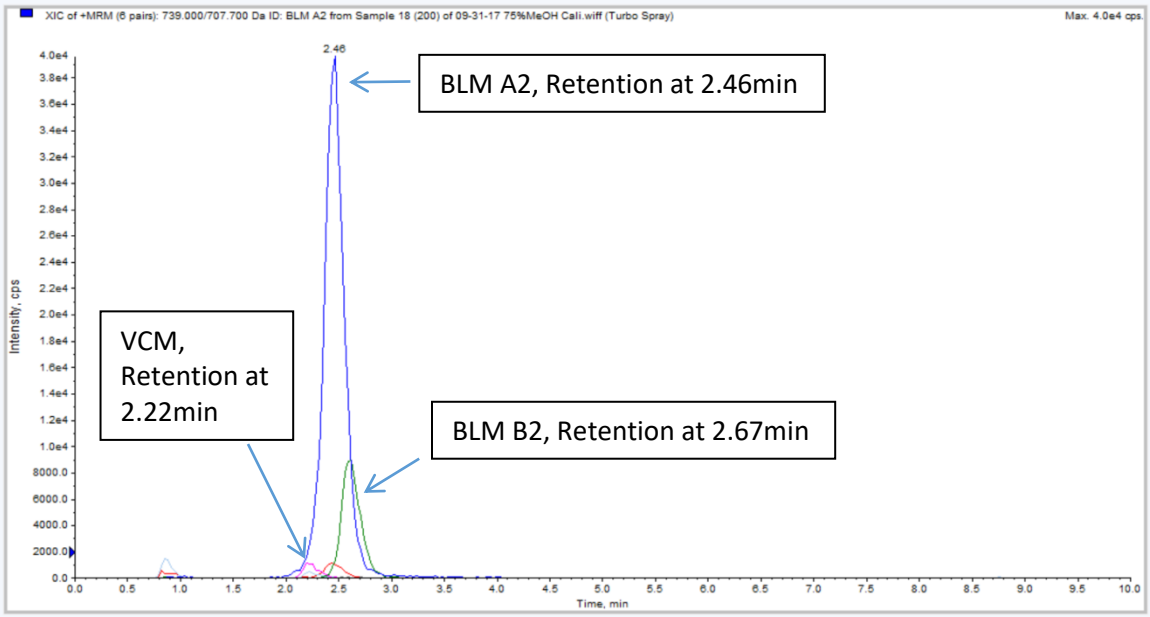

Figure 17. HPLC Chromatogram of BLM A_2 and B_2 Using a Synergi Hydro-RP C18 Column.

3.3.2 Methanol Percentage in the Sample

Calibration standards solvent has the ability to affect the retention and elution of analytes subjected to HPLC. To make the standard preparation simple, the calibration standards were prepared in methanol and DI-water plus Cu^{2+} in addition. Previous research in the literature used 75-90% methanol as the HPLC solvents with their C18 columns.[4, 17, 18] However, in this method with 5µL of injection volume, it appeared to be difficult to retain by using 70% methanol, which is shown in Figure 18. In Figure 18, it shows that all analytes and internal standard indicate on retention time of 0.87 minutes. As use of 20% methanol in Figure 19, the problem of retention has been solved, even when changed to a higher volume injection of 20µL.

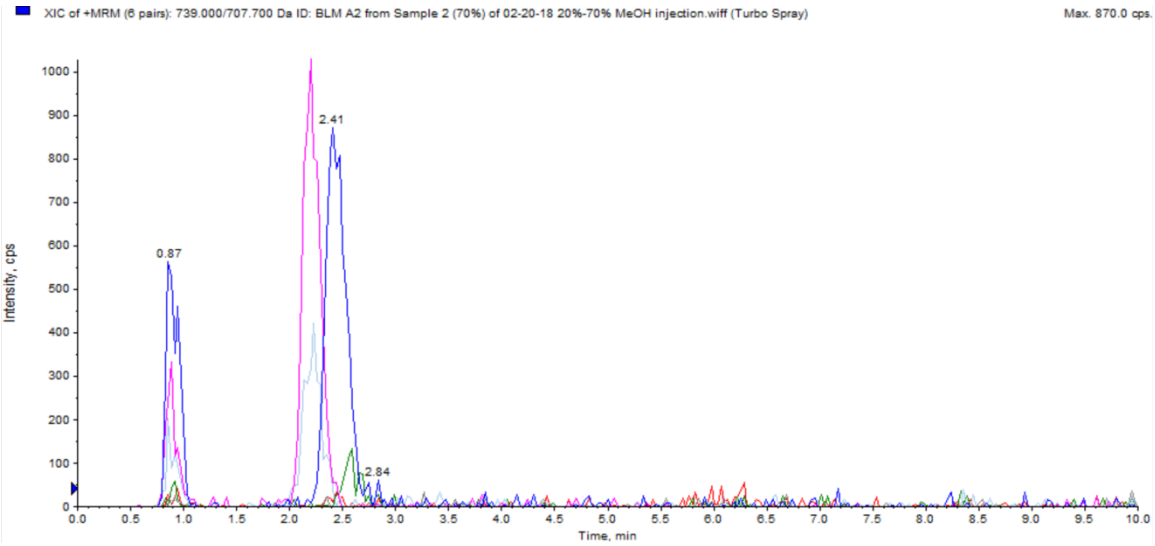

Figure 18. HPLC Chromatogram of 200ng/mL BLM in 70% methanol.

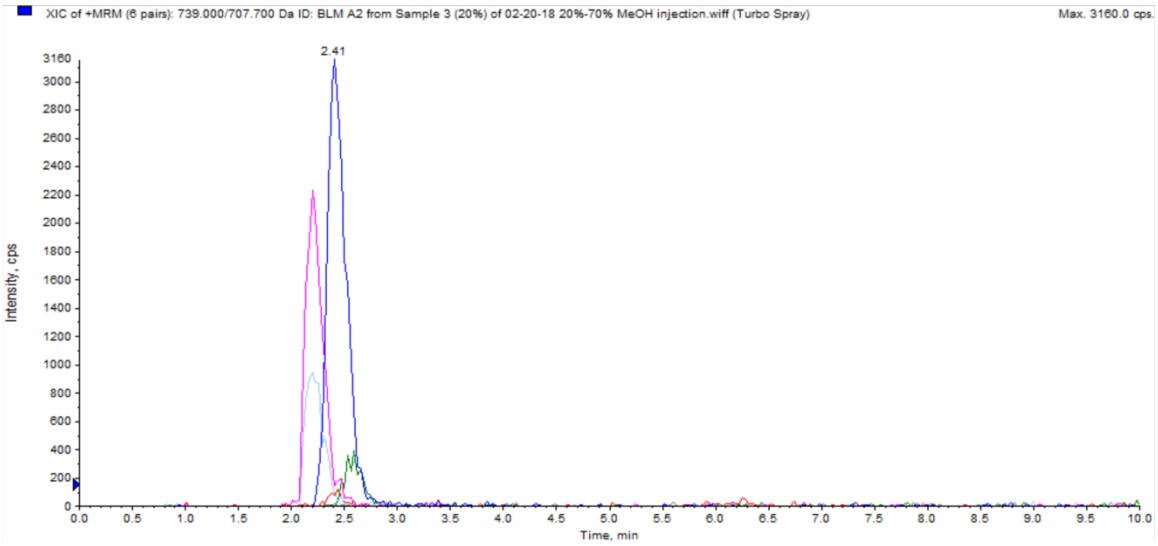

Figure 19. HPLC Chromatogram of 200ng/mL BLM in 20% methanol.

3.3.3 Mass Chromatograms of Standards Preparation from Table I

The standards were prepared according to Table I, and then protein was precipitated. 100µL aliquots of the supernatant were ice-dried, and 100µL of 20% methanol was added to dissolve. The resulting mass chromatograms are shown in Figures 20-26. BLM A_2 had a retention time of 2.4 minutes, BLM B_2 had a retention time of 2.7 minutes, and VCM had a retention time of 2.2 minutes.

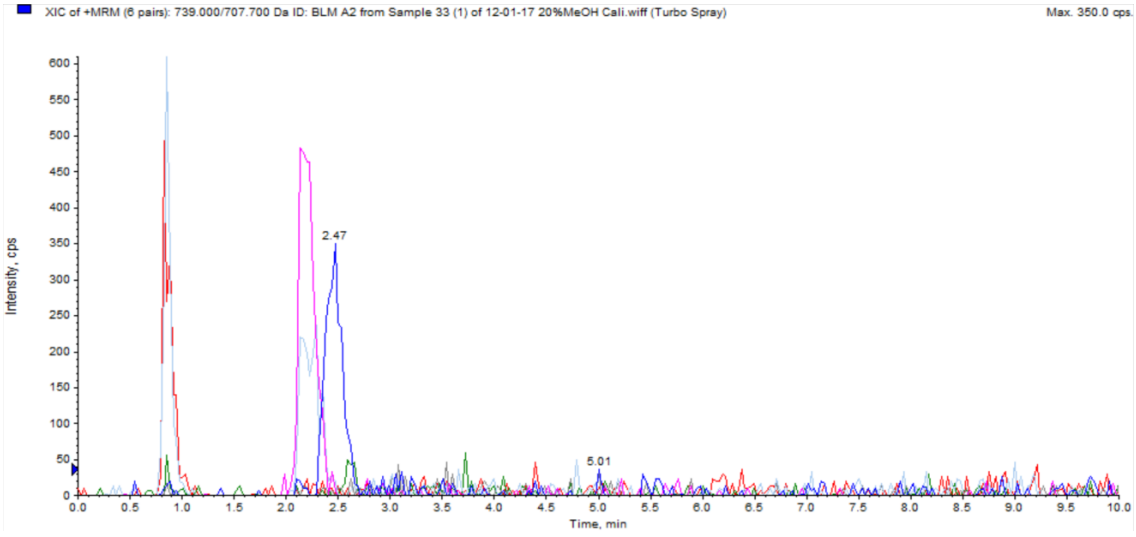

Figure 20. Mass Chromatogram of 1ng/mL of BLM and 40ng/mL of VCM in Plasma.

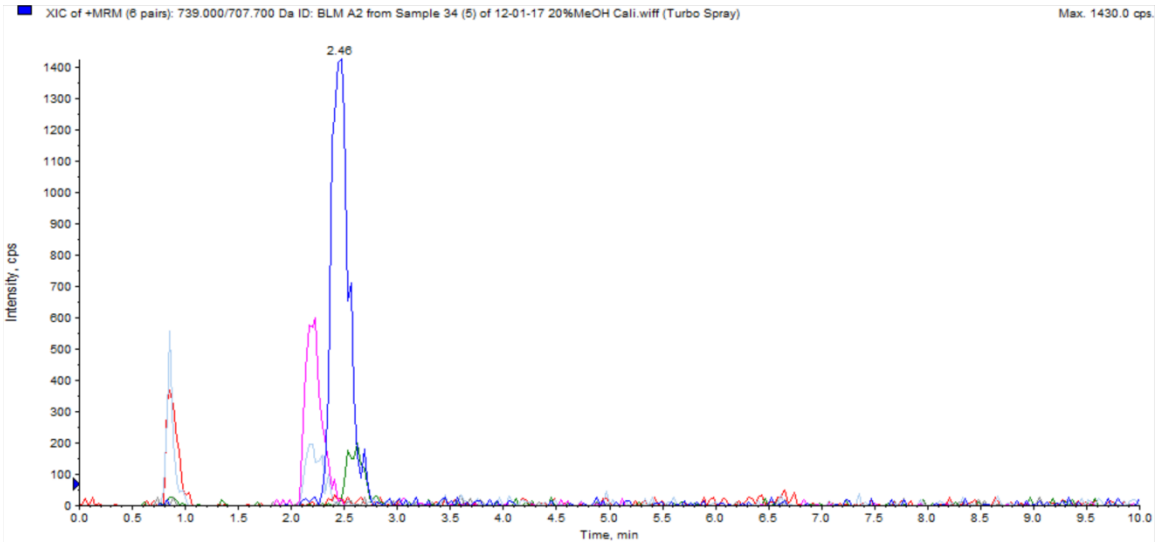

Figure 21. Mass Chromatogram of 5ng/mL of BLM and 40ng/mL of VCM in Plasma.

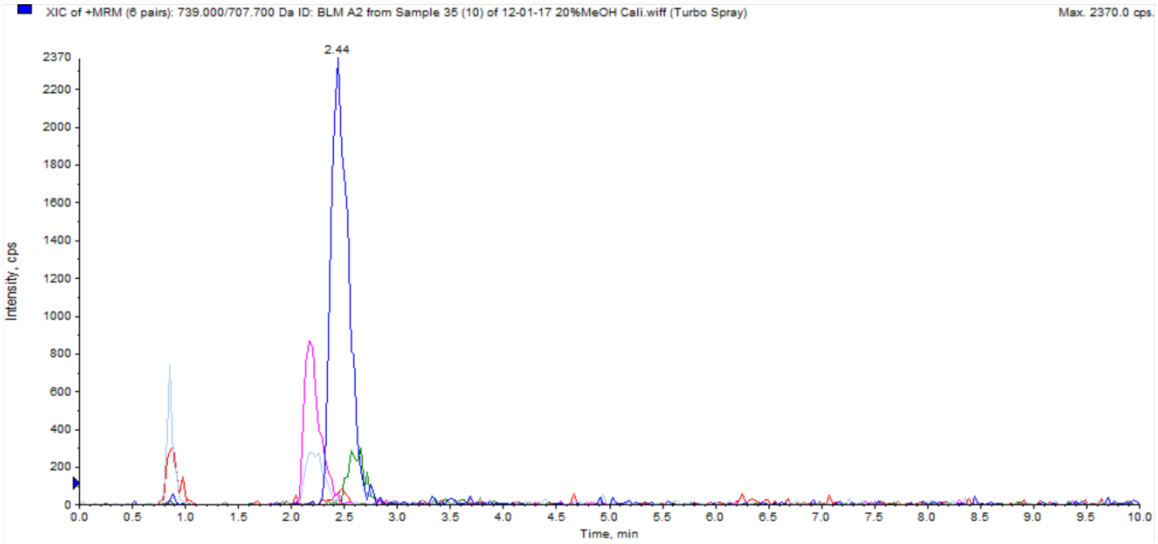

Figure 22. Mass Chromatogram of 10ng/mL of BLM and 40ng/mL of VCM in Plasma.

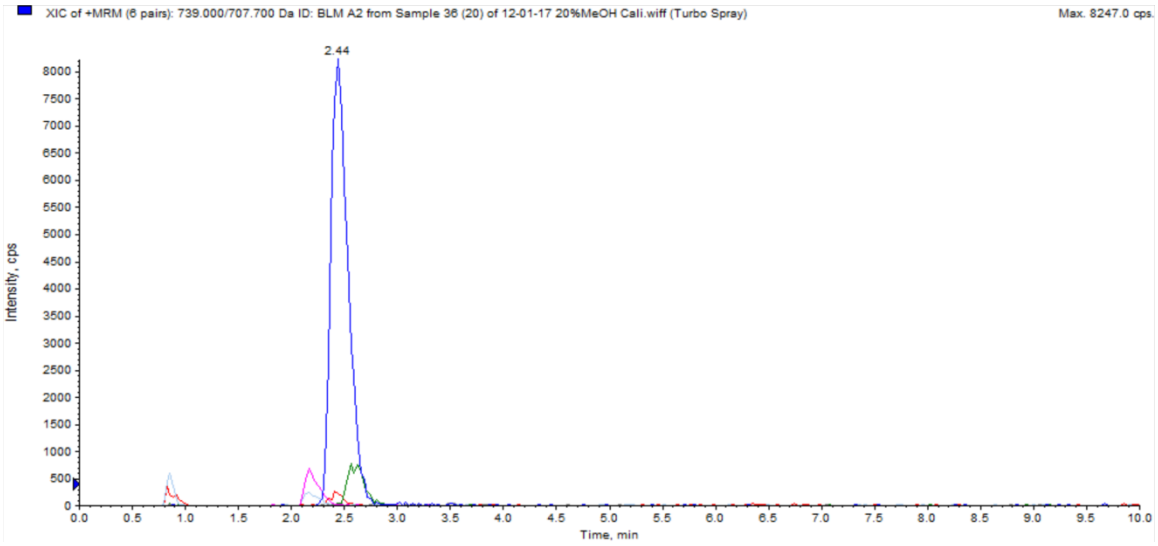

Figure 23. Mass Chromatogram of 20ng/mL of BLM and 40ng/mL of VCM in Plasma.

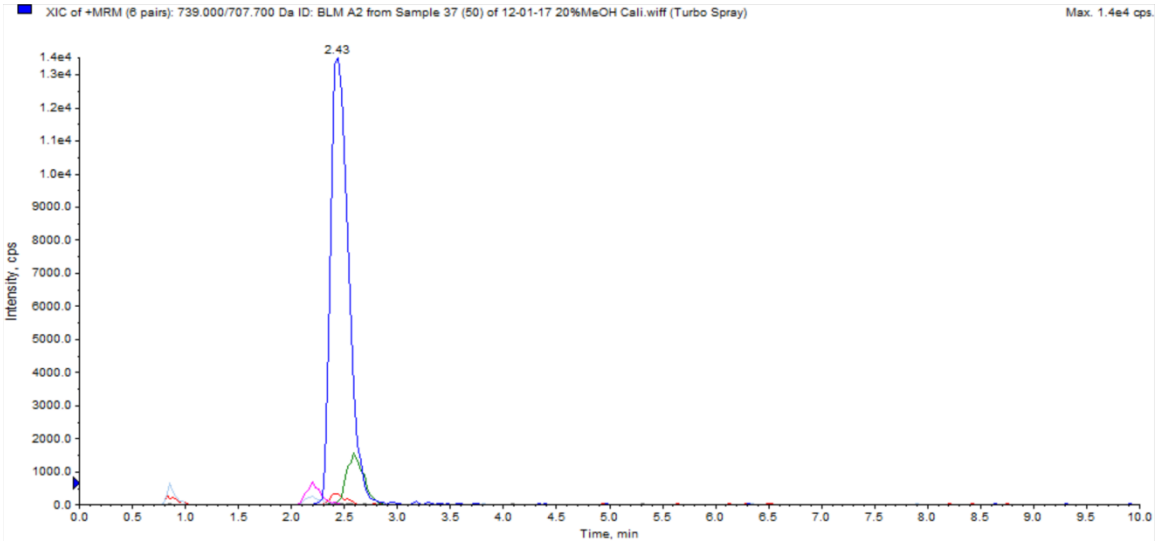

Figure 24. Mass Chromatogram of 50ng/mL of BLM and 40ng/mL of VCM in Plasma.

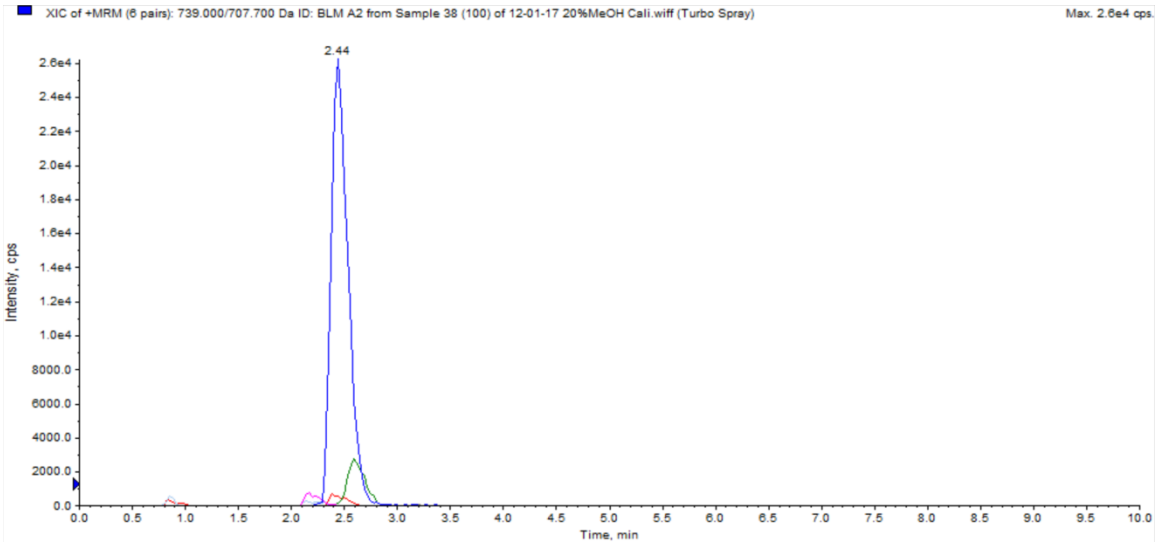

Figure 25. Mass Chromatogram of 100ng/mL of BLM and 40ng/mL of VCM in Plasma.

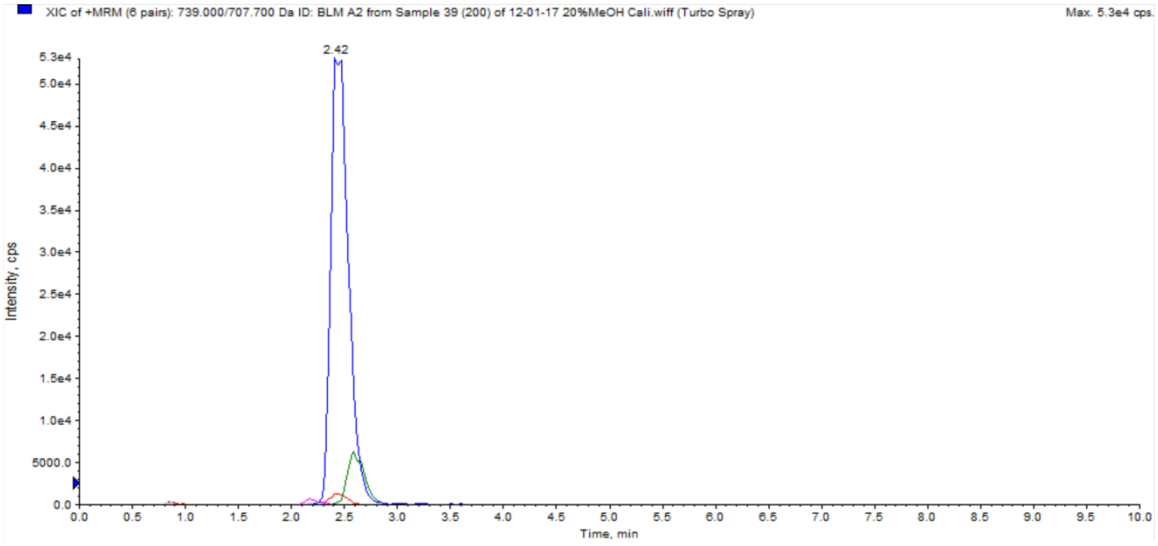

Figure 26. Mass Chromatogram of 200ng/mL of BLM and 40ng/mL of VCM in Plasma.

3.3.4 Calibration Curve of Standards Preparation from Table I

The calibration curves were constructed using seven calibration standards. The linear calibration range was between 1-200 ng/mL. The calibration curve for rats' plasma spiked with BLM and VCM is shown in Figure 27 and 28.

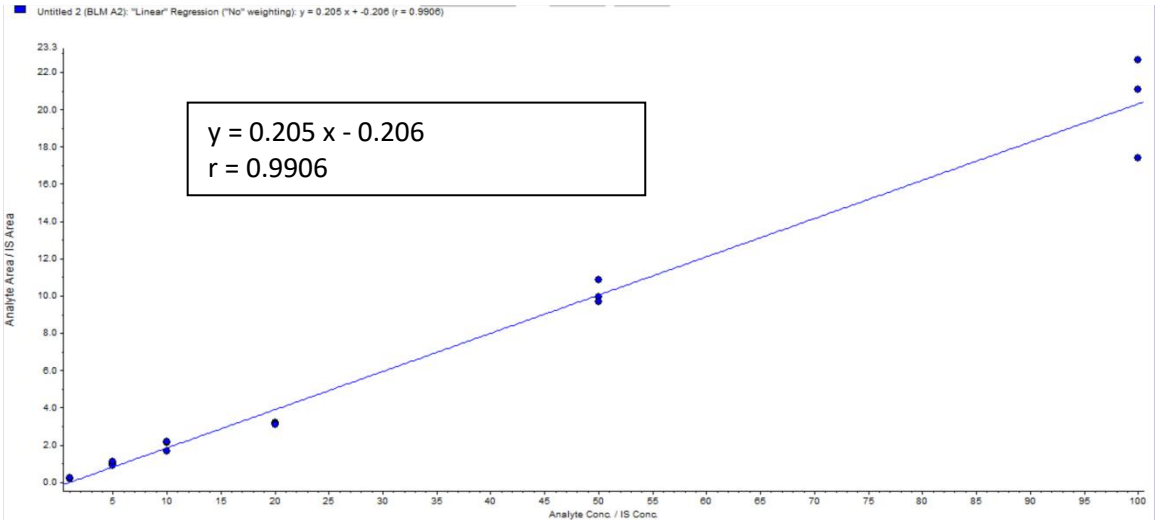

Figure 27. Calibration of BLM A_2.

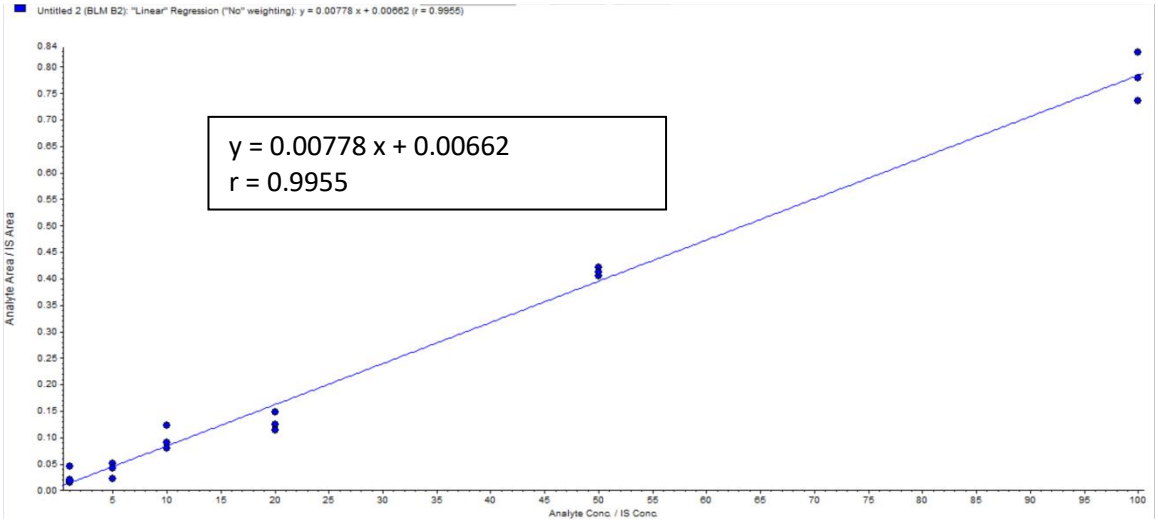

Figure 28. Calibration of BLM B_2.

CHAPTER IV

CONCLUSION

Bleomycin quantitative analysis had been developed by spiking excess of Cu^{2+} to transfer BLM into BLM-Cu complex, solving the problem of using mixed standards with exact percentage of each component to quantify clinical samples and eliminating the need for sophisticated higher resolution instruments that are costly to the lab. Simple chromatography conditions of solvent buffer was optimized, which makes future study of BLM metabolism and mechanism achievable.

In the future study of BLM, it is necessary to find the activity of each of BLM A_2 and B_2 fraction binding to Cu or other metal ions. From the calibration curves that were made during the entire research, it is obvious that the BLM B_2 is more active to bind with Cu, which was present in their spectrums of mass spectrometry Q1 scan and the quantity in calibration curves. However, the stability of the complexes would also be worth to research. Unfortunately, due to sample degradation, the method validation could not be completed in this work, however sensitivity and selectivity on the samples for the method development cohort revealed higher sensitivity and selectivity than previously published methods.

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