Stability of Ampicillin Sodium

by

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Declaration

"I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text."

Kimberley Patterson

Signed

28/02/2018

Date

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“I was raised with the idea of maximum effort: as long as you could look in the mirror and say, 'I gave it everything I had,' it was OK. But if you gave it less, that would disgrace you.”

- Mark Harmon

Publications

Presentations resulting from this research:

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Abstract

The Hospital in the Home (HITH) program has been designed to medically treat patients in their own homes. These treatments include but are not limited to intravenous (IV) infusions. For effective, long-term treatment, these infusions would be longer and at higher concentrations than those made in a clinical environment. Before drugs are used for the treatment of patients in the HITH program, research is required to determine if the drug is effective and safe as IV infusions in these conditions. Ampicillin sodium is a drug of the penicillin family, commonly used in oral and IV infusions. As this drug can fight against both Gram-negative and Gram-positive bacteria, it is of interest for the use in the program. However, ampicillin sodium has already been previously reported as unstable in aqueous solution. Therefore, for the higher concentrations in non-clinical environments, the stability of the drug needs to be investigated.

The degradation of ampicillin sodium (initial concentrations of 16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$) in water, saline solutions, and in citrate and phosphate buffers, was analysed at various temperatures, using UHPLC-UV detection and ^{1}H NMR. The results revealed that initial concentration and temperature have a large influence on the stability of ampicillin sodium; the higher the concentration and temperature, the less stable the drug. In water solutions, it was determined that the drug, at 5 ^{o}C, for the low concentration (16 $mg.mL^{-1}$), had a degradation rate of 3.59 ± 1 $seconds^{-1}$ in which it may be administered over a long period of time. The higher concentration (56 $mg.mL^{-1}$) at the same temperature, degraded faster (19.6 ± 1 $seconds^{-1}$). It was determined that water was the most viable solution for the stability of the drug to be used in the HITH program.

A proposed degradation mechanism of ampicillin sodium was produced from the identification of the degradation products, using LCMS. These degradation products included multiple stereoisomers of ampicilloic acid, ampicillin dimer and ampicillin diketopiperazine Some degradation products displayed a dependence on both temperature and concentration, some were independent to the initial concentration of ampicillin sodium, and others were independent to temperature. The IV fluid used showed that they could influence the degradation pathways for some products.

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Abbreviations	Meaning
Bioassay	Microbiological Assay
Citrate Buffer	Sodium Citrate/Citric Acid Buffer
EVA	Ethylene Vinyl Acetate
HITH	Hospital in the Home
HPLC	High Performance Liquid Chromatography
IV	Intravenous
LCMS	Liquid Chromatography Mass Spectroscopy
m/z	Mass to charge ratio
MS/MS	Mass Spectroscopy-Mass Spectroscopy
NMR	Nuclear Magnetic Resonance Spectroscopy
Phosphate Buffer	Potassium Phosphate Buffer
$t_{90\ \%}$	90 % shelf life
UHPLC	Ultra High Performance Liquid Chromatography
UV-Vis	Ultra Violet- Visible

1. INTRODUCTION

1.1 Hospital in the Home

Due to steadily increasing costs, limited availability of beds and higher risks of post-surgery infections effecting Australian wide hospital services, the Hospital in the Home (HITH) programs were developed to enable treatments in the patient's homes rather than in a hospital. These treatments include, but are not limited to, continuous intravenous (IV) infusions. Hospital in the Home intravenous therapy allows the treatment of a large range of infections and illnesses, and for the patient to receive care which would otherwise be provided as a hospital inpatient service[1, 2]. IV infusions are common practise in hospitals, generally used for the transfer of medications and fluids and allow for the medication to be transferred directly into the bloodstream. This results in faster uptake by the body than if taken orally, and thus provides the patient with quicker relief of the illness. The HITH program has been designed to reduce the length of hospitalisation or admission altogether, supplying a service of regular visits by qualified health staff and medical back up availability for 24 hours a day, seven days a week[3].

The HITH program has increased in popularity over hospital administration due to a number of issues to both government bodies and patients of hospital care[4]. The Australian Capital Territory[5], Victoria[6], Western Australia[3], New South Wales[7] and Queensland[8] governments are already allowing doctors and emergency staff to recommend HITH to patients who fulfil the requirements of authorised guidelines. In fact, the HITH has been practised in NSW since the 1980's[7]. In recent years, the South Australian Health Services has developed their own program[9], however is limited to information, rehabilitation and respiratory support services, and do not indicate the admission of IV infusions.

Caplan, *et al*[10], investigated and compared the HITH and in-hospital rates of mortality, readmission rates and associated costs. Their results showed that, overall the HITH improved health outcomes, for both carer and patient, and reduced readmission and costs. Infections are the primary source for hospital admissions and readmissions. In many circumstances IV infusions are used as the treatment to these infections, and is one area that have been increasingly used to treat infections without patient admission into the hospital. This could be applied to the South Australian HITH system, where the administration of IV infusions could be managed

suitably and safely by patient's carers when prescribed. The materials for IV set ups could be supplied either directly from the emergency department or local doctors, and can continue therapy after hospital inpatient release, or directly at home.

In 2013, 15.9 % of South Australia's population was 65 years and over[11]; higher than the National average. Patients aged over 65 typically have an increased demand on hospital services, e.g. the number of hospital beds available. South Australia also has a longer average hospitalisation period than the national average. By applying the HITH program, the number of elderly requiring hospitalisation for certain treatments could be reduced, which not only increases number of beds available in hospital for other patients, but it has been shown to potentially provide a reduction in secondary infections attained by patients in hospital.

If each South Australian patient admission was reduced by as little as one third of a day, it could free up to allow a possible 2,000 more admissions per year, for metropolitan South Australian hospitals alone[12]. large number of hospital beds per year[11]. It has been reported that public hospitals are commonly operating with an average bed occupancy rate of over 90 %[13, 14], or even above 100 %[14], often causing patients to have long waiting periods for admission. Reducing time spent in the hospitals for post treatments, and replacing that treatment with home care, would alleviate this issue.

Before use in the HITH, the stability of pharmaceutical drugs and their degradation products must be analysed and identified for the effective and safe use of the drugs. The use of many of these drugs have changed from their initial design. Due to increasing antibiotic resistance, many more infections must now be treated intravenously, and some bacteria have become highly resistant requiring longer courses of IV infusions[15]. The development of the HITH program has resulted in differing the conditions in which a drug would be administered, e.g. longer infusion times in patient's homes, opposed to short infusion times in hospitals with controlled temperatures. These long infusion times would likely be administered in common sized IV containers (50-500 mL), therefore result in high concentrations (16 to 56 $mg.mL^{-1}$) with low flow rates for effective treatments. As a result, further research into the stability and methods of increasing stability of drugs is required for conditions likely to be experienced in the HITH program.

1.2 Drug Stability

The stability of a drug is the ability of the active compound to remain within a defined percentage of the original dosage concentration, potency and purity[16], over time, and is of great concern for the medical industry. Without extensive knowledge on a drugs' stability, the patient's well-being may be inadvertently affected as a result of inadequate dosages or the potentially toxic by-products. This can lead to a number of complications from ineffective treatment of original ailment, or to further illnesses and/or the death of a patient[17, 18]. Therefore, it is crucial to determine the stability of pharmaceutical drugs[17, 19].

There are three main sources of drug instability: chemical, physical and microbial degradation[16]. Some drugs are susceptible to 'chemical' degradation e.g. hydrolysis, whereas others undergo a 'physical' degradation; leading to changes in their physical state, for example, crystallization of amorphous drugs. Physical degradation, such as crystallisation, causes a change in the crystal lattice and bond structure of the drug. This can change characteristics of the drug such as solubility. Microbial degradation is the contamination of the drug by microorganisms, e.g. contamination of open containers of eye drops[16]. The instability of a drug is not limited to one of these categories. Chemical and physical degradation of pharmaceutical drugs may change their effects, resulting in altered efficiency, as well as produce potential toxic properties[16, 20]. The preparation of the drug, e.g. as a solution, suspension, emulsion or in solid form, can strongly influence the rate and mechanism of degradation. Most drugs that degrade will do so much faster in solutions rather than their solid states, and increasingly faster in aqueous over non-aqueous solutions[16].

Stability testing of drugs involves monitoring the loss of the active pharmaceutical ingredient (the drug), as well as formation of degradation products, changes in the drugs dissolution and disintegration, loss of package integrity, and microbial contamination[18]. These tests provide evidence of how the drug product changes with time under the influence of variable factors e.g. temperature, pH, light, etc.[18]. They can also provide information on how the storage and transportation package materials and microbes can affect the drug stability[16].

Stability testing provides information on the recommended storage conditions, degradation products and the shelf life of the drug. The term "shelf life" refers to the

length of time that the drug product will last "without degrading"[18]. This is a loose term, as it is rather a known percentage (often 90 %) of the potency of the active drug remaining[16]. The shelf life is often determined by the chemical properties of the active drug and can also be limited by physical stability of the product[16]. One of the most common classes of drugs that are administered in IV infusions are β-lactam antibiotics. This class includes penicillins.

1.3 Ampicillin, Degradation, Kinetics and Stability

Penicillins are among the most widely used antibacterial agents due to their high bactericidal activity, their ability to distribute throughout the body, low toxicity and the broad range of bacterial illnesses which they can treat[21, 22]. Ampicillin (α-amino benzyl penicillin) (Figure 1 a.) is a penicillin antibiotic that is commonly used in hospitals as an IV or oral drug. Physically, ampicillin is a white, odourless, crystalline powder, which is fairly insoluble in aqueous solutions[23]. It is one of a large number of β-lactam drugs that are used for antibacterial purposes[24], and was and was first isolated in 1950[25]. The chemical structure of ampicillin differs from most other penicillins by the amine group adjacent to the benzene ring[22, 26].

Ampicillin sodium (Figure 1 b.) is a salt of ampicillin with a much higher solubility than ampicillin. Ampicillin sodium is one of the many drugs used in intravenous infusions considered for HITH, as it is suitable for treatment of for Gram-negative and Gram-positive bacteria[27]. Ampicillin sodium works by binding to the bacteria's cell membrane *via* penicillin binding proteins. In this process, the carboxyl group on the β-lactam ring is a crucial component of penicillins in affecting Gram-positive bacteria, by binding to the penicillin binding proteins, through ring opening of the β-lactam ring, which in turn disrupting the structure of the cell wall, and its function. The amino acid group on ampicillin sodium helps in the penetration of the cell membrane of Gram-negative bacteria I[28, 29].

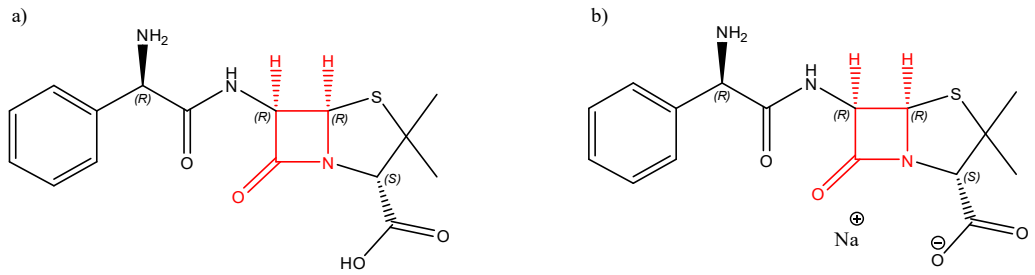


Figure 1: Structure of the antibacterial active composition for a. ampicillin and b. ampicillin sodium, β-lactam ring shown in red.

Due to the interest in using ampicillin sodium for the HITH program, the stability of the drug is of high concern. It must be determined whether the drug can be supplied at the required potency over the length of the IV infusion, and if any toxic or allergenic degradation products form from the exposed conditions. It is critical to understand the stability of the drugs due to the potential of causing mistreatment, or even death, of the patient.

1.3.1 Identification of Ampicillin Degradation Products and their Mechanisms

Penicillins can be used in many different delivery forms, e.g. oral, intravenous, intramuscular, etc., and can be influenced by a wide variety of environmental conditions. These conditions contribute to ampicillin having many different potential degradation pathways: oxidation and reduction, photolysis, racemization and hydrolysis[30], with each of these resulting in different products. Studies have shown that hydrolysis is often the main degradation pathway for drugs which have ester and amide functional groups present[20].

The identification of products and their mechanisms formed from the degradation ampicillin has been previously explored. Previous research[22, 26, 31, 32] determined that there are many degradation pathways and products produced by penicillin hydrolysis[26]. Even with the complex pathways by which degradation proceeds, it is the initial ring cleavage of the β-lactam that is responsible for the overall degradation (Figure 2)[26]. The four-membered ring (β-lactam) amide bond in the molecule is the most unstable component of the molecule, as it is highly strained and reactive[22].

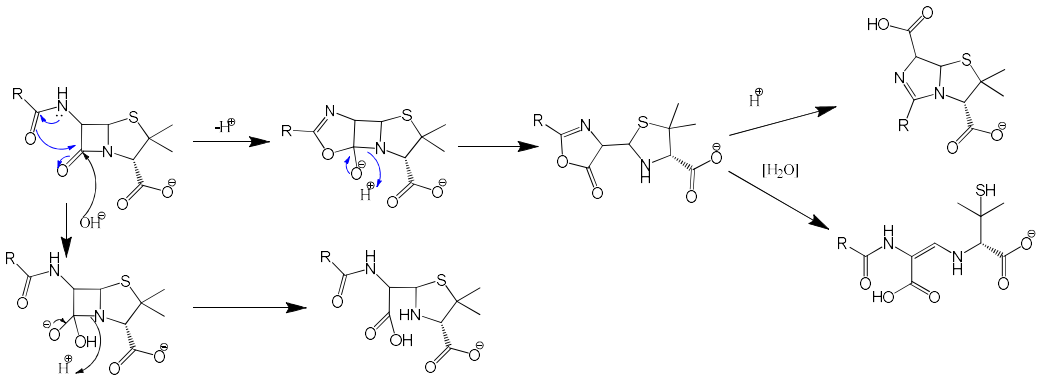


Figure 2: Intramolecular ring opening mechanism of penicillins via acyl side chain interactions[22].

Although there is a large amount of information on penicillin degradation, very few of the early research publications on ampicillin sodium proposed degradation products and mechanisms. Figure 2 gave a greater understanding for the proposed mechanism of degradation of ampicillin sodium due to acyl group influence. Figure 3 and Figure 4 below, depict the degradation mechanism of ampicillin sodium *via* acid/neutral and alkaline hydrolysis, respectively[22, 26].

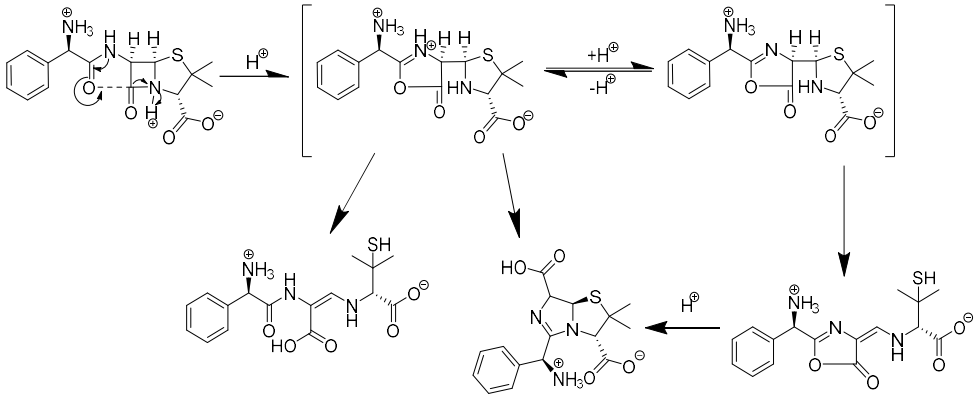


Figure 3: Hydrolysis of ampicillin sodium in acidic and neutral solutions, and degradation products.[22, 26, 31, 32]

For most penicillins, under acidic conditions, the ring breaks open, giving an assortment of degradation products, seen in Figure 3. The electron-withdrawing group decreases electron density on the side chain carbonyl group, reducing the compounds nucleophilicity[22]. Penicillins undergo rapid degradation in alkaline conditions (Figure 4), where the amide bond on the β-lactam ring opens[22], forming different products than in acidic/neutral conditions, such as penicilloic and penilloic acids[22, 26].

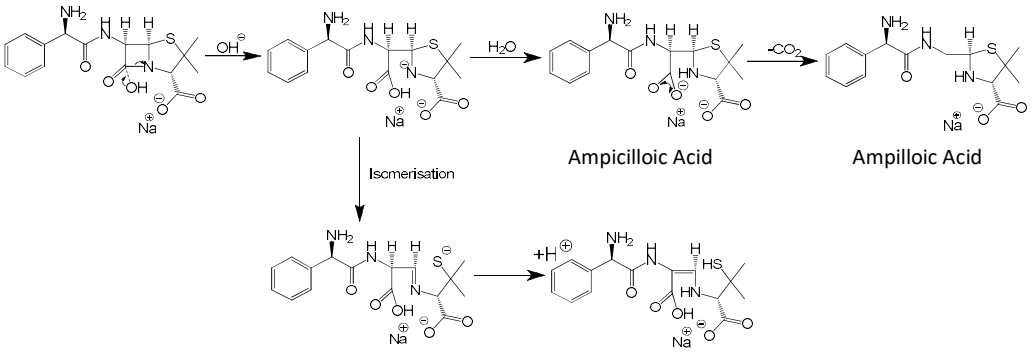


Figure 4: Alkaline hydrolysis of ampicillin sodium and degradation products.[22, 26, 31, 32]

Published in 1969, the journal article by Hou and Poole, *Kinetics and Mechanism of Degradation of ampicillin in Solution*[26] was one of the first to show specifics in the degradation pathways of ampicillin trihydrate (another soluble form of ampicillin). Hou and Poole concentrated on the effects of pH on the degradation rate of ampicillin trihydrate, controlled by citric acid and potassium citrate. It provided a useful scheme of the β-lactam ring cleavage, based on the compounds that had observed and information gathered in previous literature. Unfortunately, the schematic was not detailed and many necessary steps in the mechanism were not present, prohibiting full comprehension of the complex degradation pathways. The schematic provided a starting point on which compounds could be present in the ampicillin sodium degradation.

Other research of ampicillin stability[19, 23, 25-27] provided a generic mechanism of penicillin degradation. However, the presence of the amine side chain in ampicillin chemical structure is not existent in all penicillins, and is an important component in the acid stability of ampicillin. The side chain supplies potential interactions with water molecules and has previously been said that although it may not influence the mechanism, it does produce an effect on the rate of degradation[26, 33]. Yet, the identification of products such as an ampicillin dimerization indicated otherwise, as the amine group of the molecule is crucial for the formation of these compounds (***[figure 5] has been removed due to copyright restrictions***

Figure 5).

A number of articles[34-38], identified metabolites of dimer and other oligomeric formations of ampicillin, as well as the presence of a structure of diketopiperazines[39] [40] (***[figure 5] has been removed due to copyright restrictions***

Figure 5). The formations of the oligomeric[37] and diketopiperazine compounds from ampicillin were discovered to mainly form from high concentrations of the drug and in basic aqueous solutions[40]. As ampicillin sodium solutions, untreated, consist of a pH at approximately 8[40], it suggested that the oligomeric and diketopiperazine

compounds would be significant degradation products for the concentrations to be used in the HITH.

[figure 5] has been removed due to copyright restrictions

Figure 5: Mechanism schematic of ampicillin degradation in basic solutions provided by Robinson-Fuentes, Jefferies and Branch[40].

It has only been more recently that identification of degradation products has been analysed *via* liquid chromatography-mass spectrometry (LC-MS)[41-43]. These studies have identified many more products produced by degradation of the drug than suggested by the previously predicted mechanisms (see Table 22).

Li, *et al*.[42], degraded the drug under several conditions; heat, forced alkaline and acidic, and photolytic and humid conditions. These different conditions resulted in different degradation products, reinforcing that the environments could influence the degradation products produced. The most common products produced throughout the different tested conditions were: ampicilloic acid, ampilloic acid, diketopiperazines of ampicillin, (*Z*)-2-amino-*N*-((2-oxo-3-phenyl-2,3,6,7-tetrahydro-1*H*-1,4-diazepin-5-yl)methylene)-2-phenylacetamide, 3-phenyrazine-2-ol and several stereoisomers of these compounds. Less common were forms of ampicillin dimers and trimers. Li, *et al*. also provided possible degradation pathways for the complex and previously unidentified compounds; explaining how these compounds may have formed.

Franshi, *et al*.[43], investigated both positive and negative mass spectra of ampicillin and the formation of another degradation product; methanolyz ampicillin (for examples of these degradation compounds see Appendix 7.1). Zhang, *et al*.[41] further explored the concept of compounds related to and degraded products of ampicillin, combining and forming their own products. The samples analysed were of ampicillin in capsules, showing both already mentioned compounds; i.e. the oligomeric formations, 6-Aminopenicillanic acid (6-APA), Ampicillinyl-D-phenylglycine, D-Phenylglycylampicillin and D-Phenylglycine. The article did not show mechanisms of the formation of the compounds, but did display the mass spectroscopy fragmentation patterns of the compounds.

From the research on the degradation products and pathways produced, degradation products were observed to form by varying pH levels and buffer conditions. It has also been observed that some degradation products form with high concentrations of ampicillin, however, it is unclear what degradation products may form due to a variation in temperature, concentration and solvent for the HITH program. The identification of degradation product is necessary to understand the nature of the degradation mechanism.

1.3.2 Kinetics and Stability of Ampicillin

Previous research has shown that ampicillin sodium, after being dissolved in aqueous solutions, degrades relatively quickly compared to some other penicillins. Like other penicillins, ampicillin instability results from ring strain of the four-membered ring in the middle of the compound[22]. As ampicillin sodium is dissolved in aqueous solution, the most likely mode of degradation of the drug is hydrolysis. However, photolysis is also a possible degradation pathway. Despite various degradation pathways of ampicillin, pseudo-first order kinetic measurement has been a common method used to analyse the drug's stability for degradation by hydrolysis[26, 39, 44-47].

Kinetics is the measurement of the change in a substance's concentration over time. For example, the decrease in concentration of the reactant ampicillin over time[48][88].

$$\text{rate} \propto [\text{Amp}]^{\text{n}}$$

$$\text{rate} = \text{k}[\text{Amp}]^{\text{n}}$$

Where, k is the rate constant, [Amp] is the concentration of the reactant and n is the order of the reaction in respect to the reactant. The rate constant value is relative to the concentration of the reactants as it changes over time e.g. ampicillin sodium as the reactant. If n = 1, the overall rate order of the reactant reaction is first order reactions[48]. First order kinetics is when the reaction undergoes a spontaneous irreversible decomposition of a reactant[49, 50].

Pseudo-first order refers to when more than one reactant is present, but the concentrations of the other reactant/s e.g. the aqueous solvent, are so large than the first reactant (ampicillin sodium), the change of concentration of the reactant/s is insignificant in comparison[49].

Another common method used is reporting the decrease in concentration of a drug as a percentage potency loss over time[51-53], where the initial concentration is normalised to 100 % ampicillin (e.g. Table 1).

The rate of ampicillin degradation has been shown to vary with pH[33]. This links with previous findings where the pH affects the formation of degradation products. Ampicillin has been shown to be most stable at around pH 5[26, 54, 55]. As ampicillin sodium can act as both an acid and base in solutions, it exists in three different forms; cation, zwitterion and anion[22]. Ampicillin sodium has two different pKa values; the amino acid gives a value of 7.3, where the carboxylic acid group has a pKa value of 2.5. A pH between these two values, pH 5, would cause the ampicillin sodium to produce a zwitterion; a neutral molecule which has both a positive and a negative charge. The zwitterion produced is highly polar and has a higher water solubility. This means that the increase of water solubility of the zwitterion is proportional to hydrogen bonding[26, 56].

It is yet unknown if it is stable at high concentrations and temperatures for extended periods of time that it would be used for in the HITH program. The instability of the drug is the main reason that ampicillin sodium has not thus far been used in the HITH program.

The stability of many penicillins, including ampicillin, has been reported to be influenced by many factors and not just limited to pH. These include temperature, concentration, pH, ionic strength and light exposure, the container in which the solution is in and the structure of the drug itself[26, 27, 30, 57-59]. It has been reported that the higher the concentration and the higher the temperature the drug is exposed to, the faster the rate of degradation occurs. For example, Lynn[27] showed that significantly increasing the concentration of ampicillin by 250 %, could cause decreased ampicillin stability by a factor of 28 (at 23 ^{o}C). The trend of increasing concentration increases the rate of degradation has been reported through literature[30, 57].

Table 1: Reported stability of ampicillin exposed to varying concentrations (as % w/v) and temperatures (5 ^{o}C and 23 ^{o}C) [27]

[Table 1.] has been removed due to copyright restrictions

From Table 1, the lower temperature (5 °C) has increased the stability of the drug over the higher temperature. Again, this trend has been reported throughout a number of articles[51, 54, 59, 60].

From this, it was clear that the degradation of ampicillin sodium needs to be analysed for the concentrations in which the drug would be used for HITH. As the drug will not be used in a clinical setting, it is also necessary that the stability of the drug at these concentrations at temperatures it may experience in the home setting is known.

1.3.2.1 Frozen Studies of Ampicillin

From the results above, the lower the temperature the higher the stability of the drug. For the HITH program, it may be beneficial to store reconstituted ampicillin sodium solutions in patient's freezers. There have been a few studies that looked at the stability of ampicillin in frozen (-20 °C and below) conditions.

While Lynn[27] did not perform such a study, he did briefly summarise that normally in penicillin degradation, freezing the samples causes a 'freeze' in the rate of the degradation, and some penicillins will have very little to no degradation over several months. It was even stated that it could completely halt the formation of oligomeric formations of the penicillins. However, in the case of ampicillin, it was said to have an adverse effect, where it can increase the degradation at these lower temperatures (when compared to refrigerated samples). This phenomenon was observed in the research performed by Savello. and Shangraw[58]. They tested the drug's degradation at a concentration of 10 $mg.mL^{-1}$ (Table 2), and of 250 $mg.mL^{-1}$ (Table 3), in water. The percentage degradation of the 10 $mg.mL^{-1}$, when frozen, increased significantly compared to those samples at higher temperatures of 0 °C and 5 °C.

Table 2: Degradation percentage of 1 % ampicillin in water at -20 and 5 °C[58].

[Table 2.] has been removed due to copyright restrictions

However, the 250 $mg.mL^{-1}$ samples showed that 0 °C and 5 °C had a higher degradation than the frozen samples (Table 3).

Table 3: Degradation of ampicillin sodium (250$mg.mL^{-1}$) in pure water[58]

[Table 3.] has been removed due to copyright restrictions

Although this article by Savello and Shangraw[58] is still commonly referred to in the medical industry for their results, a number of issues with their methods and

interpretations were observed. The samples of 10 and 250 mg.mL^{-1} concentrations were discussed as though they had similar trends, however the two concentrations were calculated to have different trends. Furthermore, the method states that Savello and Shangraw froze 'an appropriate sample' and was thawed by running under room temperature water. It also specified that distilled water added to the 'frozen plug', without indicating a known dilution factor. Because of this, it was difficult to know whether their method of thawing could have influenced the drug's degradation rate. Warren, *et al*. [57] also saw a similar response, where ampicillin sodium was less stable at -20 °C than at 5 °C, in 0.9 % sodium chloride (commonly referred as saline[51, 61, 62]) solutions.

In the study by Holmes, *et al*.[63], the antibiotic activity of ampicillin sodium was tested at lower temperatures than Warren, *et al.*, and Savello and Shangraw. The stock solution used was 20 mg.mL^{-1} and the samples, in polyvinyl chloride bags and saline solution, were frozen for thirty days before being thawed by microwave. Using a bioassay method, the degradation of the drug was observed at room temperature. Similarly, as the method used by Savello and Shangraw, it was uncertain whether the microwave thawing process causes an acceleration or another effect on the rate and degradation composition of the drug. The solutions at -30 °C and -70 °C provided a promising result, where eight hours after thawing there was approximately 90 % of the initial concentration left of the drug at both temperatures. However, the -20 °C samples were degraded by roughly 70 %, after the sample had been thawed. Unfortunately, most household freezer units do not reach these low temperatures of -30 °C and -70 °C.

From these studies, it is unclear whether the increased degradation of ampicillin when frozen was caused by the low temperatures or if it was by the thawing process used. Therefore, it is unknown if freezing the drug in a commercial freezer would be a viable method of storage for the drug before administered to a patient in the HITH program.

1.4 Early Research into Ampicillin Stability

Research prior to the mid 1980's was performed on ampicillin stability using microbiological assays[53] and iodometric assays[58], as viable methods of measuring stability. After, High Performance Liquid Chromatography (HPLC) techniques[64]

became a common analysis technique. Commonly these method used a normalised percentage[51, 58] method or pseudo first order kinetics to calculate the degradation of ampicillin[29, 31, 44, 90-93]. Despite these attempts, inconsistencies within the methodology resulted in large variations in the reported stability of ampicillin sodium. This variation has complicated attempts to establish the stability of ampicillin sodium as it is difficult to compare data and between techniques. Nuclear Magnetic Resonance (NMR) spectroscopy has been used in a number of pharmacokinetics studies [24, 65-67], e.g. herapins, cefotaxime and clarithromycin, and has more recently been used in ampicillin stability. The following chapters review ampicillin stability studies using the previously mentioned analysis techniques.

1.4.1 Microbiological (Bio) Assay

Microbiological assays were once the preferred method of analysing antibiotic stability[68], supplying the baseline of stability of pharmaceutical drugs, including ampicillin. Kinetic analysis of antibiotics using bioassays consist of using agar plates for diffusion methodology. The stability of the drug is analysed by using agar plates, spiked with bacteria, commonly used are *Escericchia coli, Sarcina lutea* and multiple *Streptococcus*[69]. When the drug (of various concentrations) is added to the bacteria spiked plate, the diameter of space inhibited bacterial growth indicates the potency of the drug. This diameter is measured to determine potency. Using standards, the diameter of inhibited bacterial growth is used to determine the rate of degradation in the antibiotics[70]. It should be noted that it has been stated that biological assay have limits of accuracy[27].

Although a significant number of articles have used bioassays to determine stability of ampicillin drug, there are very few similarities observed between conditions (e.g. concentration and temperature) of analysis to compare results. Those results which can be compared have shown to have large disparities in the stability of the drug.

Four articles[27, 59], [57], [53] (Table 4) were some of the earliest work on ampicillin stability using bioassays. A variety of different conditions were analysed in each paper. These reports produced conflicting results. The results of ampicillin sodium in one IV solution: saline, with similar temperatures: refrigerated and room temperature (5 ^{o}C, and ranging between 23 and 25 ^{o}C respectively), were used for comparison.

Table 4: Comparison of ampicillin stability using bioassay methods in four journal articles[27, 53, 57, 59]

Paper	Method	[Ampicillin] ($mg.mL^{-1}$)	Temp. (°C)	Result $t_{90\%}$
Gallelli, *et al*.[59]	*E. coli* and *S. aureus* assays	5	5	Retained activity after 60 days
		5	25	No decrease after 14 days
Warren, *et al*. [57]	Biological assay	20	5	1-2 days
		20	25	24 Hours
Lynn[27]	Biological assay	2-4	23	24 Hours
Jacobs, *et al*.[53]	*S. aureus* and *P. aeruginosa* assays	10	20-25	Approximately 8- 12 Hours

Due to the variability in concentration and temperatures between the papers, it was difficult to draw some comparisons. At 5 °C, Gallelli *et al*. indicated that ampicillin had a shelf life of more than 60 days, where Warren *et al*. calculated that the drug would only last 1-2 days at this temperature. It was undetermined if the difference in concentration of the two solutions contributed to such a significant difference in the results, however Gallelli *et al*. stated that the solution changed colour, from colourless to yellow, suggesting a physical change in the sample had occurred. For room temperature samples, Lynn and Gallelli suggested that of concentrations between 2-5 $mg.mL^{-1}$, ampicillin sodium could last somewhere between one and 14 days, respectively. Variations in concentration between Jacobs, *et al*., Lynn and Warren, varied with 10 $mg.mL^{-1}$, 2-4 $mg.mL^{-1}$ and 20 $mg.mL^{-1}$, at the same temperature, showed no trend that concentration had an effect on the degradation rate.

It has been reported that the type of bacteria used in the method can influence the results of the kinetic analysis[57]. Some of the articles did not report on which bacteria was used, which could contribute to some of the dissimilarity of the shelf life values reported.

1.4.2 Iodometric Assay

Iodometric assay methods were developed and introduced to drug stability after bioassays, and became the new method of choice of penicillins and drug mixtures of penicillins for kinetic analysis[71, 72]. The technique was, and still is, used due to its ability to differentiate between the drug, and some of the degradation products. The assay quantitatively determines the degradation of a drug by the pharmaceutical substance oxidising potassium iodide in an acidic solution to produce an equivalent

quantity of iodine. The iodine may be further assayed by titration with a standard solution of sodium thiosulphate[17]. The lower the concentration of the drug, the less iodine forms, therefore the rate of degradation of the drug can be calculated.

Two papers explored the stability of ampicillin using this technique; Jacobs, *et al*.[60] and Savello and Shangraw[58]. For the comparison of the stability of the drug between papers, the 10 $mg.mL^{-1}$ of the drug, in saline, at room temperatures recorded as 20-25 ^{o}C[60] and 27 ^{o}C[58], were used. Taking into account the variation of the temperatures, both paper's assays produce vague values of shelf life, stating that ampicillin sodium can at least last more than 16 hours. However, in later publications, it was recognized that the iodometric method was not able to accurately measure the degradation of ampicillin. The iodometric method is able to differentiate between ampicillin and its degradation products where the β-lactam ring is open, but cannot differentiate between ampicillin and its oligomeric formations, e.g. dimer, trimer, etc.[64], therefore would underestimate the rate of the degradation of the drug.

1.4.3 High Performance Liquid Chromatography and Ultra High Performance Liquid Chromatography

HPLC and UHPLC are currently the most commonly used analytical techniques for the analysis of drug stability[71]. Both HPLC and UHPLC can be coupled with several detection methods, for example: Fluorescence, Diode Array Detectors (DAD), Electrochemical, Charged Aerosol Detectors and Mass Spectroscopy (MS). However the most commonly used for ampicillin stability are DAD[73], and MS[74]. These techniques have complimented or replaced the older techniques (microbiological and iodometric methods) due to HPLC's high reproducibility, separation power, and selectivity[75], producing more informative results. The two techniques are both a technique used to separate compounds, using solvents for the mobile phase and a set stationary phase.

The wavelength most often used for the UV detection of ampicillin sodium is at 225 nm[51, 76], due to the carboxylic acid functional group of the drug[77, 78]. Following the mechanisms (Figure 2, Figure 3 and Figure 4), many of the degradation products have the aromatic still intact, and therefore should be detectable at 254 nm as well. It has been observed that, in some drug mixtures, ampicillin sodium has a peak maxima wavelength of 268 nm[76, 79].

Many HPLC methods have been developed to detect ampicillin sodium with degradation products and ampicillin sodium in drug mixtures[29, 38, 71, 76, 80, 81]. The research has often concentrated on determining if the method is reliable in determining the degradation[76, 82-84], rather than the degradation rate of drugs, or the stability of ampicillin sodium when applied to admixtures containing multiple drugs[76]. This means that there is only a small amount of published research on ampicillin samples, to compare with older techniques. This makes it difficult to determine whether those results recorded using either older or newer techniques are comparable or consistent between themselves. Table 5 depicts comparable kinetic results of ampicillin shelf lives using HPLC techniques.

Table 5: Comparison of kinetic analysis of articles which use HPLC techniques. saline solution used as solvent in samples.

Paper	[Ampicillin] $mg.mL^{-1}$	Temp. (°C)	pH	Result $t_{90\%}$
Allwood, *et al.*[51]	5	5	Initial pH of 8.4-8.5	~2 days
	10	5	Initial pH of 8.7-8.8	~3 days
Kang and Kang[85]	30	4	Changes from 9.2 to 8.49 over 7 days	Over 7 days
	30	25	Changes from 9.10 to 8.49 over 7 days	3 days
Maher, *et al.*[61]	12	25	Initial pH of 9.2	32-41.7 hours

The results from the HPLC method suggested that temperature does have a significant effect on the degradation rate, and that ampicillin is stable for approximately 2-3 days. Allwood, *et al.*[51], at 5 °C, showed that an increase of concentration increased the stability. Similarly, between Kang and Kang[85] and Maher, *et al.*[61], the increased concentration also increased stability.

The lack of data for comparison between HPLC studies and the dispensary of the results suggests that using this technique, further research in ampicillin stability is required.

Comparison of ampicillin stability between these different techniques shows large variation. From Table 4 and Table 5, HPLC method of similar concentration and temperatures indicated more than 32 hours[61], and bioassays ranged from 8-12 hours[53]. Iodometric methods suggested that ampicillin (10 $mg.mL^{-1}$) at approximately 25 °C, had a shelf life of at least 16 hours[58, 60]. Assuming the techniques accurately determined the rate of degradation of the drug, the samples would have an approximate similar result of degradation. Overall, the variation

makes it difficult to determine whether ampicillin sodium would be beneficial to use within the HITH program.

1.4.4 NMR Drug Analysis

NMR spectroscopy, was once only used to characterise the drug and its degradation products[32, 86, 87] in the application in pharmaceutical stability analysis. Now it is more commonly used for the kinetic analysis of drugs[24, 65-67]. Although carbon, phosphorous and oxygen are commonly used, hydrogen (^{1}H) NMR is the most common type used in pharmaceutical applications[66, 67, 86, 88, 89]. In addition to the characterisation of drugs and their degradation products[32, 67, 86] NMR can be used to determine the level of impurities present and observation of degradation over time[66]. As the intensity of the peaks is proportional to the concentration of the solution, NMR can be used to calculate the decrease in ampicillin sodium intensity over time as a kinetic measurement. ^{1}H NMR is relatively quick in analysis run time and has little preparation required making it ideal for kinetic analysis of the degradation of drugs, such as ampicillin sodium.

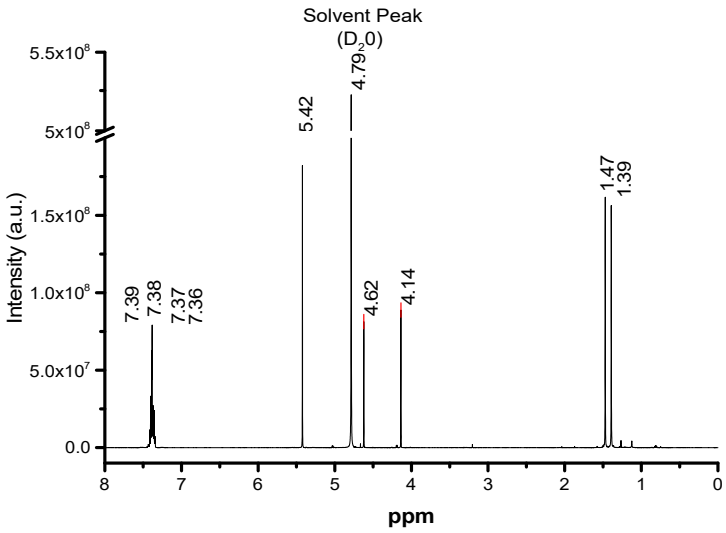


Figure 6: NMR peak assignment of non-degraded Ampicillin sodium, (16 mg.mL^{-1}) at 20 °C.

It is common in drug analysis that an internal standard is used to use the integration ratio to determine the percentage of degradation[66, 67]. For ampicillin sodium (Figure 6), the aromatic ring is significantly stable therefore the hydrogen integration is not influenced by a change in the neighbouring atoms. The only change that is observed in the aromatic peaks is a shift in the peak, caused by the changing environment. According to the degradation mechanisms (Figure 3, Figure 4 and ***[figure 5] has been removed due to copyright restrictions***

Figure 5), theoretically, the β-lactam ring-opening and double bond formation would reduce the β-lactam hydrogens peak. Chemical shifts may be observed in the peak formed by the hydrogen next to the carbonyl (Figure 6, peak at 4.137 ppm), due to the bond breakage in the β-lactam ring, causing a change in the environment of the hydrogen. However, it is unclear whether this would be observed with the degradation oligomeric formations, as they would have similar shifts.

The degradation of ampicillin sodium salt has seldom been investigated using NMR spectroscopy. Shamsipur, *et al*.[24] investigated ampicillin trihydrate, and related drugs, using proton NMR with maleic acid as an internal standard. The study explored the comparability between the percentage recovery of drug using HPLC and NMR methods. Shamsipur, *et al*., found no significant difference between the two methods used, and advised that NMR required shorter preparation and analysis time, therefore it could be argued as the better technique.

Shamsipur, *et al*., stated, due to solubility issues of ampicillin trihydrate in pure water, the NMR samples were dissolved in a mixture of deuterium and formic acid (56/44 %w/w), causing a chemical shift. Shamsipur, *et al*., also used maleic acid as the internal standard, due to its solubility and stability in the sample media. However, the was no evidence provided that the maleic acid did not influence the decomposition of the molecules, or if the addition of formic acid produced a stability influencing factor. The method used for the HPLC analysis is said to follow those from the British Pharmacopoeia (1990), which was not clearly referenced, prohibiting the reproduction of the method.

Very few articles have been published on the analysis of ampicillin kinetics *via* NMR, however, the benefits of using NMR for kinetic analysis of pharmaceutical drug stability are large. With shorter analysis times and small amount of sample preparation, the analysis of ampicillin sodium could benefit from using this technique. For this, further investigation of this technique is required.

1.5 Effects of IV Infusion Solutions, Additives and IV Container Compositions on Ampicillin Sodium Stability

A number of journal articles have been published on the influence of various IV infusion solutions on the stability of penicillin drugs. Although there are a number of

IV infusion solutions[27], the most common solutions investigated are water, saline and 5 % glucose solution, as well as pH controlling buffered solutions[40, 51, 54, 55, 57, 58, 60, 62, 90]. The following chapters review the effects the IV infusions solutions and container have on the stability of ampicillin.

1.5.1 IV Infusion Solutions

The most exstensive of studies on ampicillin stability and IV infusion solutions is that of Lynn[27], who examined eight different commonly used IV fluids, along with concentration, temperature and pH. Initial work on ampicillin stability determined that pH's of 6.5 are optimal for ampicillin stability, but that a pH between 5.5-7.5 was acceptable. However, for reasons unknown, further analysis of ampicillin stability was performed at a pH of 8.5, outside the determined acceptable range. A range of concentrations were analysed, 1 %, 5 %, 10 % and 25 % (suggested to be %w/v), at both 5 ^{o}C and 23 ^{o}C. As expected, due to the intermolecular degradation products, the low concentration solution (1 %) ampicillin sodium degraded least, and it was suggested that it could last more than 7 days at 5 ^{o}C. Increasing the temperature had a detrimental effect with the same solution where, at 23 ^{o}C, the activity of ampicillin was reduced to 85 % after three days. For 25 % ampicillin sodium, the potency was degreased past 90 % within 15 minutes.

The effect of IV fluid composition was examined for eight commonly used IV solutions. However, as noted above, the concentration is known to directly affect stability, the results cannot be reliably used as the reported concentrations in each solution was different.

Table 6: Percentage of ampicillin sodium degradation in assortment of IV solutions[27]

[Table 6.] has been removed due to copyright restrictions

Without providing an exact concentration for each test, the results from Lynn[27], suggested that 5 % dextrose, when added to any solution, would result in more rapid degradation of the drug when compared to saline. The table shows that saline and Ringer's solution provided the best result of ampicillin stability. Other studies[60, 62, 90] confirmed the result that dextrose was detrimental to the stability of ampicillin and suggested that saline produced the best result out of the solutions tested, however they did not test stability of the drug in Ringer's Solution. This outcome has been

consistent throughout most research performed on ampicillin sodium and IV solutions. Evidence has been provided suggesting that the degradation of the drug increases when in 5 % dextrose solution; when compared with that of the stability of ampicillin sodium in sterile water and saline solution[58, 62]. Although the overall trend showed that some solutions were more stable over others, the trend does not provide specific information regarding the use of the drug in solutions for the HITH program.

Table 7: Comparison between saline and 5 % glucose solution solvents between different papers.

Paper	Method	[Ampicillin] ($mg.mL^{-1}$)	Temp. (°C)	Solvent	Result $t_{90\%}$
Gallelli, MacLowry and Skolaut[59]	Bioassay (E.coli and S.aureus)	5	5	5 % Dextrose	50 % after 21 days
				Saline	No loss after 60 days
		5	25	5 % Dextrose	25 % after 14 days
				Saline	No loss after 14 days
Newton[62]	-	10	5	5 % Dextrose	4 Hours (8.6%)
				Saline	4 Hours (1.0 %)
		10	25	5 % Dextrose	4 Hours (19.5 % degraded)
				Saline	0.9 % after 4 Hours
Savello and Shangraw[58]	Iodometric	10	5	5 % Dextrose	4 Hours (10.1 %)
				Saline	Over 24 Hours (3.3 %) (1.0 % at 4 Hours)
		10	25	5 % Dextrose	21.3 % after 4 Hours
				Saline	8.3 % after 24 Hours
Hiranaka, Frazier, and Gallelli[52]	Colorimetric assay	10	5	5 % Dextrose	4 Hours
				Saline	Over 7 days
		10	25	5 % Dextrose	Less than 4 Hours
				Saline	24-72 Hours
		20	5	5 % Dextrose	7 Hours
				Saline	Over 72 Hours
		20	25	5 % Dextrose	Less than 4 Hours
				Saline	24 Hours
Warren *et al.*[57]	Bioassay	20	5	5 % Dextrose	Approximately 8 Hours
				Saline	1-2 days
		20	25	5 % Dextrose	30 minutes
				Saline	24 Hours

Table 7 displays a comparison between studies performed in saline and 5 % glucose solutions. Newton[62], and Savello and Shangraw[58] showed similar degradation percentages of ampicillin sodium (10 $mg.mL^{-1}$), in 5 % dextrose and saline, between the two articles. They also confirmed that the 5 % dextrose did influence the degradation of the drug. The results from Hiranaka Frazier and Gallelli[52], and Warren, *et al.*[57] showed the same trend of ampicillin being less stable in 5 % dextrose than in saline and had similar shelf life values.

Similarly, Gallelli, MacLowry and Skolaut[59], determined that saline was more stable than 5 % dextrose. However, a decrease in the pH of the saline solutions, after 60

days, was observed. This change in pH is an indication of a change in the chemical conformation of the solution; usually observed with a decrease in ampicillin potency. It was also noted that a colour change was observed in the 25 ^{o}C samples, another indication that a change in the samples had in fact occurred.

From these studies (see Table 7) it was indicated that the sugar/carbohydrate IV fluids e.g. dextrose/D-glucose, influenced the rate of degradation of ampicillin and therefore should not be used in the HITH. However, saline is a good IV fluid for the stability of the drug and could be considered as a viable fluid.

Although it has been determined that saline is one of the better IV fluids to use for ampicillin stability, there have been some conflicting results, for the stability of ampicillin in water. Kang and Kang[85], compared the stability of ampicillin in sterile water and saline IV fluids. Their results indicated that the stability of ampicillin was improved with saline when compared to water samples of the same conditions. However, Savello and Shangraw[58] suggested that this was not the case, that saline may increase the instability of the drug. Stiles, Allen and Prince[91], indicated that there was no significant difference in the degradation of the drug between these IV fluids.

1.5.2 Additives

To increase stability of ampicillin sodium in water and saline, some studies have been performed in analysing ampicillin solutions with additives, such as buffers. The effects of pH on the stability on ampicillin has been thoroughly investigated in a number of studies[39, 55]. The hydroxyl groups, on various penicillins, are known to accelerate the rate of degradation of penicillins, such as ampicillin, at neutral and alkaline pH. Allwood *et al*.[51] saw a reduction of degradation in buffered saline samples, in Viaflex Minibags, when compared to un-buffered saline samples. Several articles[26, 54, 55] have concluded that the ideal pH for stability of ampicillin is between 4.5 and 6; a larger range than what was suggested by Lynn[27], and therefore buffered samples in this pH range improve stability of ampicillin.

Another additive to attempt to improve ampicillin stability is cyclodextrins. Using NMR, Maffeo, *et al*.[47] provided a section on the hydrolysis of ampicillin for the analysis of the effect of selected cyclodextrins on the stability of the drug, using NMR. Ampicillin degradation was observed using the integration of the proton signals of

the methyl groups or the protons on the β-lactam ring. The ampicillin solutions were held at a pH of 7 with sodium phosphate buffer, at 30 °C. Regarding the sample in which only contained ampicillin sodium with no cyclodextrins, Maffeo, *et al*., stated that within one day, approximately 10 % of the drug had degraded. However, whether or not this was a reliable value was in question as the authors used pseudo-first order kinetics (Appendix 7.2.1), the graph plotted (Figure 7) produced a slope which logarithmically increased; not the expected linear curve for the function.

[Figure 7.] has been removed due to copyright restrictions

Figure 7: Degradation of ampicillin sodium on its own (◆) and in the presence of an excess of a range of cyclodextrins 3c (○), maltoheptaose (▲), 4c (□) and 5c (×)[47].

1.5.3 IV Infusion Containers

A number of the previous articles did not discuss in which container type was used in their analysis of the drug's stability. There is a potential that the stability of drugs could be influenced by the various compositions of intravenous infusion containers. These can be made of several different types of polymers, these include polypropylene (PP), polyethene (PE) and ethylene-vinyl acetate (EVA). With varying oligomeric structures, the differences in mechanical properties could influence the interaction of the drug with the polymer containers.

A few studies have analysed the effect of container material type on the stability of the drug. As previously stated, Allwood *et al*.[51] analysed the stability of ampicillin in one type of IV bag, however it was not clearly identified whether or not that the Viaflex had an influence on the stability of the drug.

Muller and Haker[92] investigated the effect of the IV container Freeflex® on the stability of six penicillins, including ampicillin. Freeflex®, consisting of a flexible composition of polypropylene and is a commonly used infusion bag. Muller and Haker compared the results of the of the Freeflex IV container to glass vial samples in the same conditions. The results reported in this study suggested that the Freeflex® bags had no significant hindrance on the stability of the drug, however, due to a lack of error bars, it was difficult to determine whether the IV bags could have improved the stability of ampicillin. There was no significant difference observed between the two container types.

Kang and Kang[85], also investigated the effects of an infuser device (Accufuser®) on the stability of ampicillin sodium (30 $mg.mL^{-1}$), both in saline and sterile water solutions. Using HPLC-UV techniques to test the stability of ampicillin, the drug was tested in an infusion device, in sterile water and saline solutions at 4 ^{o}C and 25 ^{o}C. The result suggested that ampicillin could be stable at the two temperatures, in both saline and water, for a minimum of approximately 3 days. However, it is unclear whether the Accufuser® had an effect on the stability of the drug, as there was no comparison between this and another container

Stiles, Allen and Prince[91] studied the stability ampicillin sodium, and other antibiotics, when stored and used in an insulating pouch during the administration of a portable infusion pump. They also provided comparative data without use of the infusion pump. In the study, a high concentration of ampicillin sodium (60 $mg.mL^{-1}$) was measured in sterile water and saline solutions. The stability was tested in both "fresh solutions", where they were made and placed at 30 ^{o}C for 24 hours, and "stored solutions" where samples were placed in a temperature of 5 ^{o}C for 24 hours, and then at 30 ^{o}C for a further 24 hours, in both the pouch and glass containers. The pouch setup was shown not to significantly hinder, and in some cases improved the stability of the drug. It was also determined to be able to last over a 24 hour infusion for both stored and freshly made samples. However, as the type, manufacture or material of the pouch was not disclosed, the experiment could not be repeated for the HITH setting.

From previous studies, it is possible that the IV container type could improve or potentially hinder the stability of ampicillin sodium. Therefore, the stability of the drug must be analyzed and compared in IV bag containers which may be used for the HITH program.

1.6 Project Aims

It is clear that a large amount of research has been performed on the stability of ampicillin. However, it is still uncertain whether the drug would be able to last in the conditions in which it would experience in the HITH program. Many of the conditions that have been researched have conflicting results relating to the stability of ampicillin sodium. There has been research previously performed on ampicillin at the concentrations (16 $mg.mL^{-1}$ to 56 $mg.mL^{-1}$) in which the HITH requires for efficient supply of the IV infusion over long periods of time. However, much of this research on ampicillin kinetics has used techniques that have large limitations in analysis of the stability of ampicillin.

The research presented in this thesis will use UHPLC and NMR to determine the stability of ampicillin sodium. The drug will be reconstituted in various IV solutions, in concentration and temperature conditions which simulated those experienced in the home. These include two concentrations of 16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$, which are the lowest and highest concentrations to be used in the HITH; four temperatures of -20 ^{o}C, 5 ^{o}C, 20 ^{o}C and 37 ^{o}C that the reconstituted drug may reside in for storage and usage; and four commonly used IV solutions (water, saline, citrate buffer and phosphate buffer). For continuous use of the drug, no less than 90 % of the original concentration of ampicillin sodium can be injected into a patient[30, 58]. Therefore, the time taken for the drug to no longer be stable enough for injection will be determined. The products produced by the degradation of the drug will also be investigated, to understand the degradation mechanism of ampicillin sodium at these concentrations. As some products have been identified to cause anaphylaxis in patients, understanding the degradation pathways of the drug can help identify the any risk in the use of the drug for the HITH purpose.

The aims of this thesis will be achieved by the following project components:

1. Developing and comparing NMR and UHPLC methods for analysing the kinetics of the drug.
2. Analysing the instability of ampicillin sodium at the two concentrations, four temperatures, and in IV solutions, and;
3. Develop a mechanism of ampicillin degradation by determining products from the instability of the drug.

2. MATERIALS AND GENERAL PROCEDURE

2.1 Materials and Reagents

Ampicillin sodium (Aspen Pharmacare Aust Pty Ltd, vials of 1g ampicillin sodium, and Juno Pharmaceutics, vials of 500 mg ampicillin sodium), sterile water and saline injection solvents, and the EVA (ethylene-vinyl acetate) and Freeflex® containers were supplied by CPIE Pharmacy Services, Adelaide, South Australia. Acetonitrile, citric acid ($C_6H_8O_7$), sodium citrate dihydrate ($C_6H_5O_7 \bullet 3Na \bullet 2H_2O$), deuterium oxide ($D_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \bullet H_2O$) and sodium phosphate monobasic (Na_2HPO_4) were bought commercially from Sigma-Aldrich®, Australia. Formic acid, and Optima® UHPLC grade water and methanol were bought commercially from Thermo Scientific™, Australia.

2.2 Sample Preparation

Samples of ampicillin sodium were made to 16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$, to a maximum of ± 0.5 mg per sample, by reconstituting ampicillin sodium in aqueous solutions of:

- D_2O,
- Water (Sterile water for injection, Freeflex®, and Optima® UHPLC grade water),
- 0.9 % sodium chloride in water (Sterile saline for injection, Freeflex®),
- 0.3 and 3.0 M sodium citrate buffers (pH 5 and 7), or;
- 0.01, 0.1 and 0.2 M phosphate buffers (pH 5 and 7).

These samples were placed in temperature regulators at -20 °C, 5 °C, 20 °C or 37 °C, for analysis of ampicillin sodium stability at different temperatures.

The various buffers were prepared accordingly:

- Saline solution was used as supplied (CPIE Pharmacy Services).
- 0.3 and 3.0 M sodium citrate were made by dissolving sodium citrate dihydrate ($C_6H_5O_7 \bullet 3Na \bullet 2H_2O$) in water (Sterile water for injection, Freeflex®) and adjusted the pH (5 or 7) with citric acid.
- 0.2 M phosphate buffer was made by dissolving 0.4 M of $NaH_2PO_4 \bullet H_2O$ and 0.4 M of Na_2HPO_4 in water (Sterile water for injection, Freeflex®), and diluting to 0.2 M and pH 7.

- 0.01 and 0.1 M phosphate buffers were made by dissolving 0.2 M of $NaH_2PO_4 \bullet H_2O$ and 0.2 M of Na_2HPO_4 in water (Sterile water for injection, Freeflex®), and diluting to the correct concentration at pH 5 and 7.

2.3 Kinetic Analysis of Ampicillin Sodium

Drug kinetics was analysed by using either the integration values of the peak signals of ampicillin sodium obtained by proton NMR, or the peak areas of ampicillin sodium from the UHPLC. From this, the data was normalised to the initial value (equalling 100 %), as a decrease of percentage of ampicillin over time. Data plotted as of the percentage remaining of ampicillin versus time. The following exponential regression was used to fit the calculated data:

Equation 1:

$$[Amp] = Ae^{-k} + [Amp]_{\infty}$$

Where $[Amp]_{\infty}$ is the value in which ampicillin concentration reaches equilibrium, *A* corresponds to the amplitude (the difference between the y-intercept: $[Amp]_0$, and $[Amp]_{\infty}$) and *k* equates to the slope; the drug's degradation rate.

The first order reaction equation assumes that the drug follows a perfect system, where ampicillin sodium reacts into completion ([Amp] = 0). This would mean that the amplitude (*A*) would be the only variable present (equivalent to $[Amp]_0$, where it is possible that amplitude can equal 100 %). However, ampicillin sodium does not perfectly fit the first order kinetics system (identified to follow pseudo-first order kinetics). The exponential decay function is mathematically calculated to take into account that ampicillin sodium may reach a reaction equilibrium, therefore not fully converting into degradation products (A ≠ 100 %); which describes the asymptotic decay. The amplitude and this y offset are added together to equal the y-intercept of the trend line.

By rearranging the exponential decay fit curve (Equation 1), the shelf life (when, y = 90) of the drug is remaining could be calculated.

2.3.1 Kinetic Analysis by Nuclear Magnetic Resonance Spectrometry

A Bruker Avance III 600 (^{1}H 600 MHz and ^{13}C 150 MHz) and a Bruker Avance III 400 (^{1}H 400 MHz) were used for proton (^{1}H) and carbon (^{13}C) characterisation, and ^{1}H

kinetics analysis. Temperatures used were 5 ^{o}C, 20 ^{o}C, and 37 ^{o}C. Data was analysed using Bruker Topspin instrumentation.

All samples were made up in D_2O. Each sample was prepared in triplicate. A sample of ampicillin sodium with an initial concentration of 56 mg.mL^{-1}, at temperature of 20 ^{o}C, was analysed by ^{1}H NMR spectroscopy over a period of 168 hours (7 days). Acetonitrile was used as an internal standard to for determination of the stability of the integration of ampicillin sodium proton peak signals. The integration (Bruker Topspin software) of the peaks in the ^{1}H NMR spectra was calculated relative to the acetonitrile peak, set to a value of three hydrogens. The integration of the drug's proton peak signals was used to calculate the percentage of ampicillin sodium remaining in the sample. The data was fitted with an exponential curve function (Equation 1), and the time taken for 10 % of the drug to degrade was calculated. The error calculated in all samples was by standard deviation (± 1 S.D)., where number of replicates equalled 3 (n=3).

2.3.2 Kinetic Analysis by Ultra High Performance Liquid Chromatography

All data concerning the results of method development, the comparison between the effect of concentration and temperature on ampicillin sodium in water, and saline samples, were obtained using a PerkinElmer Flexar™ FX-15 UHPLC instrument coupled with Ultra Violet (UV) Diode Array detection (DAD) (PerkinElmer, Waltham, MA, USA). Separation occurred on an Agilent Zorbax Eclipse Solvent Saver XDB-C18 (3 x 100 mm, 1.8 µm) column. All aqueous solvents were filtered with 47 mm, 0.22 µm PVDF hydrophilic Millipore Durapore (Sigma Aldrich, Australia) filter paper. Peak area was analysed *via* Chromera® (PerkinElmer, USA) and Origin® Pro 9.0 (Northampton, MA, USA).

IV container and remaining solvent comparisons were performed with a Thermo Scientific™ Vanquish UHPLC^{+} Focused system, with a Vanquish Diode Array Detector (Thermo Scientific, Australia). Separation occurred on a Thermo Scientific™ Accucore Vanquish C18+ (2.1 mm x 100 mm, 1.5 µm) column. Solvents were filtered through 47 mm, 0.22 µm PVDF hydrophilic Millipore Durapore (Sigma Aldrich, Australia) filter paper. Data analysed by Thermo Scientific™ Chromeleon™ 6.8 Chromatography Data System (CDS), and Origin® Pro 9 (Northampton, MA, USA) software.

Variation of UHPLC instrumentation required alteration of method. Both UHPLC systems operated with a gradient method with mobile phases consisting of an aqueous solvent: 0.1 % formic acid in water (pH 2.7); and organic solvent: methanol. Other operating parameters of the instruments were composited in Table 8 below.

Table 8: UHPLC method parameters for the analysis of ampicillin sodium using a PerkinElmer Flexar™ FX-15 UHPLC and a Thermo Scientific™ Vanquish UHPLC+ Focused system

Parameters	PerkinElmer UHPLC System	Thermo Scientific™ Vanquish System
Injection Volume (µL)		
56 mg.mL^{-1}	0.2	1
16 mg.mL^{-1}	0.5	1
Dilution step	-	1:10
Column	Agilent Zorbax Eclipse Solvent Saver XDB-C18 (3 mm x 100 mm, 1.8 µm)	Thermo Scientific™ Accucore Vanquish C18+ (2.1 mm x 100 mm, 1.5 µm)
Oven Temperature (°C)	40	40
Flow Rate (mL.min^{-1})	0.5	0.3
UV-Vis Detection (nm)	225 and 254	225 and 254
Run Time Length (minutes)	20	20
Equilibrium Length (minutes)	6	10
Mobile Phases Gradient 0.1 % formic acid in H_2O: Methanol ratio	93:7 to 0:100 linearly over 20 minutes	93:7 to 0:100 linearly over 20 minutes

All samples were filtered through 0.22 µm PTFE syringe filters (Adelab Scientific, Thebarton, SA).

2.4 Degradation Product identification by Liquid Chromatography Mass Spectrometry (LCMS)

Two instruments were used in the qualification of degradation products.

1) Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) with a Waters Acquity Photodiode Array (PDA) connected in series with an Electrospray Ionisation (ESI) Waters Synapt HDMS (Waters, Manchester, UK) mass spectrometer. Separation was achieved using an Agilent Zorbax Eclipse XDB-C18 (4.6 mm x 160 mm, 5 µm). Column solvents, 0.1 % formic acid in H_2O and Methanol, were filtered through a vacuum through a 47 mm, 0.22 µm PVDF hydrophilic Millipore Durapore filter paper (Sigma Aldrich, Australia).

Separation was provided by a Waters Acquity UPLC with UV detection using a Waters Acquity PDA connected in series with the MS detection. A degraded sample of ampicillin sodium (initial concentration of 56 mg.mL^{-1}) was analysed using a modified method developed for the PerkinElmer UHPLC (Table 8). These modifications

included: dilution of sample in 1:250 in water (Freeflex® sterile water) for a 1 µL injection, an increase in run time to 21 minutes, flow rate of 0.45 mL.min^{-1}.

Mass spectra were obtained using an ESI mass spectrometer. Mass spectrometer operated in both negative and positive ion mode with a capillary voltage of ± 3 kV and a sampling cone voltage of ± 40 V. The extraction cone voltage, was set to ± 4.0 V, ion source temperature was of 80 °C; desolvation temperature at 350 °C; and the nitrogen desolvation gas flow rate was 500 L.h^{-1}.

Data acquisition was carried out using the Waters MassLynx (V4.1) program. Positive ion and negative ion mass spectra were acquired in the V resolution mode over a mass range of *m/z* 100–800 using continuum mode acquisition.

2) Thermo Scientific™ UltiMate 3000 RSLCnano HPLC system coupled with a Thermo Scientific™ Q Exactive Plus Oribitrap mass spectrometer, supplying accurate mass spectrometry using tandem mass spectrometry (MS/MS). Separation occurred on a Thermo Scientific™ Accucore™ Vanquish™ C18+ (2.1 mm x 100 mm, 1.5 µm) column, with 0.1 % formic acid in H_2O, and 100 % Methanol solvents.

The UHPLC system used the method of the Thermo Scientific™ Vanquish™ system (Table 8). Freshly made and degraded samples of ampicillin sodium (16 mg.mL^{-1}) were frozen with dry ice for interstate transport for analysis. Ampicillin sodium samples were diluted by a factor of 1000 with the aqueous mobile phase (0.1 % formic acid in water (pH 2.7)).

Mass spectra were obtained using an Orbitrap MS instrument. Mass spectra were acquired in the H-ESI (heated electron spray ionisation) positive ion mode with an ESI spray voltage of 3.9 kV and the ion source capillary set at 320 °C. The nitrogen desolvation gas flow rate was 35.2 L.min^{-1} with a desolvation temperature of 350 °C. Collision energy was 25 eV.

Full Scan mode was used, with a range of *m/z* 100-1100, resolution 70,000, and MS/MS resolution of 17,500. Data was analysed using Xcalibur 4.0 and Mass Frontier 7.0 SR2 Master software (Thermo Scientific, Australia).

3. METHOD DEVELOPMENT

3.1 Synopsis

The instability of ampicillin has been investigated for a number of decades, using numerous techniques. Much of the research of the stability of ampicillin has been from techniques such as microbiological assays[27, 53, 57, 59] and iodometric methods[60], [58]. As previously mentioned in Chapter 1.4, these techniques have limitations in the accuracy in measuring the degradation of ampicillin. It was found that there was no consistency in the stability of ampicillin when comparing results between articles of similar methods, and results between the different methods. Over time, more sensitive techniques, such as HPLC and NMR have been developed and used for analysis of drug degradation. However, in the case of ampicillin, much of the research using these techniques are of mixtures of the drug with other compounds[29, 38, 71, 76, 80, 81], and very few on solely ampicillin.

NMR has gained momentum in the kinetic analysis of drugs[66, 67, 89], however there are only few articles published on the kinetic analysis of ampicillin using NMR[24]. ^{1}H NMR is a quick, non-invasive technique, requiring little preparation prior to samples being inserted and can analyze large concentrations. This technique would be beneficial to be used for determining the stability of the drug. UHPLC is a more common in stability-indicating techniques, used in determine the degradation of many drugs[24, 46, 71, 76, 85].

This chapter focuses on developing simple, valid methods for the kinetic analysis of ampicillin sodium using NMR and UHPLC.

3.2 Results and Discussion

3.2.1 Comparison of Kinetic Methods

3.2.1.1 UHPLC Method Development

A UHPLC method was developed for the kinetic analysis of ampicillin sodium degradation using the PerkinElmer UHPLC system. A degraded sample of ampicillin sodium was used to develop a method that provided good separation between the drug and its degradation products (for example, see Figure 8). As noted in Moldoveanu, *et al.*, methylene selectivity, or hydrophobicity selectivity, is a parameter that was developed to characterize the stationary phase capability to separate non-polar molecules in reverse phase chromatography [93]. Certain factors influence the hydrophobicity of molecules, including polarity and steric bulk of the solute. In reverse phase chromatography, as the hydrophobicity of solute increases, the longer the retention time[94]. This means that the more polar solutes will elute faster than non-polar compounds, and that larger molecules would have a long retention time. Therefore, with the case of ampicillin, the compounds that elute before ampicillin sodium, were likely to be more polar than the drug. The compounds which elute after the drug are either less polar, or larger in molecular size. Some degradation products were unable to be completely separated from each other. Using mobile phases of formic acid in water and methanol allowed the method to be transferrable to LCMS used for the identification of ampicillin degradation products.

As ampicillin sodium was analysed in high concentrations (16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$), it was necessary to develop a method without overloading of the column. Due to this, preliminary method development was performed for the lowest concentration, 16 $mg.mL^{-1}$. The optimal injection volume for this concentration was determined to be 0.5 µL. For the highest concentration, 56 $mg.mL^{-1}$, the injection volume needed to be reduced to 0.2 µL. However, the ampicillin sodium peak at this injection volume remained large, with peak tailing. The 0.2 µL dilution was set as a compromise between the kinetic analysis of the drug and the ability to detect degradation products at 225 nm.

A blank of each IV fluid (e.g. water, saline, 5 % glucose, citrate buffer and phosphate buffer solutions) was injected with each run to reduce solvent matrix effects.

Calibration curves of ampicillin sodium were produced; and showed that the peak area of ampicillin sodium was directly proportional to the concentration of the drug. The molar absorptivity of the degradation products was unknown. The degradation products could have had a higher or lower molar absorptivity than ampicillin at the measured wavelengths. Without this data or standards to produce a calibration curve, the concentration of the degradation products at each time interval could not be calculated.

Background effects were observed in the chromatograms of the PerkinElmer UHPLC instrument (Figure 8 a.). A ripple in the baseline and a decrease in absorptivity of the chromatogram could be seen over time. These effects were caused by the duel pump system of PerkinElmer UHPLC instrument, and the gradient method. Water can absorb around 225 nm, which would have caused the decrease in the baseline's absorbance. As the gradient changes, the amount of water is reduced, therefore causing a decrease in the absorbance. The influence of the baseline is significant to the method in which the concentration is calculated. Both peak height and peak width at 50 % can be impacted by the shape of the baseline slope. However, the peak area is not influenced by the baseline slope and was therefore used to calculate the degradation of the drug. Figure 8 is the chromatogram with baseline correction using Origin® 9.0 (Northampton, MA, USA).

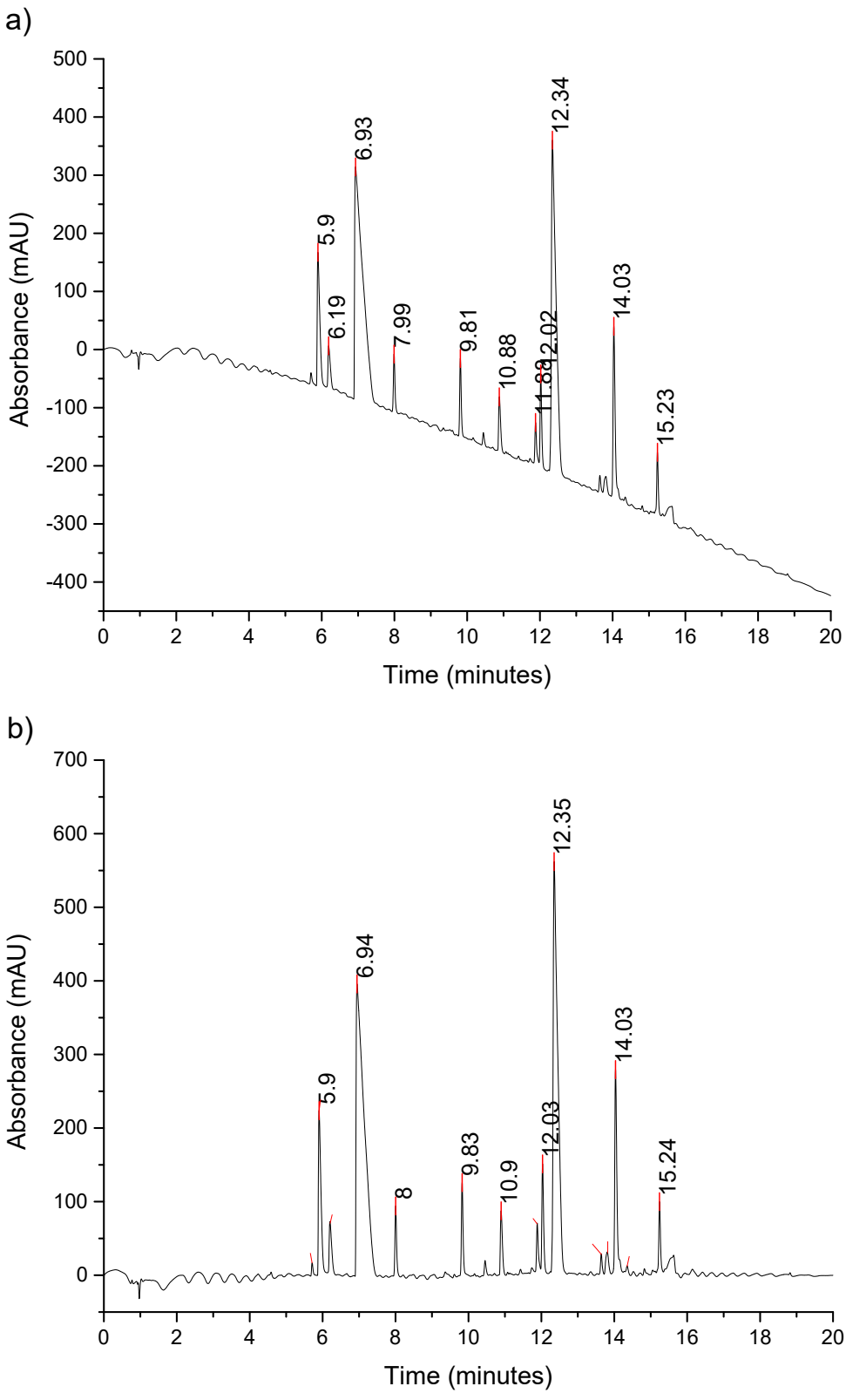


Figure 8: UHPLC chromatogram of a. degraded (215 hours) ampicillin sodium at 6.94 minutes, (initial concentration of 56 mg.mL^{-1}) at a wavelength of 225 nm, and b. with the baseline subtracted.

The contour 3D field plot (Figure 9) indicated how strongly a compound absorbed at a particular wavelength. The blue colour was of a low absorbance and white was strong absorbance. Two wavelengths were selected due to the two main chromophores of ampicillin that absorb in these regions- the carboxylic acid at 225 nm, and the aromatic ring at 254 nm. Figure 9 identified that ampicillin sodium strongly absorbed at 225 nm, and less strongly at 254 nm. Therefore, the 225 nm wavelength was used to obtain the peak area of the drug for the kinetic analysis. However, not all degradation products absorbed at the 225 nm wavelength, but do absorb at 254 nm. Therefore, 254 nm was used to monitor the degradation products.

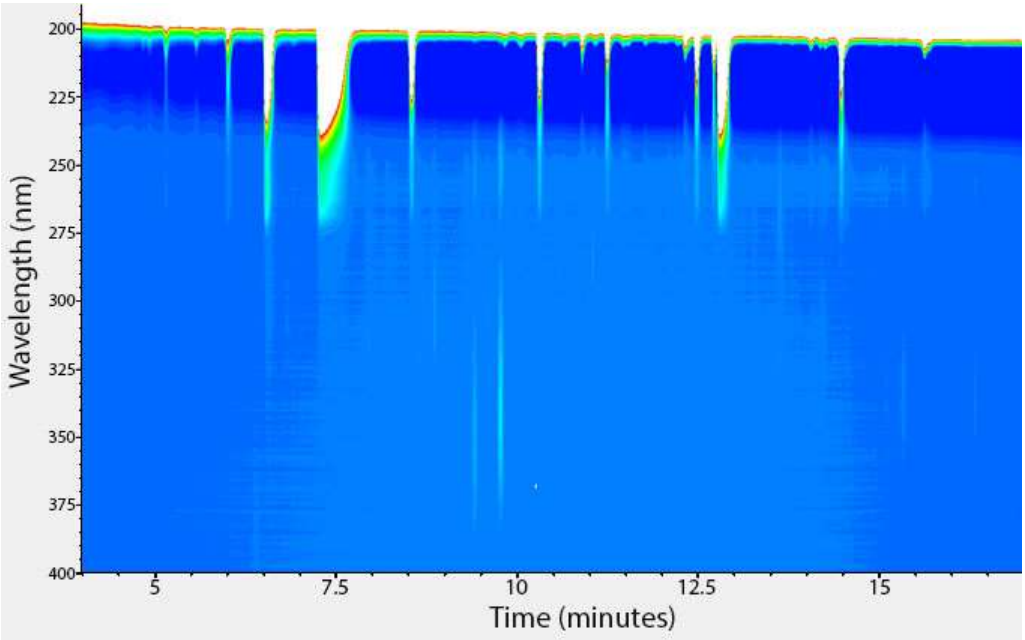


Figure 9: Contour plot of a chromatogram of highly degraded sample of ampicillin sodium, where increasing absorbancy was shown with colour from blue to white .

A change in instrument made it necessary to amend the method. The Thermo Scientific™ Vanquish™ UHPLC system has different parameters e.g. pressure, column pore size and sensitivity. To use this instrument, changes to the PerkinElmer UHPLC method were required. Some of these changes (Table 8) were made to reduce potential damage to the column by overloading the column with the high concentrations of the drug, while reducing the systematic error of a lower volume injected. Comparison of the kinetic analysis of ampicillin sodium using the original method and a new dilution method can be seen in Figure 10 below.

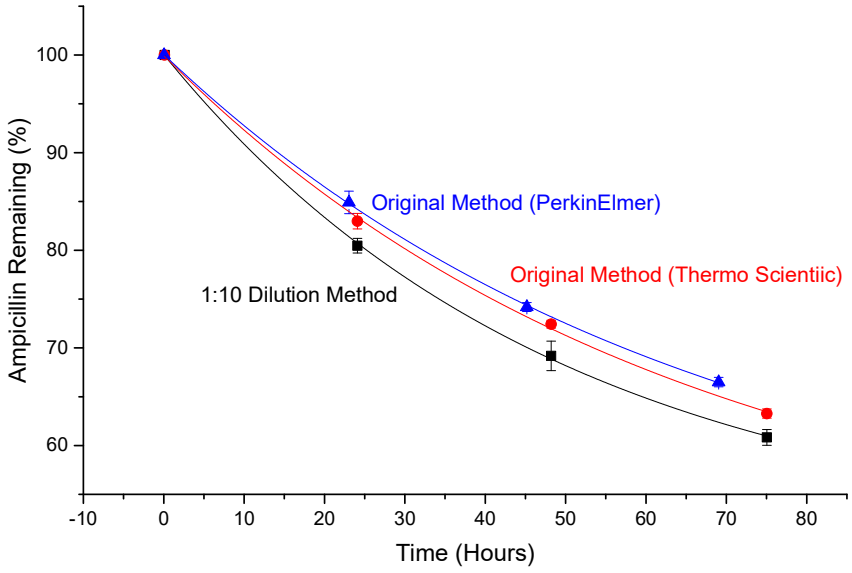


Figure 10: Comparison of the change in UHPLC method between the original method on the PerkinElmer instrument, original method using the Thermo Scientific™ UHPLC instrument and the new method of a 1:10 dilution on the Thermo Scientific™ UHPLC instrument.

For each method, the 90 % shelf life was calculated and compared to the original method in the table below.

Table 9: Shelf Life of various runs of ampicillin sodium in same conditions (16 mg.mL^{-1} at 37 °C).

Sample Method	Rate Constants (Hour^{-1})	Shelf Life Time (Hours) (Ampicillin remaining at 90 %)
Original Method (PerkinElmer)	0.0142 ± 0.004	14.2 ± 1.05
Original Method (Thermo Scientific)	0.0157 ± 0.003	13.4 ± 0.90
1:10 Dilution Method (Thermo Scientific)	0.0202 ± 0.004	11.1 ± 0.97

The original method and the new method, with a 1:10 dilution step, were analysed with samples stored and analysed together to reduce variation in kinetics. The two methods were compared (Figure 10), and showed an increase in the rate constant of the drug, and therefore a decrease in the stability. Some variation in the degradation rate the original method data between the two instruments was observed. This may have been due to additional errors in sample preparation. Overall, the differences in the kinetic rate between methods was within error, and therefore had no significant difference. This change was applied to the analysis of ampicillin sodium in IV container and buffered samples.

3.2.1.2 ^{1}H NMR Method Comparison of Ampicillin Sodium Stability

NMR has been used to characterise ampicillin sodium and degradation products of the drug[47]. However, only a small number of articles have shown NMR used for kinetic analysis of ampicillin sodium. Therefore, the UHPLC method was used to compare to stability analysis by ^{1}H NMR to determine if the method was valid for measuring the stability of the drug.

Identification of ampicillin sodium proton peaks was required for kinetic analysis. The ^{1}H NMR spectrum obtained (Figure 12 a.) was compared to spectra from previous research[24, 87] and the structure of the drug (Figure 11), to determine the ^{1}H peak shifts. The proton peaks were assigned as peaks A-F in order of lowest to highest peak shift (ppm) in the spectrum. The aromatic ring hydrogens, labelled as F, resulted in a multiplet at approximately 7.38 ppm (Figure 12 a.). The peak at 5.42 ppm, which appeared to be a singlet, was determined to correspond to the two hydrogens on the four-membered ring labelled with peak E (Figure 11). The singlets at 4.62 and 4.14 ppm were produced by the hydrogen adjacent to the carbon near the amine group (R-CH-NH_2), labelled as peak D, and the hydrogen attached to the carbon adjacent to the carboxylic acid group (R-CH-CO_2Na), was labelled with peak C. The hydrogen atoms of the methyl groups of the molecule produced two signals, both singlets with a shift of 1.47 and 1.39 ppm. From the figure, the methyl groups have two different chemical shifts. This was likely due to the shielding effects caused by the sulphur on the closest methyl group. Although this is more commonly seen with interactions of hydrogens and nitrogen atoms, the orientation of the lone pair of the electrons on the sulphur atom has been shown to have a significant impact in both ^{1}H and ^{13}C NMR. It is possible that the lone pair interact with the methyl hydrogens *via* hyperconjugation[95]. In this case, the sulphur atom of ampicillin caused the hydrogens closest to the sulphur to have been deshielded, which caused an increase in the chemical shift of the hydrogen signal (1.47 ppm). Therefore, the hydrogens signals, at 1.47 and 1.39 ppm were labelled as peak B (deshielded) and peak A (shielded), respectively.

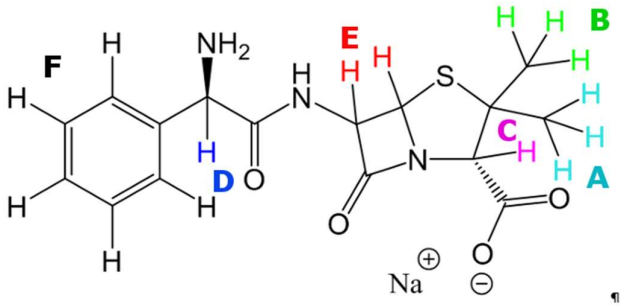


Figure 11: Ampicillin Sodium with ^{1}H NMR peak assignment.

Figure 12 compared ampicillin sodium at a. initial time and b. after 24 hours. After 24 hours, the degradation of ampicillin sodium was observed by a reduction of the intensity of the drug's hydrogen signals, and the increase in number of hydrogen signals observed (Figure 12 b.). As the sample degraded, proton peak D had a shift to the left of the spectrum. Over a longer period of time e.g. over 166 hours, this shift increased. A shift of a peak in the spectrum is an indication of a change in chemical environment. As other peak signals indicated that there was remaining drug, and that there was no hydrogen peak at 4.62 ppm, this shift was likely due to the protonation of the amine group.

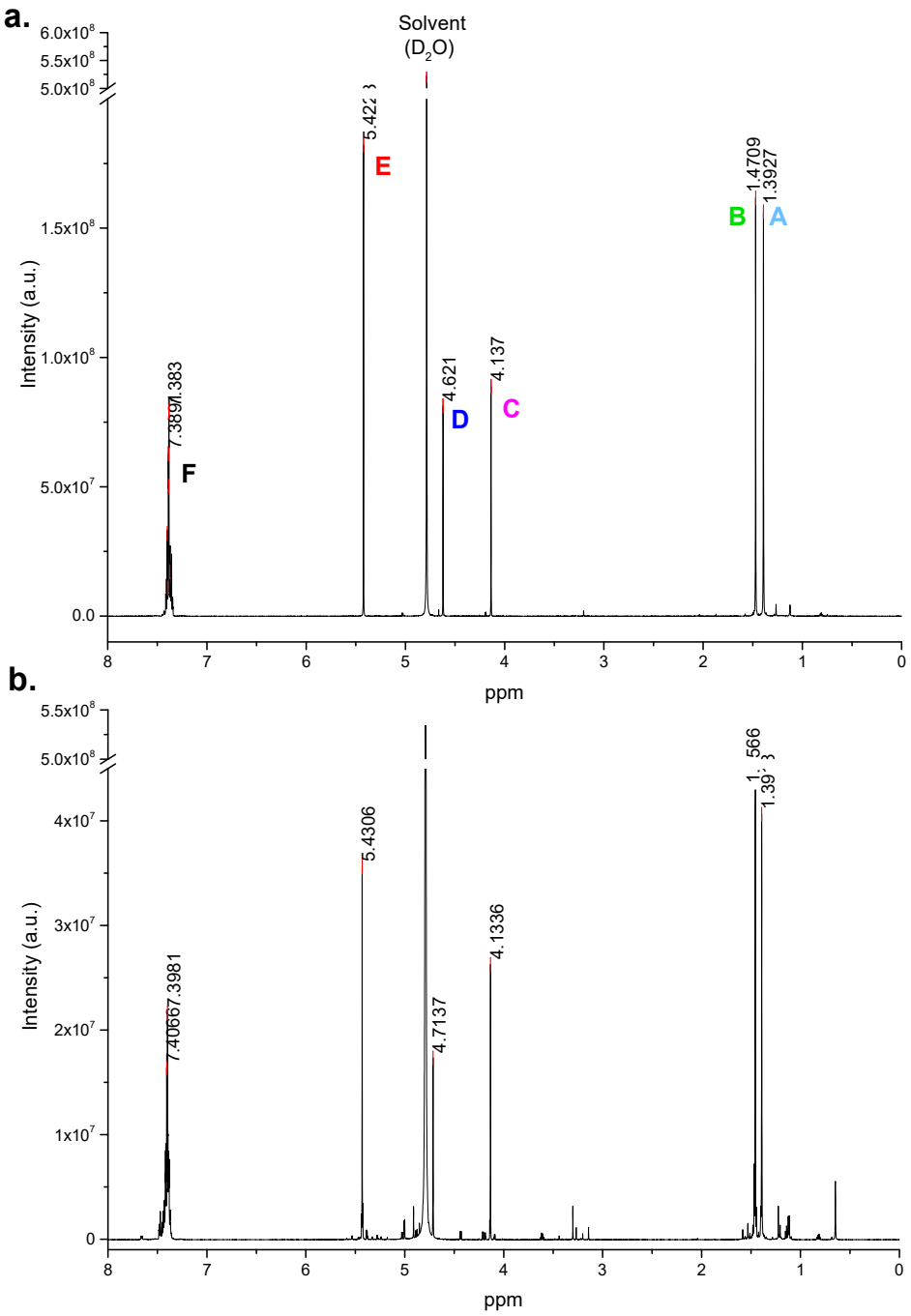


Figure 12: ^{1}H NMR spectra peak assignment of a. non-degraded and b. degraded (24 hours) ampicillin sodium (16 mg.mL^{-1}), at 20 ^{o}C.

An internal standard of acetonitrile (2.1 ppm) was used to analyse all ampicillin proton peaks. As ampicillin sodium degraded the shape and chemical shift (increase in ppm) of peak F changed (Figure 13), and an increase in the number peaks was observed. However, the peaks produced by the aromatic hydrogens had no loss in the overall intensity of the signal. Aromatic rings are known to be highly stable structures, therefore unlikely to break apart during hydrolysis. The shift was likely to

be caused by a change in the chemical environment of these hydrogens; caused by ampicillin sodium degradation.

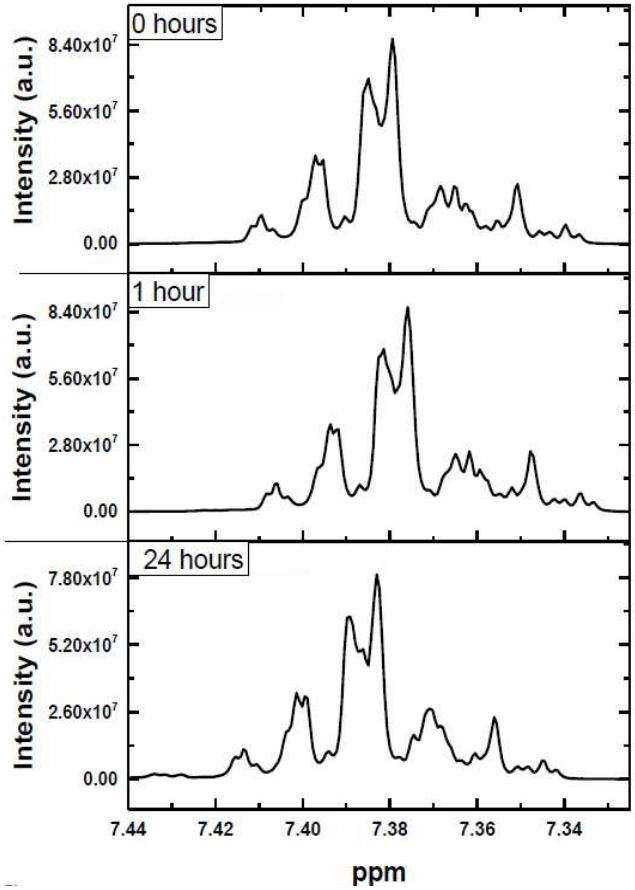


Figure 13: ^{1}H NMR spectra of the change in peak intensity, number of peaks and chemical shift of proton multiplet (peak F) of ampicillin sodium over 24 hours.

For the kinetic analysis, the data for each hydrogen peak was gathered and fitted to an exponential decay. A sample of 56 mg.mL^{-1}, at 20 ^{o}C, was used to display the comparison of the two kinetic analysis techniques (Figure 14). According to the analysis of ampicillin sodium using the ^{1}H NMR method, the drug had a significantly slower rate than that analysed using the UHPLC method (Table 10). The data obtained via the ^{1}H NMR method was calculated to have a larger S.D. (± 1 S.D.) error than that obtained by using the UHPLC method, indicating that there was a possible reproducibility issue with the ^{1}H NMR method.

As seen in previously, but over the longer period of time, there was no significant decrease in the peaks F (Figure 14). This meant that the group of peaks (F) indicated that the degradation rate calculated suggested that ampicillin sodium was “stable” in aqueous solutions. This also indicated that the degradation products of ampicillin

sodium may have had the aromatic ring attached, further discussed in Chapter 5.2.5. The loss in the intensity of the other hydrogen peak signals corresponding to the drug did reduce, and indicated that there was a change in the drug and could not be used to calculate the degradation rate of the drug. Therefore, the aromatic ring hydrogens were not used to determine ampicillin kinetics.

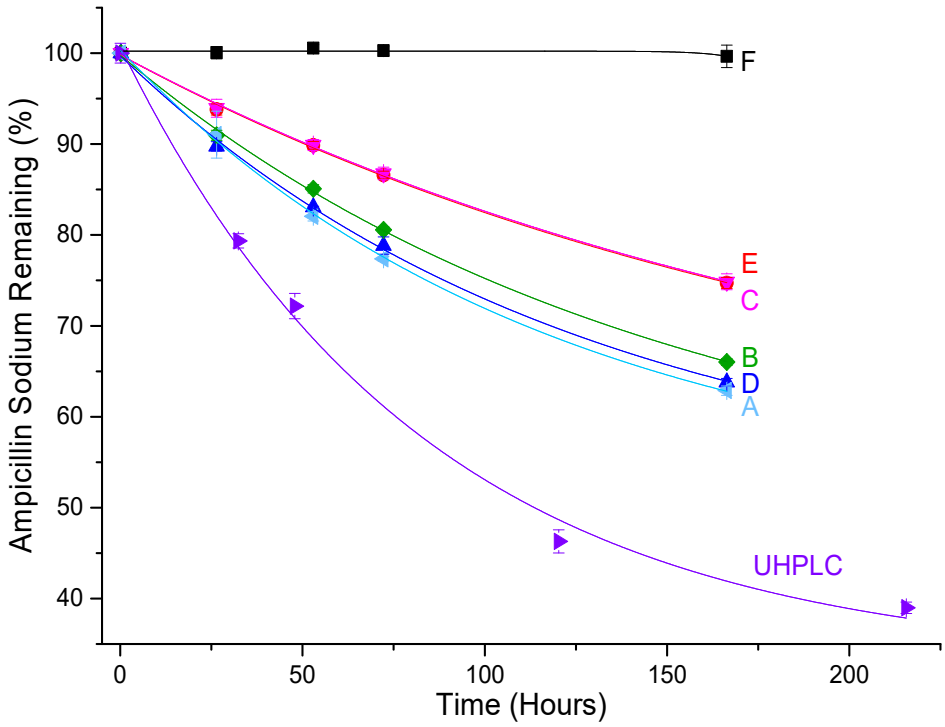


Figure 14: Comparison of NMR individual hydrogen peak kinetics and UHPLC kinetics of ampicillin sodium (56 mg.mL^{-1}) analysed at 20 ^{o}C. Errors calculated as ± 1 S.D.

The shelf life of ampicillin sodium, measured using UHPLC, was almost double of the smallest shelf life produced *via* the ^{1}H NMR method.

Table 10: Comparison of Ampicillin degradation (56 mg.mL^{-1} at 20 ^{o}C) between UHPLC and NMR methods.

Sample/NMR Hydrogen Peak	Rate Constant (Hours^{-1})	Shelf Life (Hours)
UHPLC Method	0.0123 ± 0.0001	14.2 ± 0.28
NMR Hydrogen Peak A	0.00858 ± 0.0009	27.5 ± 2.52
NMR Hydrogen Peak B	0.00681 ± 0.0005	32.2 ± 1.16
NMR Hydrogen Peak C	0.00466 ± 0.002	51.3 ± 4.31
NMR Hydrogen Peak D	0.00792 ± 0.0004	28.0 ± 1.15
NMR Hydrogen Peak E	0.00476 ± 0.0004	50.6 ± 2.45

This slower rate, measured by ^{1}H NMR, was observed for both concentrations and at 5 °C, 20 °C and 37 °C. There are three main reasons which might cause the hydrogen peaks to be "invisible" in the spectrum, which contributed to this observed slower rate; large molecular weight caused by polymerization, rapid exchange of hydrogens and deuterium between the drug and the solvent (D_2O), or peak overlap between ampicillin and at least one of the degradation products. Polymerization is where repeating units of monomers would react with each other to form chains (see ***[figure 5] has been removed due to copyright restrictions***

Figure 5)[96]. In the case of ampicillin monomer units would be single molecules of ampicillin and its degradation products. Polymerization of drugs and natural products have been already observed in literature[35, 36]; e.g., polyphenols result from multiple monomers of flavonoids and phenolic acids in foods such as red wines and teas[97, 98]. Large molecular weight polymers that are invisible to NMR would contain a large number of monomer units, and would likely precipitate out of the solution. This was not visually observed in samples degraded by less than 50 % (Appendix 7.5.1). Therefore, the formation of degradation products with significantly high molecular weights would unlikely be the cause to this observation.

From previous research of the drug, some degradation products e.g. ampicilloic acid and ampicillin oligomers[34] (see Figure 4), have similar structure to ampicillin and therefore potentially have similar chemical shifts in the aromatic and methyl group hydrogens. It was possible that there was an overlap in the peaks of ampicillin sodium and the hydrogen peaks of degradation products. Therefore, the most likely cause to this slower degradation rate was either the hydrogen exchange, the peak overlap, or a combination of both. As the kinetic analysis via ^{1}H NMR did not accurately measure the degradation of ampicillin, the UHPLC method was used to analyse the drug throughout the rest of the research.

Although the NMR analysis did not show a similar trend as UHPLC, the ^{1}H NMR degradation indicated a possibility of two main kinetic trends. From the figure, peaks C and E formed one of these kinetic trends, and peaks A, B and D produce a separate degradation trend. These kinetic trends indicated a possibility of two main degradation pathways, where the degradation of ampicillin sodium effected different chapters of the molecule, one pathway where the molecule at or surrounding protons C and E were most effected, and another for protons A, B and D. The identification of ampicillin degradation products *via* LCMS (Chapter 5.2.1) determined what was being produced, and how those products may affect the ^{1}H NMR peak shifts observed.

3.3 Conclusions

Two methods to kinetically analyse ampicillin sodium degradation were developed. UHPLC separated ampicillin sodium peak from degradation products. Using the peak area of the drug, the degradation was able to be measured. Kinetic analysis of ampicillin sodium using ^{1}H NMR was compared to the UHPLC method. Each proton peak was analyzed, and all peaks indicated a slower degradation rate than that calculated through the UHPLC method. It was determined that the ^{1}H NMR method was not viable for kinetic analysis of ampicillin sodium.

4. KINETIC ANALYSIS OF AMPICILLIN SODIUM

4.1 Synopsis

The stability of ampicillin has been reported to be influenced by many factors such as temperature, concentration, pH, ionic strength and light exposure, the container in which the solution is in, and the structure of the drug itself[26, 27, 30, 57-59]. It has been reported that the higher the concentration and the higher the temperature the drug is exposed to, the faster the rate of degradation occurs[27]. Evidence has been provided to suggest that different IV solutions influence the rate of the degradation of ampicillin sodium[27]. It has been reported that the degradation of the drug is increased in 5 % dextrose solution; compared with the stability of ampicillin sodium in sterile water and saline solutions[62, 90]. Buffers, which are used to control the pH, have also been shown to reduce the instability of the drug[39, 55]. The intravenous containers could be a source of stabilising the drug. The containers can be made of several different types of polymers and structure compositions. The differences in mechanical properties could influence the interaction of the drug with the polymer containers.

This chapter focuses on determining the kinetic rate and shelf life of ampicillin sodium at high concentrations, and the effects of conditions simulating those which the drug would be exposed to in a patient's home; range of temperatures, IV solutions and IV container compositions.

4.2 Results and Discussion

4.2.1 Effect of Temperature and Concentration on Ampicillin Sodium in Water

Concentration and temperature can have a significant effect on the rate of degradation of ampicillin[51, 57, 59]. Here, the effects of temperature and concentration of ampicillin sodium in water has been analysed. Three temperatures: 5 °C, 20 °C and 37 °C, and two concentrations: 16 mg.mL^{-1} and 56 mg.ml^{-1} were selected. These three temperatures represent relevant conditions, namely refrigerated, room temperature and body temperature. The two concentrations are of the lowest and highest IV infusion concentrations that have been identified for the HITH program. These samples were stored in glass vials. Using the UHPLC method, the kinetic analysis of the percentage of ampicillin remaining at different time intervals for the temperature and concentration parameters was plotted (Figure 15).

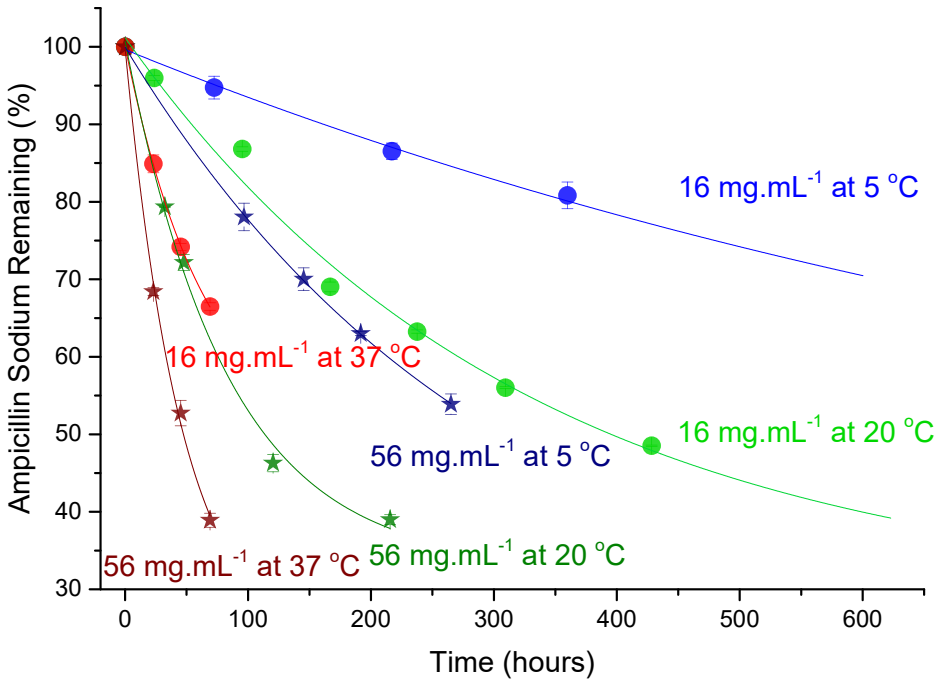


Figure 15: Percentage of remaining ampicillin sodium for the comparison of the effects of temperature (5 °C, 20 °C, and 37 °C) and initial concentration (16 mg.mL^{-1} and 56 mg.mL^{-1}) of the drug, fitted with the exponential decay. Errors calculated as ± 1 S.D.

The samples were analysed until a minimum of approximately 50 % ampicillin sodium had degraded. This was to have a sufficient amount data, to accurately calculate the shelf life (Table 11). At 5 °C, ampicillin at the concentration of 16 mg.mL^{-1} did not

degraded over 50 %, however the samples were analysed for a period of over 600 hours. The rate of degradation was significantly slower and the trend line resembled a linear line. However, this sample was treated the same as the others with the exponential curve function. The combination of these factors, linearity and slow degradation rate, resulted in a larger error when calculating the rate constants and shelf lives.

An increase in concentration and temperature led to a higher degradation rate of the drug. This result was expected, as the increase in concentration allowed for more molecules of the drug to interact within solution, producing a larger decrease in the remaining ampicillin concentration. As the concentration decreases, less interactions occur, therefore slowing down the rate.

The higher temperatures, accelerated the rate of the degradation, which has been commonly observed in the degradation of penicillin drugs. The trends observed (Figure 15) showed that initial concentration had a significant influence on the rate of degradation. Although the exponential decay fit is similar to first order kinetics the result supports that degradation of the drug is not a simple mechanism. This has also been described in the proposed mechanism derived from ampicillin degradation products identified *via* LCMS (Figure 42 in Chapter 5.2.1). From the rate constants of the samples, the corresponding shelf lives were calculated (Table 11). It was determined that at 5 ^{o}C both initial concentration, 16 mg.mL^{-1} and 56 mg.mL^{-1}, would last a 24 hours infusion period. Similarly, 16 mg.mL^{-1} at 20 ^{o}C could also last for this period. The 24 hours infusion is ideal for the HITH program.

Table 11: Comparison of the calculated, rate constant, shelf life and pH values, of ampicillin sodium at two initial concentration (16 mg.mL^{-1} and 56 mg.mL^{-1}) and three temperatures (5 ^{o}C, 20 ^{o}C, and 37 ^{o}C).

Sample (initial concentration and temperature)	Rate Constant	Shelf Life Time (90 % of drug remaining)	pH		
	Seconds^{-1}	Hours	Initial	Final	Difference
56 mg.mL^{-1} at 5 ^{o}C	19.6 ± 1.08	40.7 ± 3.45	9.46	8.19	1.27
56 mg.ml^{-1} at 20 ^{o}C	43.6 ± 0.63	14.2 ± 0.28	9.49	7.96	1.53
56 mg.mL^{-1} at 37 ^{o}C	76.8 ± 6.69	6.69 ± 0.24	9.87	7.96	1.91
16 mg.mL^{-1} at 5 ^{o}C	3.59 ± 1.07	163 ± 22.4	9.17	8.37	0.80
16 mg.mL^{-1} at 20 ^{o}C	10.0 ± 0.21	49.3 ± 8.91	9.18	7.93	1.25
16 mg.mL^{-1} at 37 ^{o}C	54.3 ± 14.4	14.2 ± 1.05	9.47	7.98	1.49

The pH of the samples was analysed to determine if it was a significant factor in ampicillin degradation. It has been previously reported that different degradation pathways take place in different pH conditions[40, 42]. Ampicillin has been reported to be more stable in some pH ranges more than others[26]. The initial and final pH was reported, to the difference in pH over the degradation analysis. The initial pH was observed to vary between the two concentrations, with a pH of 9.17 and 9.46 for 16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$, respectively, at 5 °C. This was most likely due to the higher concentration of ampicillin sodium causing an increase in the pH. The high temperature, 37 °C, also showed an increased the pH of both concentrations, at 9.47 and 9.87 for 16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$, respectively. This may have been caused by a higher solubility of the drug, as higher temperatures can increase solubility of compounds in aqueous solutions.

Over the course of the degradation the pH of the solution decreased. From the difference in pH, the results showed that the it is relative to the amount of degradation of the drug. Both 20 °C and 37 °C ampicillin sodium (56 $mg.mL^{-1}$) degraded at different rates, the two samples were analysed to have a final pH of 7.39 when ~39 % of ampicillin sodium was remaining. The sample at 37 °C had a larger pH change over the course of its degradation period. This indicated that final pH may be proportional to the percentage of ampicillin remaining. Ampicillin has a pKa value of 2.5 and 7.3 for the carboxylic acid group and amine group, respectively [99]. If the amine group was being hindered or consumed and there were more free carboxylic acid groups in solution, this could explain the increase in acidity of the samples.

4.2.1.1 Analysis of Ampicillin Sodium Degradation When Frozen

It was determined that the rate of degradation of ampicillin sodium is influenced by temperature; the lower the temperature, the greater the stability. The stability of ampicillin sodium in freezing temperatures (- 20 °C) was analysed to determine if freezing the samples would increase the stability. Aliquots of solutions of ampicillin sodium (16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$) in water were frozen at -20 °C. These aliquots were defrosted immediately prior to dilution and injection of the samples into the UHPLC instrument. Figure 16 showed the kinetic trend of the stability of ampicillin sodium.

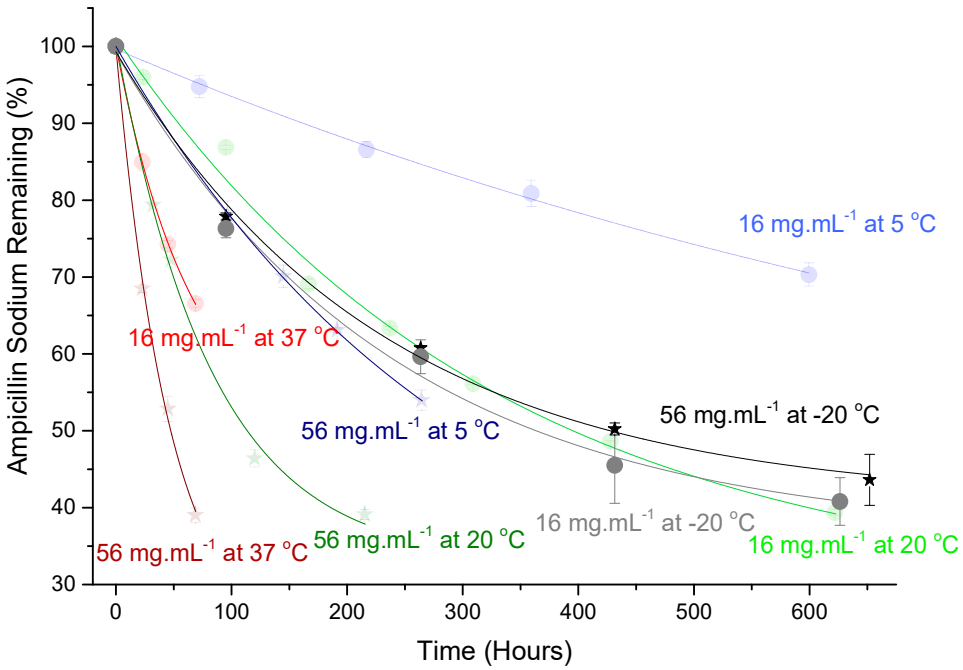


Figure 16: The calculated degradation trends of 16 and 56 mg.L^{-1} ampicillin sodium, at -20 °C, compared to degradation of the drug at temperatures of 5 °C, 20 °C, and 37 °C, with calculated exponential decay trends. Errors calculated as ± 1 S.D.

The rate constants and shelf lives were calculated (Table 12). The lower initial concentration of 16 mg.mL^{-1} showed a decrease in stability when compared to the refrigerated temperatures. The higher initial concentrations of the drug (56 mg.mL^{-1}) had a similar shelf life and rate constant, within error for both. However, the degradation of the drug at – 20 °C produced different values final pH.

Table 12: Rate constant, shelf life and pH values of ampicillin sodium (16 mg.mL^{-1} and 56 mg.mL^{-1}) analysed at temperatures of -20 and 5 °C

Sample (initial concentration and temperature)	Rate Constant	90 % Shelf Life	pH		
	Seconds^{-1}	Hours	Initial	Final	Difference
56 mg.mL^{-1} at 5 °C	19.6 ± 1.08	40.7 ± 3.45	9.46	8.19	1.27
56 mg.ml^{-1} at -20 °C	15.7 ± 2.52	40.1 ± 1.94	9.52	8.81	0.71
16 mg.mL^{-1} at 5 °C	3.59 ± 1.07	163 ± 22.4	9.17	8.37	0.80
16 mg.mL^{-1} at -20 °C	15.1 ± 0.12	37.8 ± 2.42	9.21	8.72	0.49

As ampicillin sodium is a salt, it was possible that it behaved in a similar fashion as salt water. Salt water freezes by the water freezing, pushing the salt into a smaller area, increasing concentration. This continues until the solution completely freezes into a non-homogenous solid; with the salt in the centre of the solid. If ampicillin sodium behaved the same as salt water, ampicillin would increase in concentration, and therefore increase degradation rate prior to forming the fully frozen solid. This

method could explain the possible mechanism in which the low concentration has a similar degradation rate as the high concentration at – 20 °C. The 56 mg.mL^{-1} initial concentration is near the reported solubility threshold of the drug[99], and the 16 mg.mL^{-1} initial concentration samples could be forced into saturation.

Chromatograms of samples stored at -20 °C, for both concentrations, were compared to a sample stored at 37 °C, Figure 17. The results indicated that the mechanism in which ampicillin degraded was affected by the change in temperature, as a number of degradation products were not observed. Some degradation product peaks seemed to show a decrease in peak area, while others increased in peak area, when compared to the higher temperature sample. The pH of samples stored at -20 °C, had small differences between the initial and final pH values, which indicated that the change in the mechanism may affect the change in pH. Again, the two pKa values of the functional groups could be influencing this pH. If these compounds that are forming from the degradation of ampicillin have very little interaction at the amine group, the pH of the solution is less likely to increase in acidity.

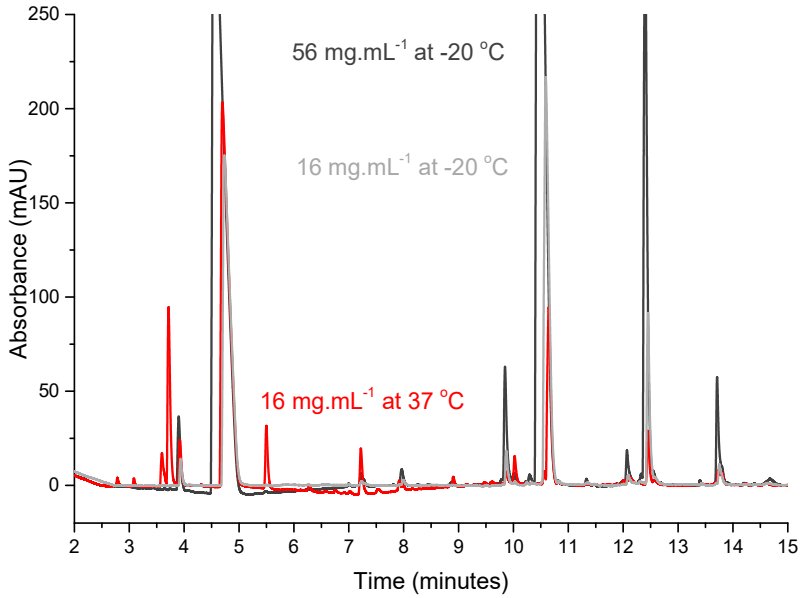


Figure 17: UHPLC chromatograms depicting the degradation products present of ampicillin sodium stored at a temperature of – 20 °C (16 mg.mL^{-1} and 56 mg.mL^{-1}) and compared to the chromatogram of a sample of ampicillin sodium (16 mg.mL^{-1}) at 37 °C, in water. Errors calculated as ± 1 S.D.

4.2.2 Effect of IV Fluids on the Degradation of Ampicillin Sodium

A test (Figure 18) of a low concentration of ampicillin sodium was performed and analysed to compare to previously reported results in different commonly used IV

solutions[27]. From these previous studies, it had been recorded that saline and citrate buffer (pH 7) increase the stability of the drug when compared to water, however 5 % glucose solution would decrease the stability[58, 62]. Figure 18, showed that the citrate buffer (pH 7) did increase the stability of ampicillin sodium, increasing the shelf life by approximately 5 hours over water (Table 13). The 5 % glucose solutions did decrease the stability of the drug, producing the least stable result out of the four solutions tested. The saline solution also had a large decrease in ampicillin sodium's stability, in comparison to water.

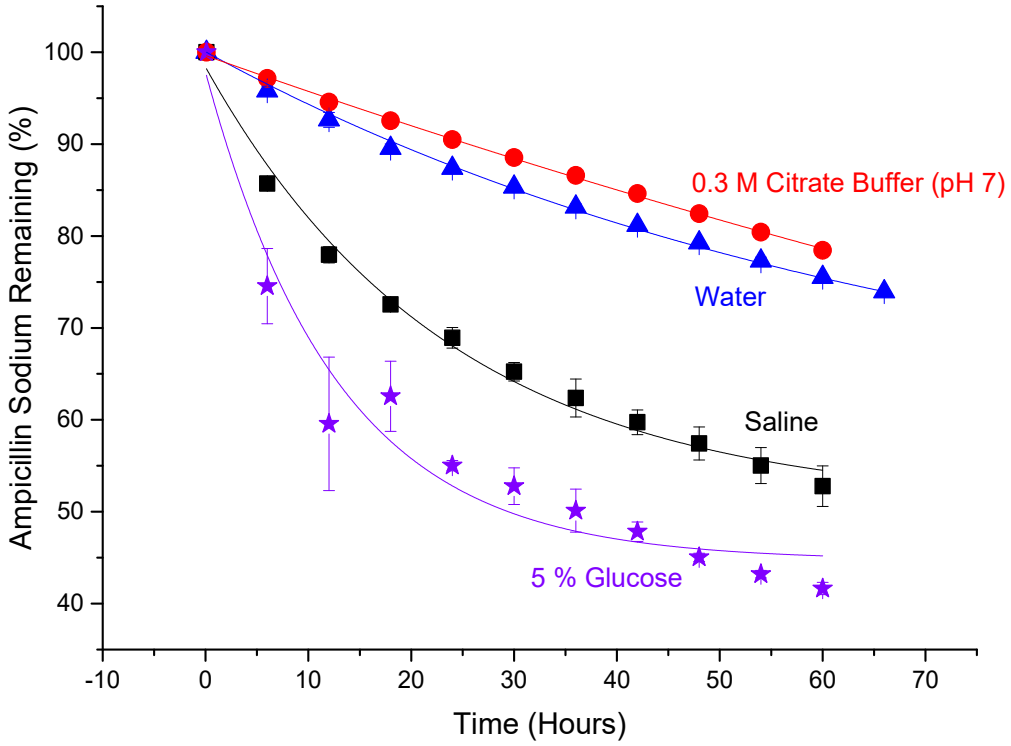


Figure 18: Comparison of degradation kinetics of ampicillin sodium (1 mg.mL^{-1}) at 37 °C in solutions of water (blue), saline (black), 5 % glucose (purple) and citrate buffer at pH 7 (red), and each data's exponential decay fit. Errors calculated as ± 1 S.D.

Table 13: Rate constant, shelf life and pH profile of ampicillin sodium (1 mg.mL^{-1}) in water, saline, citrate buffer (pH 7.5) and 5 % glucose IV solvents.

Sample (IV Solvent)	Rate Constant	90 % Shelf Life	pH		
	Seconds^{-1}	Hours	Initial	Final	Difference
Water	51.1 ± 2.88	18.2 ± 0.219	8.92	7.35	1.57
saline	137± 3.6	4.60 ± 0.202	8.63	7.45	1.18
0.3 M Citrate Buffer pH 7.5	20.1 ± 14.4	25.4 ± 0.644	7.90	7.66	0.24
5 % Glucose	269 ± 36	2.00 ± 0.341	8.37	6.86	1.51

As the pH of the solutions were analysed, further insight to the results was provided. The initial pH of the solvents all increased after the ampicillin sodium was added to make a 1 mg.mL^{-1} solution. The saline and water increased from pH 6.86 (saline) to 8.92 (saline + ampicillin) and pH 6.28 (water) to 8.63 (water + ampicillin), respectively. The citrate buffer also had a slight increase in the pH from 7.5 to 7.9 with the addition of ampicillin sodium to the solution.

The pH was taken at a similar time point, and not the same percentage of ampicillin sodium remaining for the samples. From Table 13, the citrate buffer sample had the lowest pH change, and suggested that the buffer was controlling ampicillin sodium. This was likely due to the buffer ranging closer to the pKa value of the amine group, causing a higher stability than other solvents. The 5 % glucose and the water solvents had the largest change from the initial to final pH. The result for the 5 % glucose solvent was expected, as it degraded the fastest out of the different solutions. Ampicillin sodium in water was the second most stable sample analysed, however it had the greatest pH change. To understand why this result occurred, the chromatograms of the four different solvents were analysed in Figure 19.

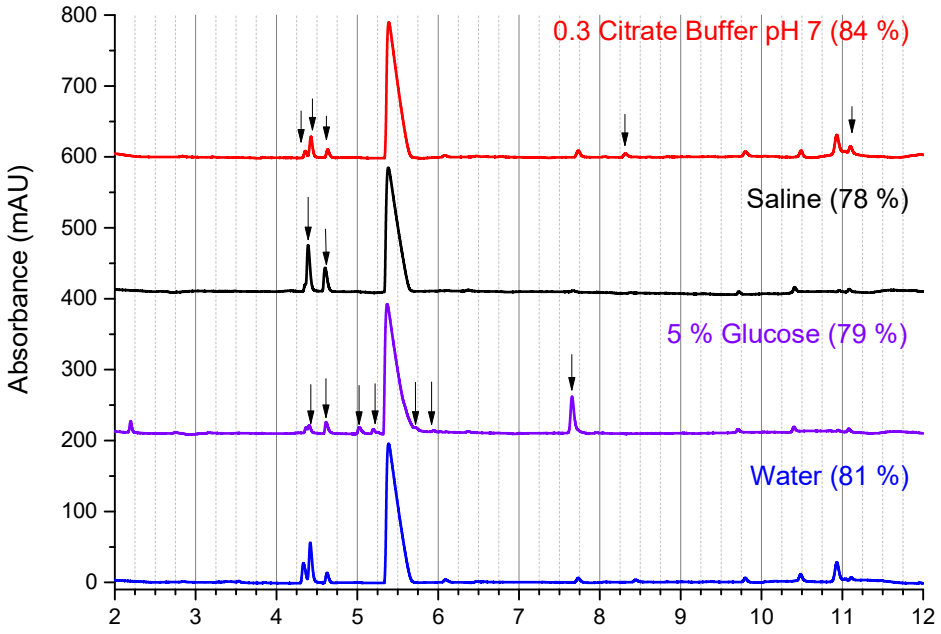


Figure 19: UHPLC chromatograms of 1 mg.mL^{-1} ampicillin sodium at 37 °C in water, saline, 5 % glucose and citrate buffer (pH 7) solvents, at recorded remaining percentages of ampicillin sodium. Arrows indicated differences of degradation products compared to those observed in the ampicillin sodium in water sample.

The concentration of the drug is over 10 times lower than that of the concentrations analysed in Chapter 4.2. Due to this, it was assumed that it was possible that not all degradation products would be the same as those identified using LCMS on the higher concentrations; as concentration is a factor of some of the products formations, for example ampicillin oligomers[40]. Additional peaks were detected in samples of 0.3 M citrate buffer at 8.28 minutes, and 5 % glucose were at 5.01, 5.2, 5.73 and 5.9 minutes.

The chromatograms showed that the 5 % glucose solution had a significant increase of one degradation product at 7.79 minutes, over the water, saline and citrate buffered samples. The exponential curve also did not fit the data of 5 % glucose samples well. This was likely due to the slower retention time of the sample in the UHPLC instrument. The ampicillin sodium peak in the 5 % glucose solution has a slower retention time than those in the other three solvents tested, as well as a different peak shape. The peak shape and retention time difference was most likely caused by the 5 % glucose solution injected onto the column. A number of degradation product peaks were identified within the tailing of this peak (at 5.73 and 5.9 minutes), which would influence the peak areas, and therefore underestimate the degradation rate of the drug. Due to the issues with the exponential fit caused by

the 5 % glucose IV fluid and the agreement with previous research, samples containing ampicillin sodium in 5 % glucose was not tested further for the HITH program.

The 0.3 M citrate buffer had the highest percentage of ampicillin remaining and therefore, a reduced formation of most of degradation products, over the other IV fluids. Ampicillin sodium in saline showed an increase in the peak areas of the degradation products which have retention times of 4.4 and 4.6 minutes, with a lower peak area of the degradation product at 4.35 minutes. These compounds were identified in Chapter 5.2.1.1. The pH change was not as significant in the saline samples, when compared to that observed with the water and 5 % glucose samples, it is thought that this degradation may not have caused a significant change in the pH. However, certain degradation products may influence the change in the pH over others.

4.2.2.1 Kinetic Analysis of Ampicillin Sodium in Saline Solvent for HITH

Saline solutions are one of the most common IV infusions solutions used[100, 101]. For the HITH, ampicillin sodium was analysed at higher concentrations (16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$), at temperatures of 5 ^{o}C and 37 ^{o}C. The results of ampicillin in saline solutions were compared to the results of the water IV fluid, and was found to have decreased the stability of ampicillin sodium. This was not unexpected due to previously observed results in Figure 18. With the larger concentrations of ampicillin sodium (16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$), there was a larger decrease in the kinetic trend of the drug, for the lower concentration (1 $mg.mL^{-1}$).

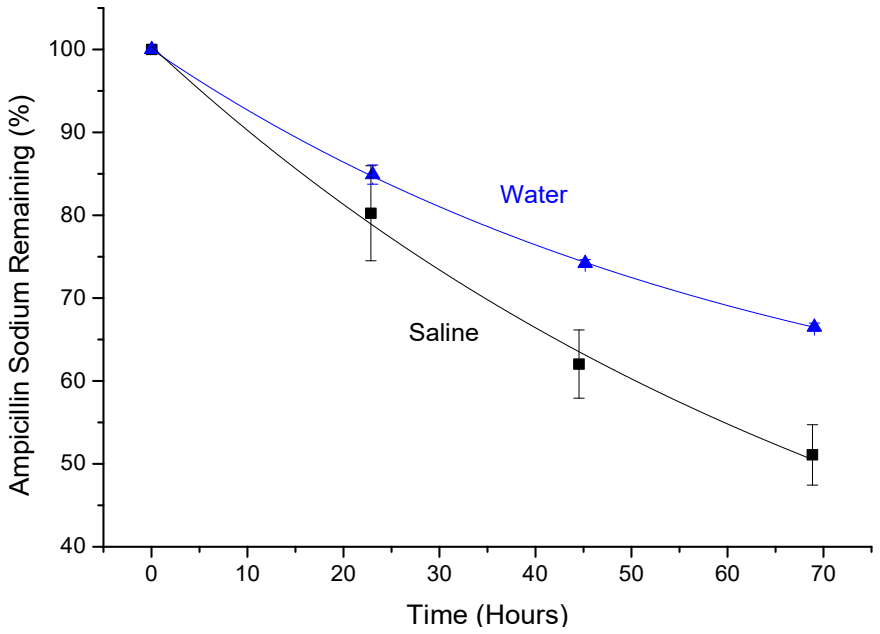


Figure 20: Comparison of the degradation calculated for ampicillin sodium (16 mg.mL^{-1}) degradation in water and saline solutions, at 37 °C, fitted with an exponential decay. Errors calculated as ± 1 S.D.

Table 14: Comparison of ampicillin sodium rate constants and shelf life values between samples of water and saline solutions.

Sample (initial concentration and temperature)	Rate Constant (Seconds^{-1}) and 90 % Shelf Life (Hours)			
	Water		Saline (0.9 % NaCl)	
	Rate Constant	$t_{90\,\%}$	Rate Constant	$t_{90\,\%}$
56 mg.mL^{-1} at 5 °C	13.4 ± 1.80	40.7 ± 3.45	19.0 ± 1.80	27.5 ± 3.56
56 mg.mL^{-1} at 37 °C	76.7 ± 7.2	6.69 ± 0.35	91.1 ± 18.0	4.79 ± 0.50
16 mg.mL^{-1} at 5 °C	3.59 ± 1.08	163 ± 22.4	8.14 ± 1.80	121 ± 13.0
16 mg.mL^{-1} at 37 °C	51.1 ± 14.4	14.2 ± 1.05	94.0 ± 1.80	10.5 ± 2.69

The rate constants of the saline solutions were larger than that of the water samples. The saline solutions negatively impacted the stability of the drug. However, the shelf life, calculated using the exponential fit showed little difference of the stability; the number of hours between the saline and water. At 37 °C, the both 16 mg.mL^{-1} and 56 mg.mL^{-1} samples of ampicillin sodium showed no significant difference. At 5 °C, the shelf life of the drug with an initial concentration of 56 mg.mL^{-1} had a significant decrease when compared to water samples of the same temperature.

Table 15: pH studies of ampicillin sodium at various temperatures and concentrations in saline IV solvent.

Sample (initial concentration and temperature)	pH		
	Initial	Final	Difference
56 mg.mL^{-1} at 5 °C	9.38	8.27	1.11
56 mg.mL^{-1} at 37 °C	9.70	7.82	1.88
16 mg.mL^{-1} at 5 °C	9.25	8.25	1.00
16 mg.mL^{-1} at 37 °C	9.38	7.60	1.78

The pH values of water and saline were recorded as pH 6.86 and pH 6.28, respectively. Throughout all concentration and temperature variables, the initial pH of ampicillin sodium in saline (Table 15) was lower than the initial pH of water (Table 12). The difference in pH between these solvents became larger with the addition of ampicillin sodium; saline solvent controlled the change in pH better than water. Previous reports suggested that ampicillin sodium is less stable at a higher pH, and is more stable in acidic and neutral pH; most stable at approximately pH 5[26], due to the pKa of the amine group and carboxylic acid group. Therefore, if the pH had a large influence on the degradation rate, with the lower pH, ampicillin sodium in saline solutions should have increased the stability of the drug. However, this was not the case. It was thought that the difference in pH was not a significant factor in the degradation of ampicillin sodium in saline. Although it remained a factor in the degradation of the drug, the pH was determined to not have a significant influence in these samples; it was thought that the ionic strength of the solution could have played a larger role in the decrease in the concentration of ampicillin sodium.

Previous research[102] has indicated that the ionic strength of the solutions can affect the stability of drugs. Using potassium penicillin G, Lindsay and Hem[102], showed that changing the ionic strength and pH of sodium chloride solutions did influence the kinetic rate of the penicillin. Hou and Joole[26] showed that salt of similar charges could have an effect on the kinetic rate of ampicillin. Therefore, it was possible that saline solutions have an increase in the ionic strength of the solution.

**With simple S_N2 nucleophilic reactions, Pham and Westaway[103] indicated that the transition state of some reactions could be influenced by the ionic strength of the solvent. A number of previously identified degradation products of ampicillin have reactions at the benzene adjacent amino group[42], e.g. oligomeric and diketopiperazines (*[figure 5] has been removed due to copyright restrictions*

Figure 5). A higher ionic strength was shown to increase the charges of nucleophilic atoms, effecting the polarity of molecule. It was observed that the more ionic

transition state was found at the higher ionic strength, as it was more stable in the ionic solvent, which also increased solubility. If this system was applied to the case of the ampicillin sodium in saline samples, it was surmised that the charge on the amine group increased, causing a change in the stability of the compound, and formation of degradation products.

As the ionic strength could have influenced the solubility of the drug, initial peak areas of ampicillin in the various conditions were analysed. The peak areas (mAU*minute) of samples at initial concentrations of 16 $mg.mL^{-1}$ and at the same temperatures e.g. 37 ^{o}C, gave no indication of insolubility of the drug when compared to water. However, this may have occurred for the 56 $mg.mL^{-1}$ samples, which had a higher peak area of the initial (100 % ampicillin) samples in water than in saline. Hou and Joole also determined that the effect of ionic strength on the stability of ampicillin was dependant on pH[26]. Therefore, the pH would have an indirect influential factor on the stability of the drug.

4.2.2.2 Kinetic Analysis of Ampicillin Sodium in Sodium Citrate Buffer

Previous research has determined that the degradation rate of ampicillin is often slowed with the addition of a buffer[26, 39, 54, 55]. Sodium citrate was chosen as a buffer due to the availability of the pre-made IV bags for the pharmaceutical company and for the pH range the chemical can control. Similarly, to saline and water samples, the pH of the solution was observed. Previous research has indicated that ampicillin was the most stable at a pH range of 4.5-6[26, 54, 55]. For IV infusion, the fluid is more versatile at a neutral pH, so that it can be used at many more IV location sites on the body. Therefore, the citrate buffer was made and tested in pH 5 and 7.

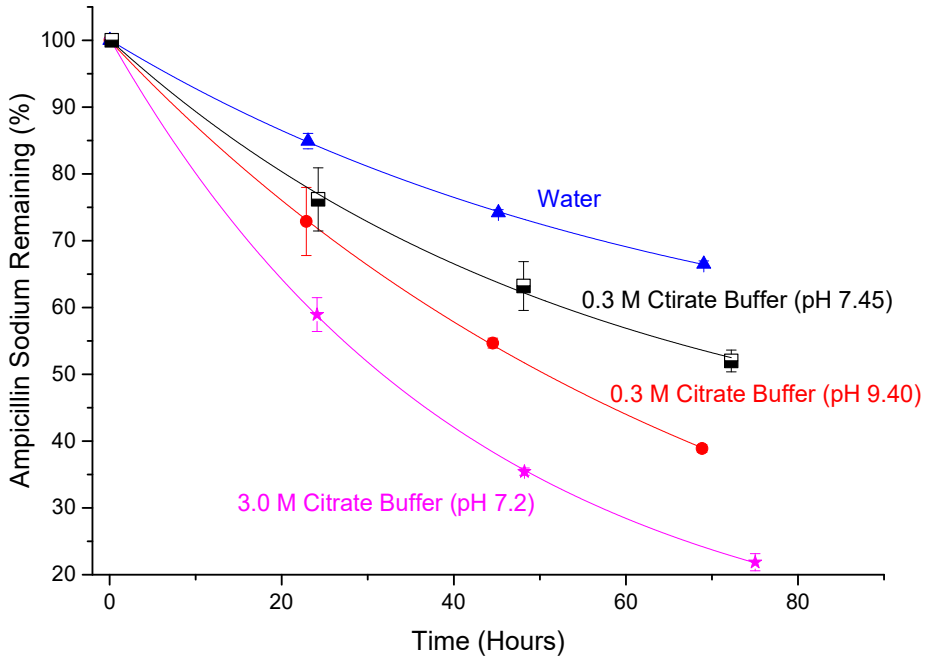


Figure 21: Degradation of ampicillin sodium (16 mg.mL^{-1}), at 37 ^{o}C, in varying conditions of citrate buffer at pH 7; 0.3 M citrate buffer (pink), 3.0 M citrate buffer (red), 0.3 M citrate buffer where the pH was controlled after addition of ampicillin sodium (black), and water (blue), fitted with exponential decay. pH values in brackets represent initial pH of solution after ampicillin added. Errors calculated as ± 1 S.D.

The current medicinal method of buffering drugs in IV containers, is that the concentration of citrate buffer is made up to the correct pH, and then the drug is added. Using this method, the citrate buffer (0.3 M) (Figure 21, in red) caused an increase in the degradation rate of ampicillin when compared to water. This method of the buffer did not control the pH of the samples. With addition of ampicillin sodium to the solution, the neutral (pH 7) solution increased to a pH of 9.40. In attempt to improve the stability of the drug, the pH was corrected with citric acid after the drug had been reconstituted (Figure 21, in black). This difference in preparation of the sample displayed an increase in the stability, however did not improve over the stability identified in the water samples.

A sample with an increase in the buffer concentration, by a factor of 10, was tested to determine if increasing the buffer concentration could improve the stability of the drug. The 3.0 M citrate buffer still had an increase in the pH after ampicillin was added, from pH of 7.2 to 8.39. This increase in the pH could be contributed to the starting pH (7.2), as it was on the high end of the pH range that citrate can be used effectively as a buffer. A significant result for this sample was that the increase in

citrate concentration produced a precipitate. A similar effect occurred in samples of 0.3 M citrate buffer at a pH 5.2, where a white precipitate formed in both concentrations and temperatures measured. This precipitate was collected, dried, redissolved in D_2O and characterised by proton NMR, see Figure 22.

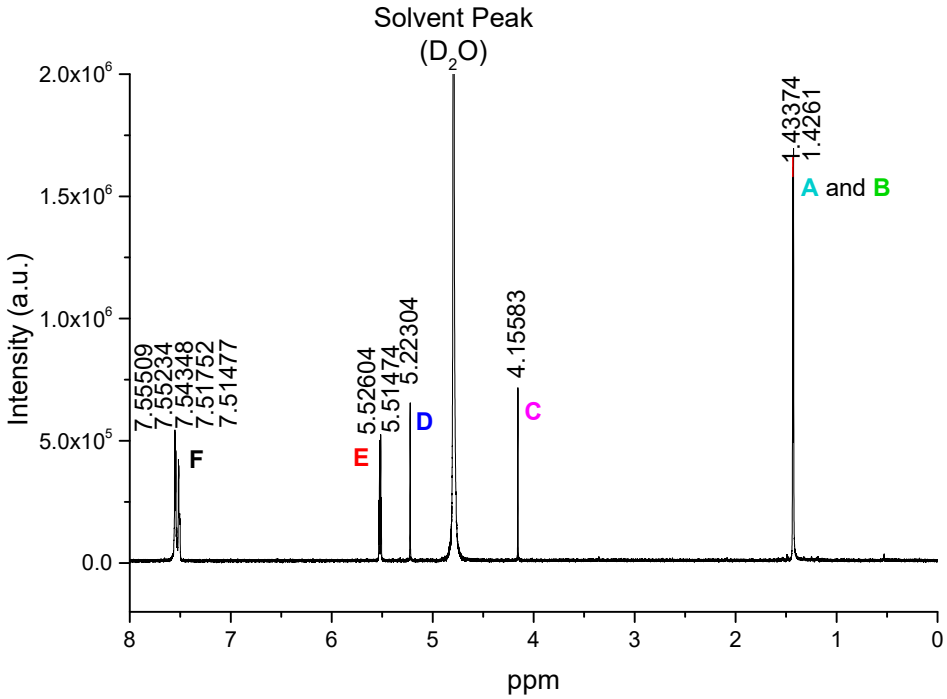


Figure 22: Proton NMR of precipitate formed in solution of ampicillin in sodium citrate buffer at pH 5.5.

The compound observed has a similar structure to ampicillin sodium; peak shift similar to that of the Peaks A, B, C and E from proton NMR of ampicillin sodium, see Figure 12 a. in Chapter 3.2.1.2 for comparison. The proton peaks F and D seem to have a significant chemical shift, from approximately 7.4 to 7.5 ppm for the aromatics and 4.6 to 5.2 ppm for Peak D. The methyl groups formed one peak at 1.43 ppm, rather the than two peaks seen in the ampicillin sodium proton NMR spectrum, Figure 12 a. These chemical shifts indicated that a change in the chemical environment of these protons had occurred. It was likely that the precipitate was produced by the interaction of ampicillin with the sodium citrate/citric acid buffer. It was surmised that the decrease in pH caused the protonation of the amine group in ampicillin, allowing the binding of the citrate to the molecule, and therefore causing a shift in the proton Peak D (Figure 22). Due to the close proximity, a shift was also observed in the aromatic proton peaks (F).

This precipitate formation caused a significant decrease the concentration of ampicillin sodium in solution, by altering the solubility, therefore reducing the amount of the drug remaining in solution. As a precipitate formed, these solutions could not be used as IV infusions.

Table 16: Shelf lives of ampicillin sodium with varying temperatures and initial concentrations, in sodium citrate buffers.

Sample (initial concentration and temperature)	Shelf Life Time (Ampicillin remaining at 90 %) Time (Hours)			
	Water	**0.3M Citrate (pH 6.5)**	**0.3M Citrate (pH 5.2)**	**3.0 M Citrate (pH 7.2)**
56 mg.mL^{-1} at 5 ^{o}C	40.7 ± 3.45	23.82 ± 1.31	Precipitate formed	Precipitate formed
56 mg.mL^{-1} at 37 ^{o}C	6.69 ± 0.35	4.23 ±2.74	Precipitate formed	Precipitate formed
16 mg.mL^{-1} at 5 ^{o}C	163 ± 22.4	-	Precipitate formed	Precipitate formed
16 mg.mL^{-1} at 37 ^{o}C	11.1 ± 0.97	8.22 ± 0.42	Precipitate formed	4.80 ± 0.36

The shelf lives calculated for ampicillin sodium in citrate buffer (pH 6.5) at 5 ^{o}C and 37 ^{o}C, for both concentrations (Table 16), indicated that there was no improvement of the stability of the drug compared to water samples. However, at an initial concentration of 16 mg.mL^{-1} and at 5 ^{o}C, the concentration of the drug did not decrease in an exponential decay that was commonly seen prior in the water and saline samples. The sample resulted in a repeatable step like trend, which had no decrease in the concentration of the drug after 95 hours, but was then observed to have had a drop between two data points (Figure 23). This drop was of approximately 40 % of the concentration of the drug. This would have significantly affect the accuracy of the exponential trend line, and therefore the rate constant and shelf life were not determined. The cause of this rapid decrease in degradation was unknown. However, one possible cause could be related to the precipitate formation seen in the 3 M citrate buffer and 0.3 M citrate buffer (pH 5.2) samples.

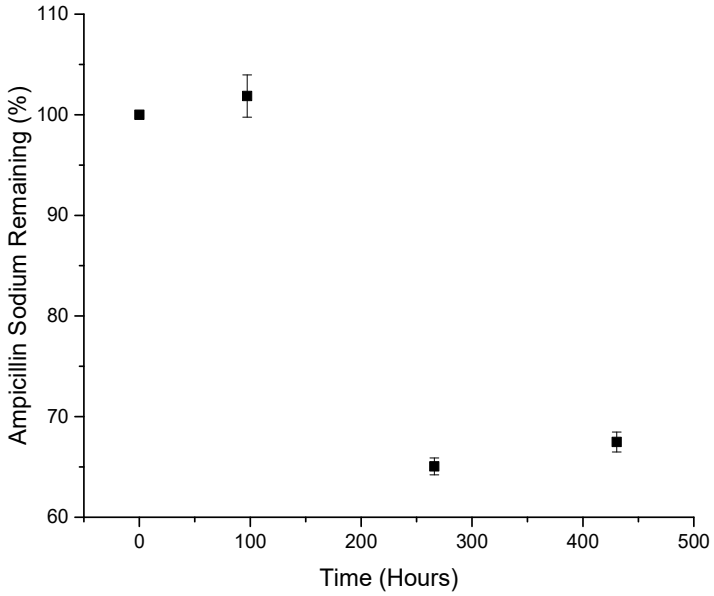


Figure 23: Kinetic analysis of ampicillin sodium (16 mg.mL^{-1}) in 0.3 M citrate buffer (pH 6.5), at 5 °C. Errors calculated as ± 1 S.D.

As previously stated, the buffer did not seem to control the pH of ampicillin. Again, the higher the amount of degradation had occurred, the larger the difference of pH (Table 17).

Table 17: pH of samples in 0.3 M sodium citrate buffer (pH 6.5)

Sample (initial concentration and temperature)	pH After Ampicillin Sodium Added		
	Initial	Final	Difference
56 mg.mL^{-1} at 5 °C	8.96	8.38	0.58
56 mg.mL^{-1} at 37 °C	9.35	7.73	1.62
16 mg.mL^{-1} at 5 °C	8.66	8.20	0.46
16 mg.mL^{-1} at 37 °C	9.40	8.07	1.33

The pH of the ampicillin solution in citrate buffer (Table 17) did show a decrease in the initial pH measurement, compared to the drug in water (Table 12). However, the change in the pH indicated that there was no significant trend. It could not be determined if pH was a contributing factor in the decrease of the concentration of the drug. The most likely explanation of the decrease in concentration was that it was caused by the same insolubility effect seen in samples tested in the 0.3 M citrate buffer (pH 5.2) and 3.0 M citrate buffer (pH 7.2).

4.2.2.3 Kinetic Analysis of Ampicillin Sodium in Phosphate Buffer Solvent

The use of phosphate buffers in IV solutions have been previously researched. Phosphate buffers have been one of the most commonly used buffer to test stability

of pharmaceutical drugs. A beneficial factor to the phosphate buffer was that ampicillin did not form a precipitate with the buffer present in solution. Several concentrations of the phosphate buffer were analysed at 16 $mg.mL^{-1}$ and 37 ^{o}C, to determine which concentration improved the stability (Table 18).

Table 18: Comparison of the rate constant and shelf life of ampicillin sodium (16 $mg.mL^{-1}$) in variable concentrations of phosphate buffer (0.01, 0.1, 0.2 M), and water, at 37 ^{o}C. pH of samples after ampicillin sodium added to solution at start and end of the kinetic analysis of these samples, and the difference between the two pH values.

Sample (phosphate buffer concentration)	Shelf Life Time (ampicillin sodium remaining at 90 %)		pH		
	Rate Constant ($Seconds^{-1}$)	$t_{90\,\%}$ (Hours)	Initial	Final	Difference
Water	73.1 ± 1.44	11.1 ± 0.97	9.47	7.98	1.49
0.01 M (pH 6.82)	51.1 ± 25.2	7.78 ± 1.71	7.61	7.54	0.07
0.1 M (pH 6.96)	91.4 ± 3.60	4.59 ± 0.09	7.45	7.05	0.40
0.2 M (pH 6.68)	155 ± 14.4	2.72 ± 0.22	6.80	6.68	0.12

The concentration of the phosphate buffer seemed to influence the degradation of ampicillin sodium. Similar to the citrate buffer, with the increase in concentration of the buffer, the faster the rate. The increase in concentration of the phosphate buffer also did not decrease the change in the pH over the degradation period. The 0.01 M phosphate buffer at pH 6.82, was observed to have had the lowest pH difference of the different concentrations of these solvents.

The phosphate buffer showed a large influence on the formation of the degradation products. The change in concentration of the phosphate buffer indicated that it influenced the degradation of ampicillin sodium. The increase in buffer concentration showed that the buffer changed the degradation pathways. This will be further discussed in (Chapter 5.2.4). The lower concentrations of the phosphate buffer had a similar kinetic trend of the pathway to the water samples.

For these reasons, analysis of the kinetic effects of the phosphate buffer on the drug was continued with the lowest concentration of phosphate buffer (0.01 M) was analysed at pH 5.5 and 6.82. The rate constant and shelf lives were calculated and compared to that of the water in Table 19.

Table 19: Comparison of the rate constant and shelf life of ampicillin sodium (16 mg.mL^{-1} and 56 mg.mL^{-1}) at temperatures of 5 °C and 37 °C, for solvents of 0.01 M phosphate buffer (pH 5 and pH 7), and water.

Sample (initial concentration and temperature)	Rate constant (Seconds^{-1}) and shelf life time (Hours)					
	Water		0.01M (pH 5.5)		0.01 M (pH 6.82)	
	Rate Constant	$t_{90\,\%}$	Rate Constant	$t_{90\,\%}$	Rate Constant	$t_{90\,\%}$
56 mg.mL^{-1} at 5 °C	13.4 ± 1.80	40.7 ± 3.45	7.20 ± 0.36	58.4 ± 5.90	13.9 ± 1.80	40.4 ± 3.18
56 mg.mL^{-1} at 37 °C	76.7 ± 7.20	6.69 ± 0.35	96.8 ± 0.72	5.09 ± 0.08	87.8 ± 21.6	5.66 ± 1.00
16 mg.mL^{-1} at 5 °C	3.59 ± 1.08	163 ± 22.4	8.53 ± 3.60	206 ± 39.7	-	-
16 mg.mL^{-1} at 37 °C	73.1 ± 1.44	11.1 ± 0.97	52.2 ± 10.8	12.0 ± 1.68	59.8 ± 21.6	10.9 ± 1.47

Compared to the water samples, the phosphate buffer was calculated to have no significant effect over the degradation rate of ampicillin sodium at 37 °C at either pH tested. At 5 °C and buffer of a pH of 5.5, the samples showed an improvement in stability. However, the samples of ampicillin sodium, 16 mg.mL^{-1} at 5 °C produced similar trend to the 0.3 M citrate buffer (pH 6.82), however this drop was of smaller amplitude. As was observed with the citrate buffer, this was a repeatable occurrence. The trendline for ampicillin sodium (16 mg.mL^{-1}) at 5 °C and pH 6.82 could not accurately fit the data. This meant an accurate value for the rate constant and shelf life could not be calculated.

The pH of the solutions can be seen in Table 20. The pH of the phosphate buffer (5.5 and 6.82) changed after the addition of ampicillin sodium. The buffer at pH 5.5, had a larger increase in the pH than the buffer at pH 6.82. However, both phosphate buffers seemed to control the pH as the samples degraded. Again, this was likely due to the close pH values being similar to the pKa values of the functional groups of ampicillin.

The phosphate buffers and water showed a similar degradation trends in most samples tested (Table 19). However, the pH values did not reflect this. Both phosphate buffers, had a smaller pH difference than water. The final pH measured within the same time frame for sample conditions e.g. 16 mg.mL^{-1} at 37 °C after approximately 72 hours, showed no correlation between this value and the percentage of remaining ampicillin sodium. This again indicated that the pH of the solution did not have a significant influenced the overall kinetic rate of the degradation of the drug for these samples.

Unlike what was observed in the chromatograms of the 1 mg.mL^{-1} samples, the samples in the phosphate buffers (pH 5.5 and 6.82), did not indicate a change in the degradation product formation, compared to the water samples (see Chapter 5.2.4 for further discussion). Conflictingly, as there was no difference in degradation products formed, the degradation products may not have had a significant influence on the change in pH.

Table 20: Change in the pH of samples in water, and 0.01 M phosphate buffer at pH 6.82 and 5.5

Sample (initial concentration and temperature)	pH After Ampicillin Sodium Added								
	Water			0.01M (pH 5.5)			0.01 M (pH 6.82)		
	Initial	Final	Difference	Initial	Final	Difference	Initial	Final	Difference
56 mg.mL^{-1} at 5 °C	8.92	7.35	1.57	8.49	8.10	0.39	8.74	8.23	0.51
56 mg.mL^{-1} at 37 °C	8.63	7.45	1.18	8.45	7.70	0.75	7.90	7.70	0.20
16 mg.mL^{-1} at 5 °C	7.90	7.66	0.24	7.86	7.75	0.11	8.11	8.00	0.11
16 mg.mL^{-1} at 37 °C	8.37	6.86	1.51	7.88	7.49	0.39	7.61	7.54	0.07

As previously stated, there was a possible increase in the stability of ampicillin sodium was observed for the lower temperature (5 °C) in both concentrations. However, for application in the HITH program, the phosphate buffer was not considered as the most viable option. The 0.01 M phosphate buffer is not readily available in pre-made IV solutions for easy usage in HITH. The buffered samples did not significantly improve the stability of ampicillin sodium. Therefore, it was determined that the water samples were the most stable and viable solvent to use.

4.2.2.4 Effect of IV Container Material on the Stability of Ampicillin Sodium

A number of different types of IV containers are used for infusions of drugs. Two readily available containers were selected for the analysis; EVA and Freeflex®. These IV containers were of different polymer compositions: ethylene vinyl acetate (EVA) and polypropylene (Freeflex®).

Water was determined to be the solvent ampicillin sodium was most stable, therefore it was used to determine if the IV container had an effect on the stability of the drug. In the IV bags, ampicillin sodium was analysed at the two concentrations (16 mg.mL^{-1} and 56 mg.mL^{-1}) and at temperatures of 5 °C and 37 °C. The kinetic trends of ampicillin sodium in these containers were compared to those previously performed (Chapter 4.2) in glass containers. There were no new degradation peaks observed in the IV containers. Figure 24 showed that at 56 mg.mL^{-1} and at 37 °C, both

EVA and Freeflex® containers had no significant effect on the degradation rate and shelf life of the drug. This trend was seen throughout most of the range of concentrations and temperatures tested (Table 21).

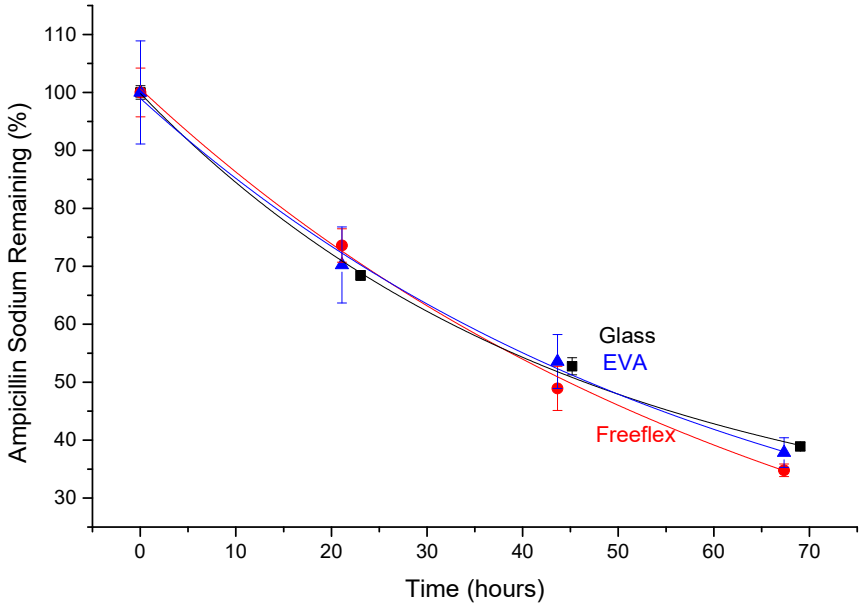


Figure 24: Comparison of the decrease of ampicillin sodium (56 mg.ml^{-1}) concentration while stored in glass, Freeflex® and EVA IV containers at 37 °C. Errors calculated as ± 1 S.D.

Ampicillin sodium at 16 mg.mL^{-1} at 37 °C in Freeflex® was calculated to have a faster degradation rate and therefore a smaller shelf life than the glass and EVA containers. The Freeflex® containers had a shelf life of 7.19 ± 1.19 hours, compared to that of glass and EVA containers of 11.1 ± 0.97 and 10.5 ± 0.87 hours, respectively. As the other three sample variations in the Freeflex® containers having similar degradation rates to the glass and EVA containers (Table 21), the results supported that the 16 mg.mL^{-1} at 37 °C was an outlier. It was considered that the lower shelf life may have been caused due to preparation of ampicillin sodium for injection. The third data point of the graph was calculated to have less ampicillin than that of the fourth data point, in all replicates. The third data point was omitted and therefore the calculated shelf lives and degradation rate was produced by only three data points. This affected the accuracy of the degradation rate and shelf life values.

Table 21: Rate constant and shelf life comparison between glass, EVA and Freeflex containers for ampicillin sodium stored at 16 mg.mL^{-1} and 56 mg.mL^{-1} at temperatures of 5 °C and 37 °C.

Sample (concentration and temperature)	Rate constant (Seconds^{-1}) and 90 % Shelf Life (Hours)					
	Glass		EVA		Freeflex®	
	Rate Constant	$T_{90\,\%}$	Rate Constant	$T_{90\,\%}$	Rate Constant	$T_{90\,\%}$
56 mg.mL^{-1} at 5 °C	13.4 ± 1.80	40.7 ± 3.45	10.8 ± 1.80	41.03 ± 2.37	10.0 ± 1.80	42.8 ± 3.30
56 mg.mL^{-1} at 37 °C	76.7 ± 7.20	6.69 ± 0.35	91.8 ± 18.0	5.47 ± 0.43	50.4 ± 25.2	7.24 ± 1.35
16 mg.mL^{-1} at 5 °C	3.59 ± 1.08	163 ± 22.4	3.89 ± 7.20	131 ± 34.2	3.82 ± 1.44	149 ± 18.5
16 mg.mL^{-1} at 37 °C	73.1 ± 1.44	11.1 ± 0.97	52.6 ± 14.4	10.5 ± 0.87	114.48 ± 25.2	7.14 ± 1.19

4.3 Conclusions

The kinetic analysis of ampicillin sodium (16 $mg.mL^{-1}$ and 56 $mg.mL^{-1}$), stored in glass, was analyzed in temperatures of -20 ^{o}C, 5 ^{o}C, 20 ^{o}C and 37 ^{o}C, and in water solutions. These results showed that ampicillin sodium has a dependence on initial concentration and temperature. An increase in both these variables significantly increases the rate of degradation. At refrigerated temperatures (5 ^{o}C), ampicillin sodium at both initial concentrations could be used for a long infusion period (over 24 hours). Samples of 16 $mg.mL^{-1}$ were also stable at 20 ^{o}C. Rapid degradation occurred at the higher temperatures and concentration, 56 $mg.mL^{-1}$, and would not be suitable for long infusion periods. The IV containers; Freeflex® and EVA, showed no impact on the stability of the drug.

Frozen solutions of ampicillin sodium had varying results. At an initial concentration of 56 $mg.mL^{-1}$, ampicillin had an increase in stability than the higher temperatures. However, the was not the case for samples at 16 $mg.mL^{-1}$. The stability at this initial concentration decreased when compared to samples analyzed at 5 ^{o}C. This result was possibly caused by the solutions freezing non-homogenously, concentrating the drug, causing ampicillin to degrade rapidly. Further analysis of frozen samples is required to confirm this.

The stability of ampicillin sodium was dependent on the IV fluid. Compared to water, saline and sodium citrate buffer showed a decrease in the stability of the drug. However, at low temperatures (5 ^{o}C), 0.01 M phosphate buffer had evidence of improving stability, but there was no difference in the stability of the drug at 37 ^{o}C.

The samples analysed indicated that pH may not have significantly influence the degradation rate of ampicillin sodium in saline or the buffered solutions. It is likely that ionic strength had a larger influence in the stability of the drug. However, to determine this, analysis on the influence of ionic strength is required.

5. IDENTIFICATION AND ANALYSIS OF DEGRADATION PRODUCTS

5.1 Synopsis

The identification of products and their mechanisms formed from the degradation ampicillin has been previously explored[22, 26, 31, 32]. Previous research determined that there are many degradation pathways produced by penicillin hydrolysis[26]. Numerous articles[39, 43, 104] have been published on the identification of degradation products of ampicillin sodium. Some compounds which have been identified as common occurrences in hydrolysis of ampicillin include ampicilloic and ampilloic acids, polymer and oligomer formations and diketopiperazines. Environmental factors have shown to influence the formation of certain degradation products of ampicillin. For example, it has been reported that the oligomeric[37] and diketopiperazine compounds from ampicillin were discovered to mainly form from high concentrations of the drug, and in basic aqueous solutions[37, 40].

This chapter focuses on identifying the degradation products of ampicillin sodium by LCMS, and producing a mechanism of the degradation. Analysis of the kinetic trends of these degradation products and further investigation effects on the products from the change in temperature and IV solutions will also be performed.

5.2 Results and Discussion

5.2.1 Identification of Ampicillin Sodium and Degradation Products

The identification of the degradation products of ampicillin sodium produced by the conditions the drug is exposed to in the HITH program, is an important part of understanding the stability of the drug. The formation of degradation products have been reported to be dependent on factors, such as concentration and temperature[27]. For example, oligomeric formations such as the dimer have been previously reported to form in high concentrations of ampicillin sodium. Some of these compounds have been connected to cause allergic reactions in some patients[22]. For HITH, the drug will likely be stored and used at high concentrations. The identification of the products could lead to identifying methods to reduce or inhibit the rate of degradation of ampicillin sodium.

Two LCMS instruments with tandem mass spectrometry (MS/MS) were used to determine the main degradation products formed from ampicillin sodium: a Thermo Scientific™ Vanquish UHPLC coupled with a Q Exactive Plus Orbitrap MS, and a Waters Synapt HDMS system. Due to differences in parameters of each LC instruments, e.g. column dimensions and flow rate, the method for analysis was not the same in each system.

Mass spectra of ampicillin sodium and its degradation products were collected using two different degraded samples of ampicillin sodium; both at a temperature of 37 °C and in water. Mass spectra of ampicillin in the other solvents were not obtained, due to the high concentrations and involatile nature of the salts.[105].To reduce overloading of both instruments, samples analysed by the Thermo Scientific™ Orbitrap MS and the Waters Synapt LCMS, were diluted 1000 and 250 times, respectively. The Waters Synapt MS instrument used both total ion count (TIC) and UV detection. Figure 25 is the UV detection of the separation of ampicillin sodium and its degradation products at 254 nm. Figure 26 depicts the TIC chromatogram of a degraded sample of ampicillin sodium (16 $mg.mL^{-1}$) collected from the Thermo Scientific™ Orbitrap MS system.

Ampicillin and main degradation products were ordered (1.-13.) in retention time, as seen in both Figure 25 and Figure 26.

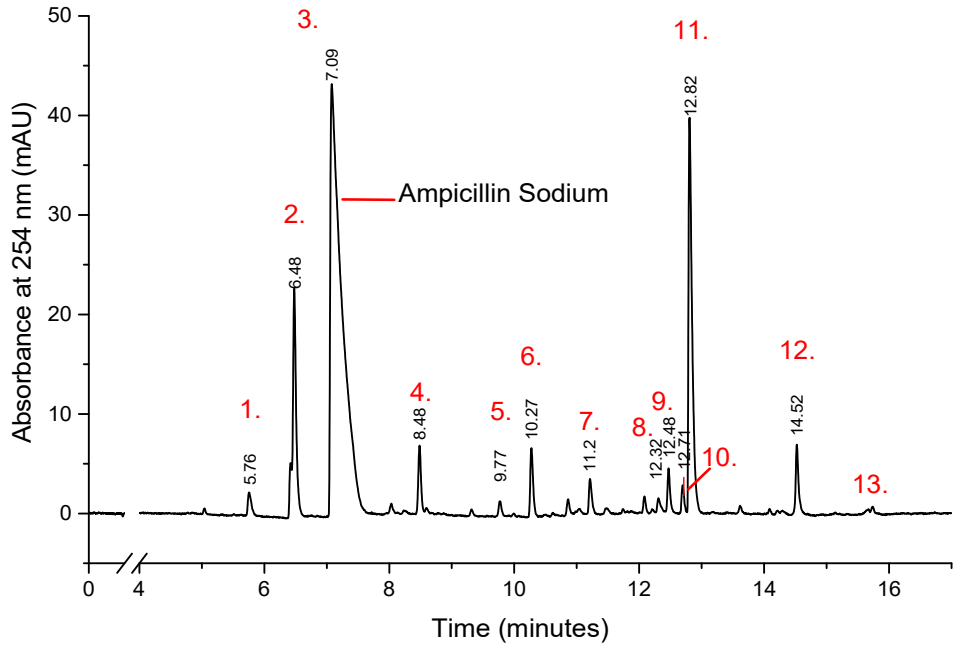


Figure 25: Degraded sample of ampicillin sodium (56 mg.mL^{-1}) chromatogram collected by a Waters Synapt system. Sample was degraded for 43.5 hours, where approximately 53 % of the drug was remaining. Sample was degraded at a temperature of 37 °C. Retention time of peaks were reported in black and compound peaks numbered in red. Detection via UV-Vis detector at 254 nm

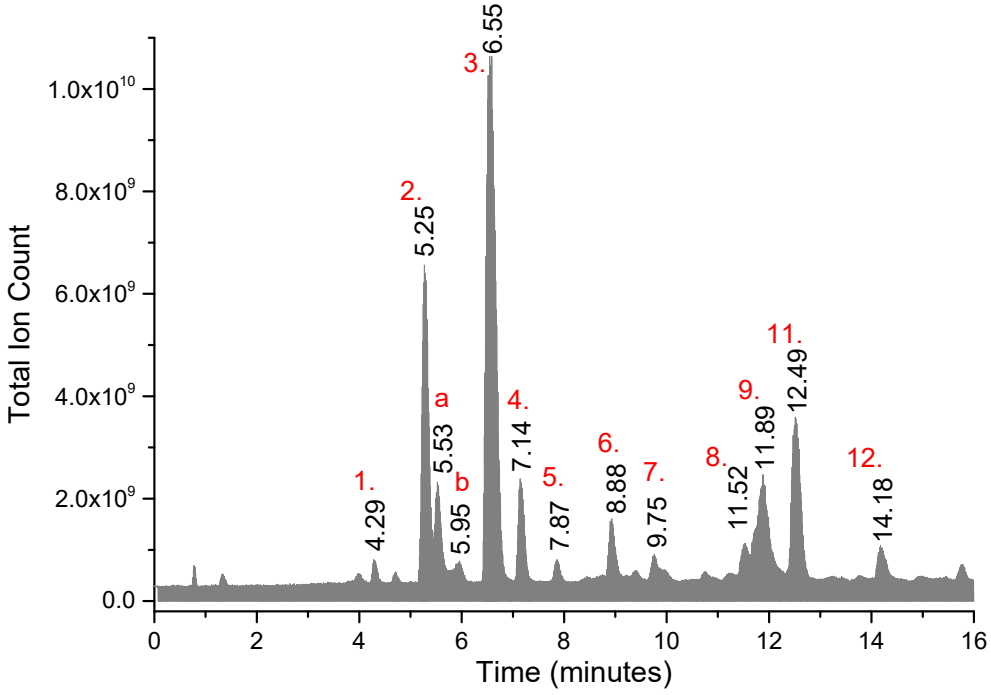


Figure 26: TIC chromatogram of degraded 16 mg.mL^{-1} ampicillin sample, diluted by a factor of 1000, with peak numbered of ampicillin and main degradation products. Chromatogram collected using Thermo Scientific™ Orbitrap instrument.

The samples analysed by the different LCMS systems had different degradation periods. The sample analysed *via* the Synapt instrument was analysed after four days, however the Orbitrap sample was analysed after two weeks. This was due to the sample of ampicillin sodium interstate travel for analysis by the Orbitrap system. Although both were kept at 37 ^{o}C for degradation, the sample analysed *via* the Orbitrap MS was frozen for interstate transportation until the sample analysed. This meant that the samples were not analysed at the same degradation point.

The concentrations of the degradation products were not known, and the main degradation products were determined by having both high UV absorbencies (225 nm and 254 nm) (Figure 25), and high TIC counts (Figure 26). Some degradation products in the 56 mg.mL^{-1} had larger peak areas in comparison to those in the 16 mg.mL^{-1} samples. This difference in the peak areas was analysed in more detail in Chapter 5.2.3.

The two chromatograms (Figure 25 and Figure 26) produced by the two LCMS systems varied; where different separation of peaks were observed. This was due to the differences in methods between the two instruments, where a different column and flow rate was required (Chapter 2.4). Standards of ampicillin sodium were used to identify the drug peak, due to differences in the chromatograms. Retention time and MS spectra of these standards confirmed the most abundant peak was ampicillin sodium. The retention times of these peaks were at 7.09 minutes (Figure 25) and 6.55 minutes (Figure 26) in the Waters Synapt and Thermo Scientific^{TM} Orbitrap systems, respectively. The mass spectra of ampicillin sodium was comparable to mass spectra in previous literature[41].

The Thermo Scientific^{TM} Orbitrap system provided a greater separation of the degradation compounds that eluted before the ampicillin sodium peak, however poorer separation occurred in the peaks that eluted after. The Waters Synapt instrument provided poorer separation of the peaks that eluted before ampicillin, but had good separation of the samples that retained longer. There was a clear overlap in identifying common peaks and compounds with similar retention times, which allowed the two analyses to be compared.

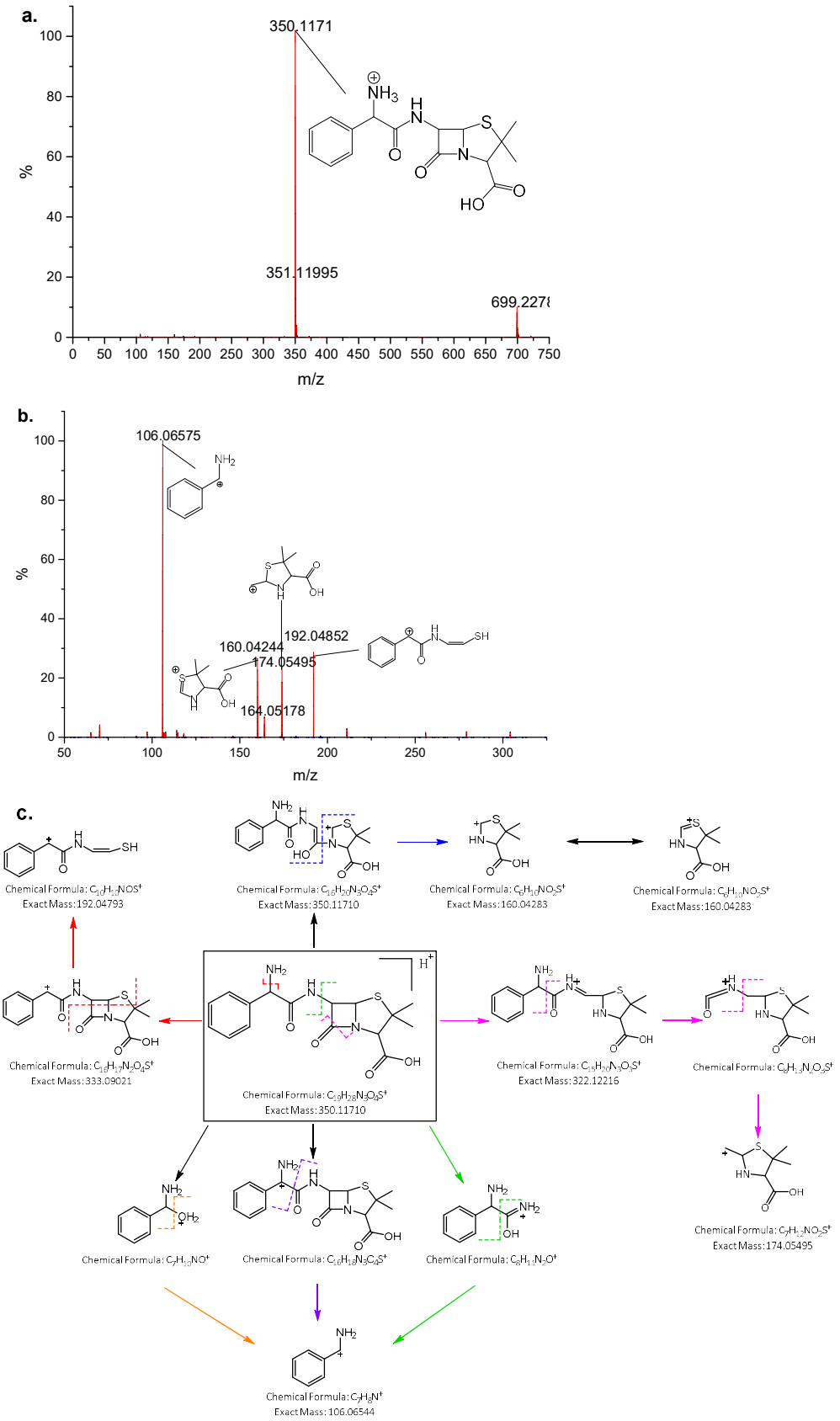


Figure 27: a. Mass spectra of ampicillin with parent peak at *m/z* 350 and b. MS/MS spectra of ampicillin sodium fragmentation, and c. fragmentation mechanism of ampicillin. Mass resolution of 63,653.18 at *m/z* 350.12.

The mass spectrum of the ampicillin peak had a parent peak of *m/z* 350 (Figure 27 a.). This mass to charge ratio corresponds to the molecular weight of ampicillin, not the sodium salt. The mass to charge ratio of ampicillin sodium would be *m/z* 372. This *m/z* 350 was observed in mass spectra obtained from both instruments. A peak at *m/z* 699 in the mass spectrum of the ampicillin peak corresponded to the dimerization of ampicillin. Dimerization of compounds can occur in mass spectrometry instrument as an artefact of the ionisation the peak[106].

Common fragments related to ampicillin sodium and its degradation products were calculated by the Mass Frontier program. The most common and abundant fragments of ampicillin were observed to have mass to charge ratios of 192, 174, 160, and 106 (Figure 27 b.). These fragments have been reported in literature as common fragments of ampicillin[41, 42, 74].

The mechanism showed a fragmentation pathway of ampicillin into the identified fragments (Figure 27 c.). Many of these fragments form *via* ionisation, cleavages and hydrogen rearrangements of the molecule, and can have multiple pathways. For example, the structure displayed for the fragmentation peak: *m/z* 106 ($C_7H_8N^+$), was able to form *via* hydrogen rearrangement around the charged site, or inductive cleavage of the bond. Fragments of the molecule could also break apart in MS/MS, producing smaller fragments. This occurred with the *m/z* 106 fragment (Figure 27 c.). There are three pathways to produce this fragment shown in the mechanism. However, these are not the only pathways in which this fragment could occur. In the mechanism, some of the larger fragments have this section of ampicillin intact, and could possibly break apart further.

Some fragments were shown to have the same chemical formula and therefore have the same mass to charge ratio. An example of this was the fragment of $C_8H_{10}NO_2S^+$ at *m/z* 160. In the fragmentation mechanism (Figure 27 c.), the structure was shown to be depended on the placement of the positive charge. The two fragments are likely to be interchangeable.

Another fragment of ampicillin had a mass to charge ratio of 192. To form this fragment, the mechanism depicted several bonds are required to break apart from the parent molecule. Several steps may be absent from the mechanism (Figure 27 c.). However, the four-membered ring is highly unstable, and would require very little

energy to break apart, therefore allowing the fragment to form. Additionally, a hydrogen rearrangement would be the likely cause for the double bond.

Some peaks were determined to not be related to ampicillin. It is common for background fragments to occur in analysis[107, 108]. These ions are often of low *m/z* ratios e.g. from solvent or gas; however, larger mass to charge ratio peaks caused by contaminants e.g. from the column, can occur.

A number of main degradation products of ampicillin sodium were identified, where several stereoisomers of these compounds were identified. Table 22 is a summary of the degradation products and ampicillin determined by the two LCMS systems.

Table 22: Main degradation products and ampicillin determined by LCMS: Thermo Scientific™ Orbitrap and Waters Synapt MS systems.

Peak Number	Compound Name	Chemical Formula	Orbitrap LCMS System		Synapt LCMS System	
			Retention Time (minutes)	Parent Peak (*m/z*) ± Error (ppm)	Retention Time (minutes)	Parent Peak (*m/z*) ± Error (ppm)
1.	Ampicilloic Acid*	$C_{16}H_{21}N_3O_5S$	4.29	368 ± 12	5.75	368 ± 891
2.	Isomer of Ampicilloic acid	$C_{16}H_{21}N_3O_5S$	5.28	368 ± 12	6.46	368 ± 619
a.	Isomer of Ampicilloic acid	$C_{16}H_{21}N_3O_5S$	5.53	368 ± 12	-	-
b.	Ampilloic acid*	$C_{15}H_{21}N_3O_3S$	5.96	324 ± 105	-	-
3.	Ampicillin*	$C_{16}H_{19}N_3O_4S$	6.55	350 ± 1	7.09	350 ± 906
4.	Diketopiperazine*	$C_{16}H_{19}N_3O_4S$	7.15	350 ± 1	8.45	350 ± 906
5.	2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal	$C_{10}H_{10}N_2O$	7.86	173 ± 2	9.77	173 ± 7923
6.	Isomer of Diketopiperazine	$C_{16}H_{19}N_3O_4S$	8.93	350 ± 1	10.27	350 ± 906
7.	Dimer*	$C_{32}H_{38}N_6O_8S_2$	-	-	11.20	699 ± 181
8.	Oligomer of Ampicilloic Acid and Ampicillin*	$C_{32}H_{40}N_6O_9S_2$	11.54	717 ± 1	12.35	717 ± 191
9.	Isomer of Oligomer of Ampicilloic Acid and Ampicillin	$C_{32}H_{40}N_6O_9S_2$	11.92	717 ± 1	12.48	717 ± 191
10.	Isomer of Dimer	$C_{32}H_{38}N_6O_8S_2$	-	-	12.71	699 ± 181
11.	Isomer of Dimer	$C_{32}H_{38}N_6O_8S_2$	12.50	699 ± 1	12.82	699 ± 181
12.	Trimer*	$C_{48}H_{57}N_9O_{12}S_3$	14.17	1048 ± 1	14.52	1048 ± 33
13.	Tetramer*	$C_{64}H_{76}N_{12}O_{16}S_4$	-	-	15.63	1397 ± 772

*degradation products previously identified in literature [34, 40-43, 74, 109]

Many of these degradation products in

Table 22 have been previously identified in literature[34, 40-43, 74, 109]. The mass spectra of these main degradation products were analysed and compared to previously recorded mass spectra[34, 40-43, 74, 109]. When the mass spectra of both LCMS analyses agreed with the identification of the product, the spectra collected from the Orbitrap system was used. However, due to the difference in samples and peak separation between the two analyses, not all degradation products were present in both chromatograms. This meant that these degradation products could not be compared. Therefore, the data presented is only collected from the instrument which it was present. An example of this was the detection of the ampicillin tetramer (

Table 22), which was only present in the sample analysed by the Waters Synapt system.

Numerous stereoisomers of the degradation products were identified by LCMS. The MS and MS/MS spectra shown are an example of each compound. For all products spectra in both the Thermo Scientific™ Orbitrap and Waters Synapt LCMS systems, see (Appendix 7.4).

5.2.1.1 Compounds 1., 2., 2.a, 2.b: Ampicilloic and Ampilloic Acid

The degradation peak in the Waters Synapt system had co-elusion of some compounds (Figure 25). This co-elution was identified by the Orbitrap system (Figure 26), where three compounds were determined to be present. The additional peaks were labelled as 2.a and 2.b. Two of the peaks, 2. and 2.a, were identified to have a parent peak of *m/z* 368 (Figure 28). This suggested that the two peaks were two compounds of the same structure with different stereocentres, and therefore were different stereoisomers, of ampicilloic acid. Compound 1. was also determined to be an isomer of ampicilloic acid.

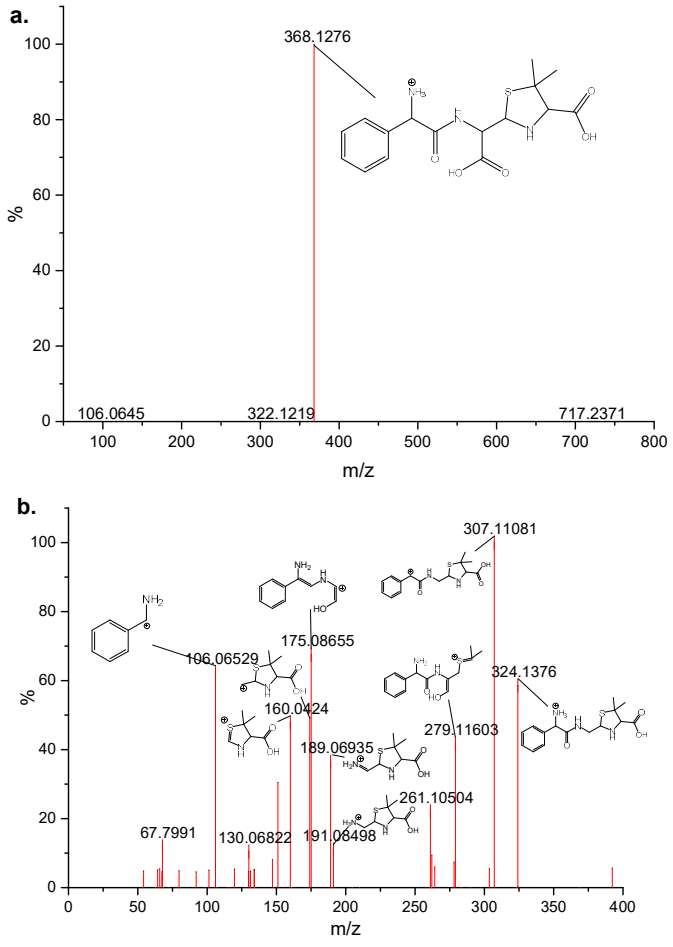


Figure 28: a. Mass spectra of compound 2. and b. MS/MS spectra of the fragmentation of the product, using Orbitrap LCMS system.

Figure 28 b. showed that the compound easily fragmented. In the figure, the corresponding fragments can be seen with these peaks. Ampicilloic acid shared a number of fragment peaks with ampicillin (Figure 27 c.), e.g. *m/z* 160 and 106, however, other fragments were detected. Some of the most abundant of ampicilloic acid's fragments included *m/z* 324, 307, 175, 174, 160 and 106. A fragmentation mechanism of ampilloic acid fragments can be seen in (Figure 29). One fragment of ampicilloic acid was $C_{15}H_{21}N_3O_3S^+$ (*m/z* 324). This resembles a positively charged fragment of the same structure as ampilloic acid. Like with ampicillin, these fragments could further break into smaller fragments. For example, the *m/z* 175 could fragment into *m/z* 106.

The fragment peak at *m/z* 191 was not a highly abundant peak (Figure 28 b.), but had multiple different fragments (Figure 29). This may have been caused by the system not supplying enough energy for this fragmentation to readily occur. In the mechanism, some intermediate fragments (Figure 29) were produced e.g. peak at *m/z* 332. These fragments were not observed in the MS/MS spectrum (Figure 28 b.). This was likely due to the fragments easily breaking apart into the smaller fragments. An example of this was the peak at *m/z* 322 (Figure 28 b.), where two fragments were shown to stem from this intermediate. The formation of the two fragments shown in the mechanism was dependent on which bond breaks to form the positively charged fragment.

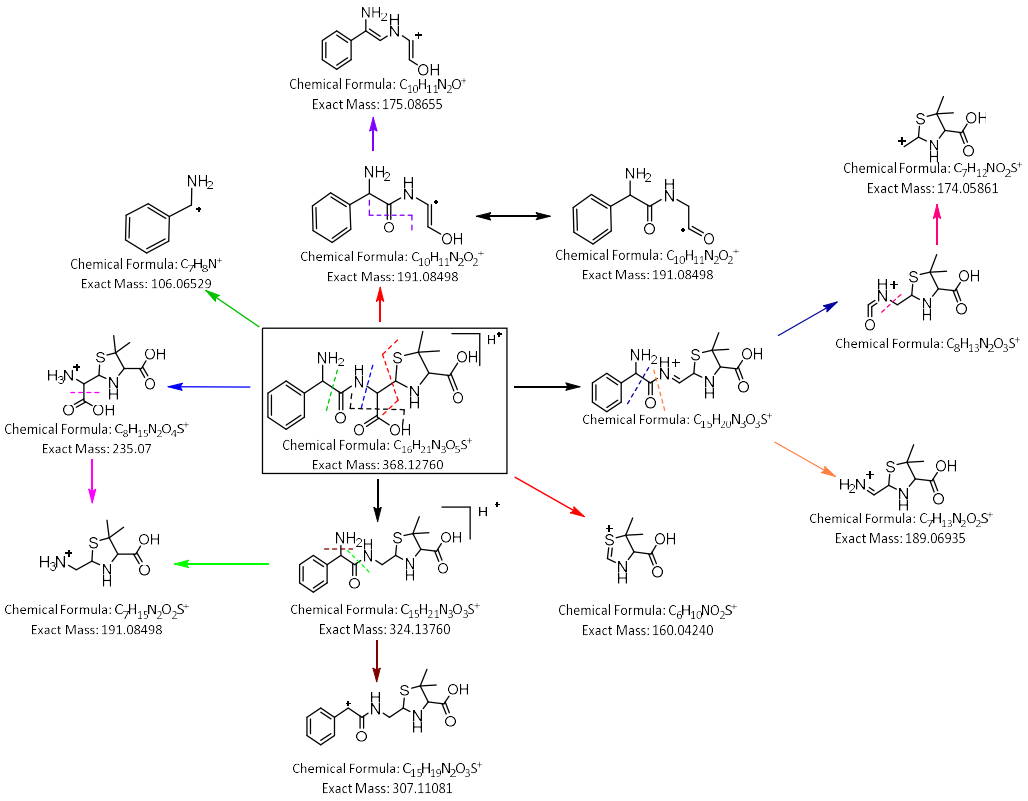


Figure 29: Mechanism of ampicilloic acid (compounds 1., 2. and 2.a.) common fragmentation peaks observed in Orbitrap MS/MS spectra.

Peak 2.b had a parent peak of *m/z* 324 (Figure 30), therefore was identified as the formation of ampilloic acid. The structure of the compound is similar to ampicilloic acid, with the loss of CO_2. Both degradation products have previously been identified in literature[34, 40-43, 74, 109].

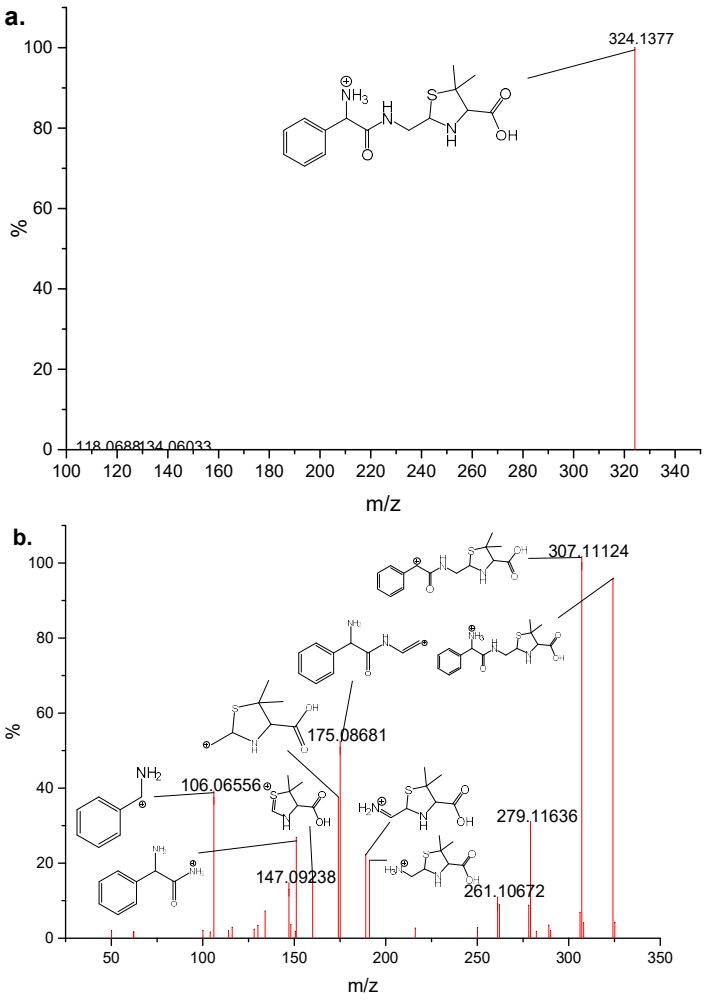


Figure 30: a. Mass spectra of compound 2.b and b. MS/MS spectra of the fragmentation of the product, using Orbitrap LCMS system.

Both ampicilloic acid and ampilloic acid showed similar peak fragments. These common fragment peaks *m/z* 307, 191, 189, 175, 174, 160 and 106. For ampilloic acid, this resulted in a similar fragmentation pattern and mechanism (Figure 29). Ampilloic acid had an unique peak at *m/z* 151 with a chemical formula of $C_8H_{11}N_2O^+$ (Figure 30 b.). This fragment formed from cleavage of the bond between a carbon and nitrogen (Figure 31).

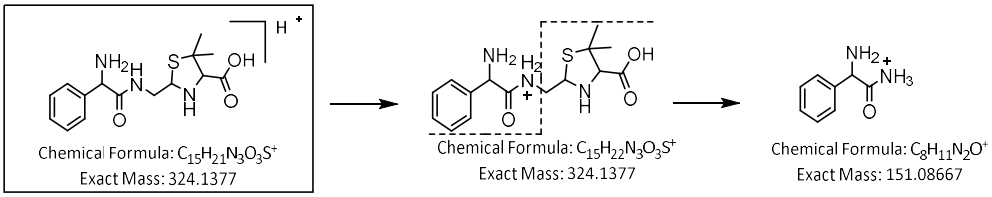


Figure 31: Mechanism of fragment $C_8H_{11}N_2O^+$ (*m/z* 151) from ampilloic acid.

5.2.1.2 Compounds 4. and 6.: Diketopiperazines (ampicillin piperazine- 2, 5- dione)

Two diketopiperazine isomers (4. and 6.) were observed in the degradation of the drug. The chemical structure of diketopiperazine is formed by an intramolecular interaction of ampicillin sodium. This meant that the parent peak of diketopiperazines had the same mass to charge ratio as ampicillin (*m/z* 350). However, the mass spectra collected from both LCMS systems showed small differences in the fragmentation between the drug and the diketopiperazines isomers. The most commen fragments of the diketopiperazines were identified as *m/z* 114, 160, and 191 (Figure 32 b.). These peaks were formed by hydrogen rearrangment and inductive cleavages of bonds. The fragmentation mechanism of diketopiperzine can be seen in Figure 32 c.

Diketopiperazine was distinguishable from ampicillin (), due to this lower number of fragments produced. This was caused by the formation of the 6-membered ring structure, produced by the intramolecular interaction as ampicillin degrades. Diketopiperazine would have to break multiple bonds to form many of the fragments observed in ampicillin's fragmentation mechanism (Figure 27 c.). For example, if molecule were to fragment into $C_7H_8N^+$ (*m/z* 106), the stable six-membered ring would be cleaved along two bonds. For this to occur, a larger amount of collision energy would be required.

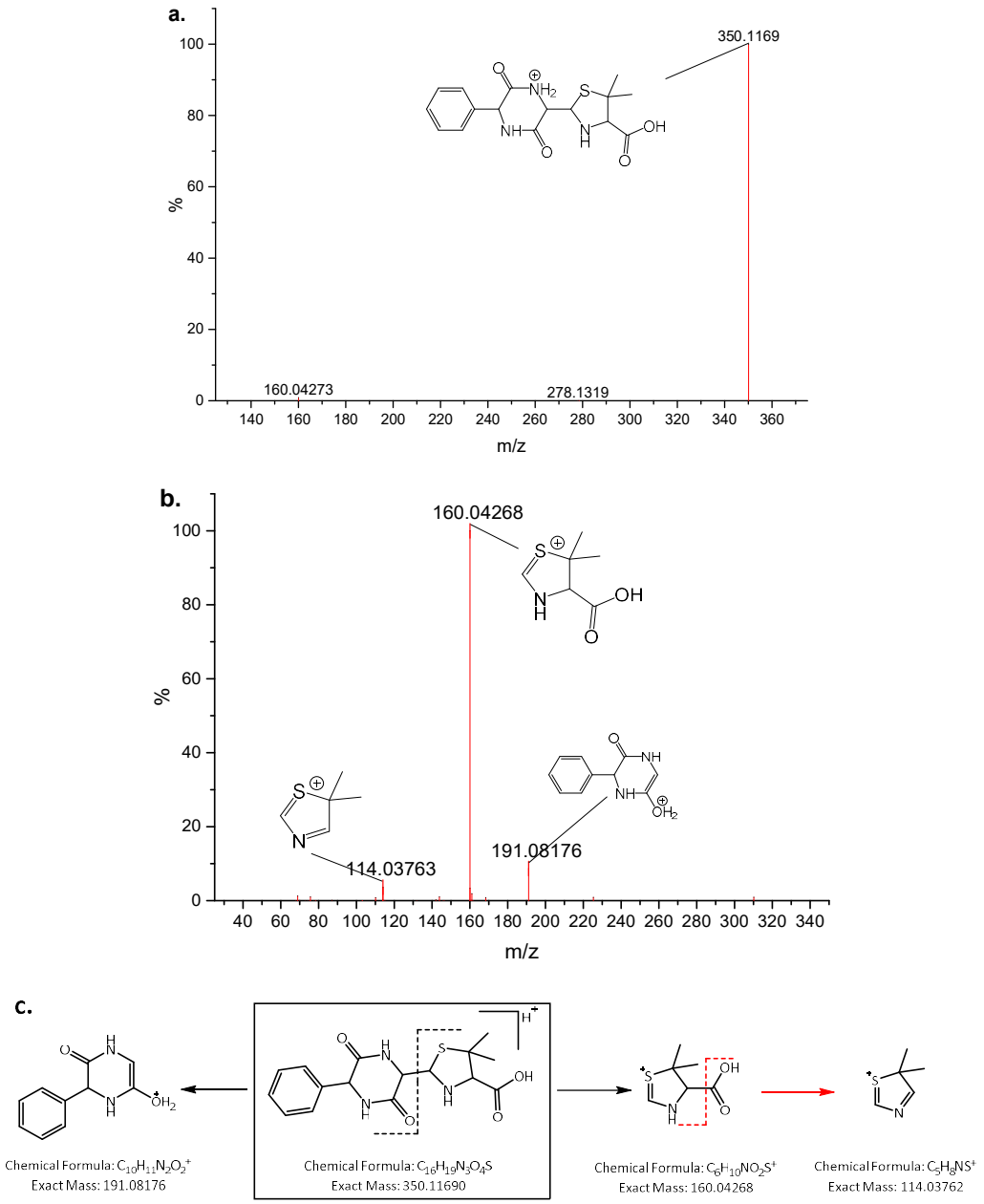


Figure 32: a. MS and b. MS/MS of compound 6. identified as diketopiperazine in Thermo Scientific™ Orbitrap LCMS system with corresponding fragment structures, and c. diketopiperazine fragmentation mechanism. The coloured, dotted lines represent where cleavage of the compound occurs to form the fragment.

5.2.1.3 Compound 5.: 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal

The mass spectrum of a compound, previously unidentified in literature was obtained using the Orbitrap MS/MS system (Figure 33). This result was compared with the MS/MS spectrum from the Water Synapt system, which showed a similar result. The parent peak at with a mass to charge ratio of *m/z* 173 was calculated to likely have a chemical formula of $C_{10}H_9N_2O$. Further investigation found that the compound had

no peaks detectable in negative electron spray mode of the Waters Synapt system. The product (5.) had very little fragmentation in the MS/MS data of both LCMS systems. As each instrument had different ionisation collision energies, this indicated that the unidentified product (5.) was likely to be highly stable.

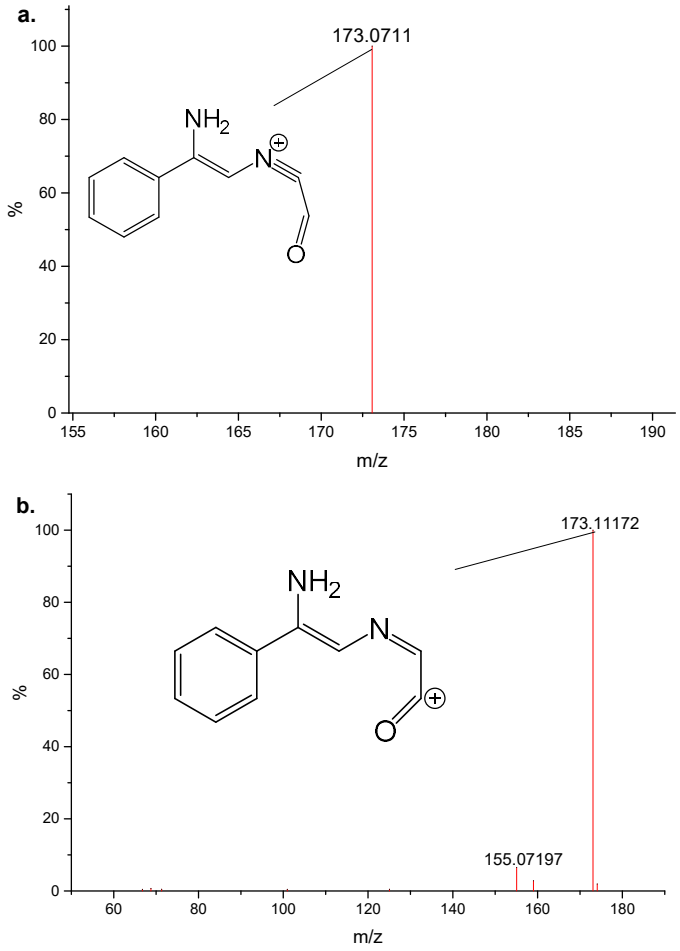


Figure 33: a. Mass spectra of compound 5. and b. MS/MS spectra of the fragmentation of the product, using Orbitrap LCMS system.

The UV contour plot (Figure 34) from the Thermo Scientific™ Vanquish UHPLC system showed the wavelengths at which the compound absorbs in the UV range. The gradient from blue to white colour, displayed a low to high absorbance of the wavelengths. Two peaks can be observed to have a strong absorbance (in red), at 249 and 340 nm. The absorbance at 249 nm indicated that the aromatic ring was still present on the molecule, and the lack of an absorbance at 225 nm suggested that the carboxylic acid chromophore was absent. Using this information, it was determined that the structure of the compound was likely to be 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal.

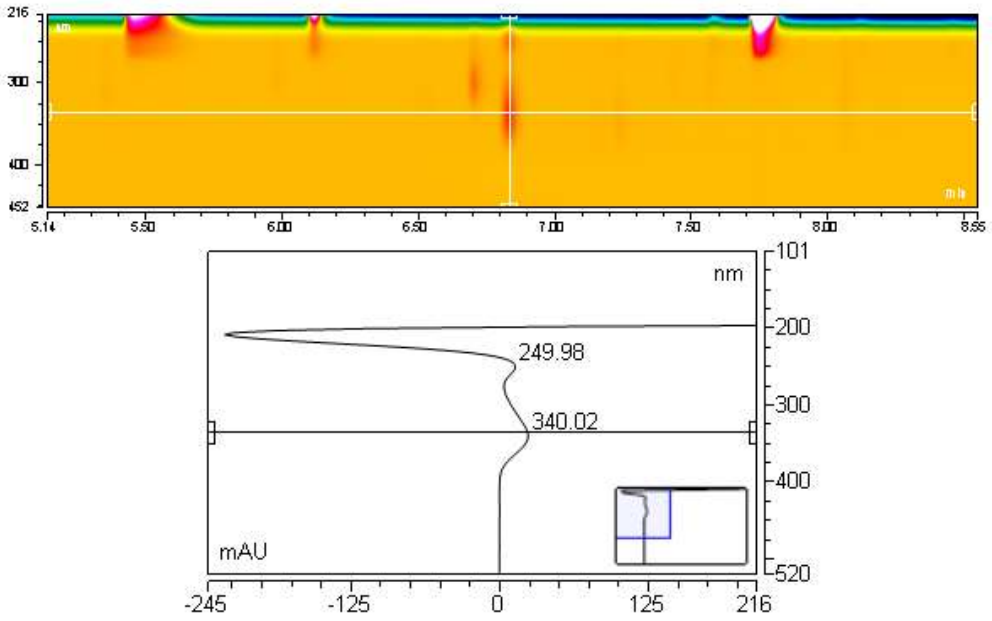


Figure 34: a. UV Contour plot of degraded ampicillin sodium, isolating compound 5., using Thermo Scientific™ Vanquish UHPLC system, for determination of wavelength of chromophores. Increasing absorbancy was shown with colour from blue to white. b. at 249.98 and 340.02 nm.

The compound 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal was modelled with GuassView to determine a theoretical UV-Vis spectrum. This determined a maximum wavelength absorbance of this structure to be at 360 nm, similar to that of the UHPLC UV contour plot data. Further characterisation is required of this compound to confirm the products structure.

5.2.1.4 Compounds 7., 10., 11., 12 and 13.: Homo-Oligomeric Formations of Ampicillin

Oligomers are similar to polymers where a small number of monomer units make up the molecule. However, they differ from polymers by having low molecular weights, consisting of a small number of monomer units[110]. Homo-oligomer formations of ampicillin are defined here as of dimer, trimer and tetramer formations. These consist of two or more of the same ampicillin molecule, where the ampicillin β-lactam ring is closed. For example, the dimer forms by two ampicillin molecules interacting; one ampicillin will force the ring opening another ampicillin molecule (Figure 35 a.).

A number of peaks was identified as homo-oligomeric compounds of ampicillin sodium; dimer, trimer and tetramer formations. Three degradation products 7., 10. and 11. were determined to be stereoisomers of ampicillin dimer (Figure 35). The MS/MS spectra from both LCMS systems showed a number of fragment peaks

previously identified in ampicillin and its lower molecular weight degradation products, e.g. *m/z* 350, 191, 160 and 106.

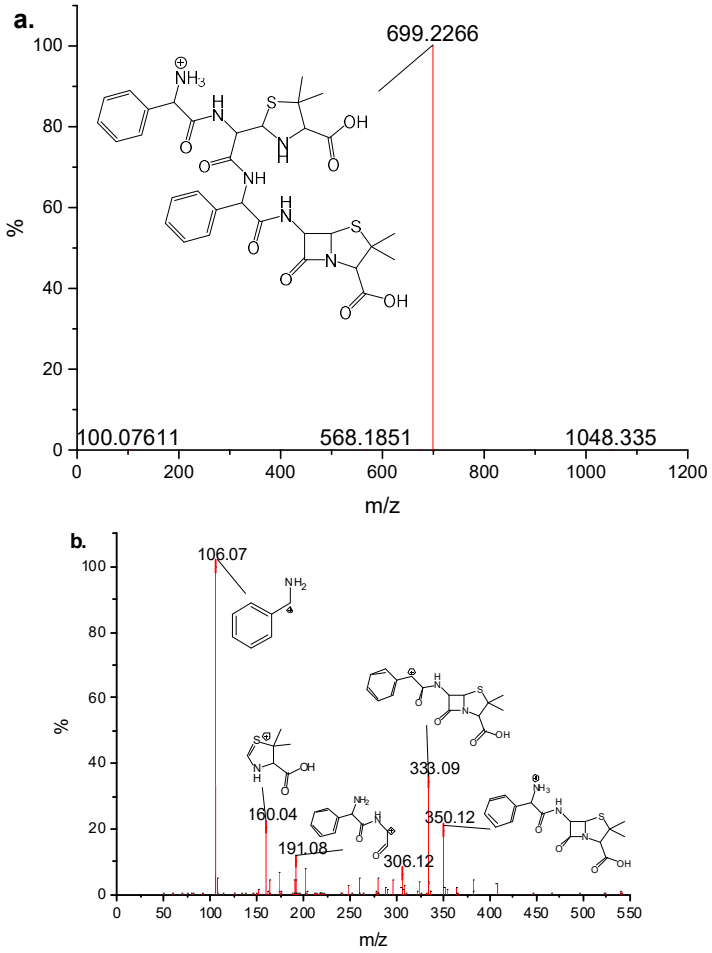


Figure 35: a. MS and b. MS/MS spectra of compound 11. obtained with a Thermo Scientific™ Orbitrap LCMS system. Peak was identified as ampicillin dimer with corresponding fragment structures.

A peak, at *m/z* 333 (Figure 35 b.), was observed that had not been seen in previous MS/MS of ampicillin or its previously identified degradation compounds. Another fragment peak that was unique to homo-oligomeric products was detected in the Synapt MS system at *m/z* 540. The structure of these fragments and how they form from the dimer can be seen in Figure 36. Both fragments were also visible in the mass spectrum of the tetramer. The MS/MS spectrum of the trimer did not contain a peak at *m/z* 540, but did have the fragment peak at *m/z* 333.

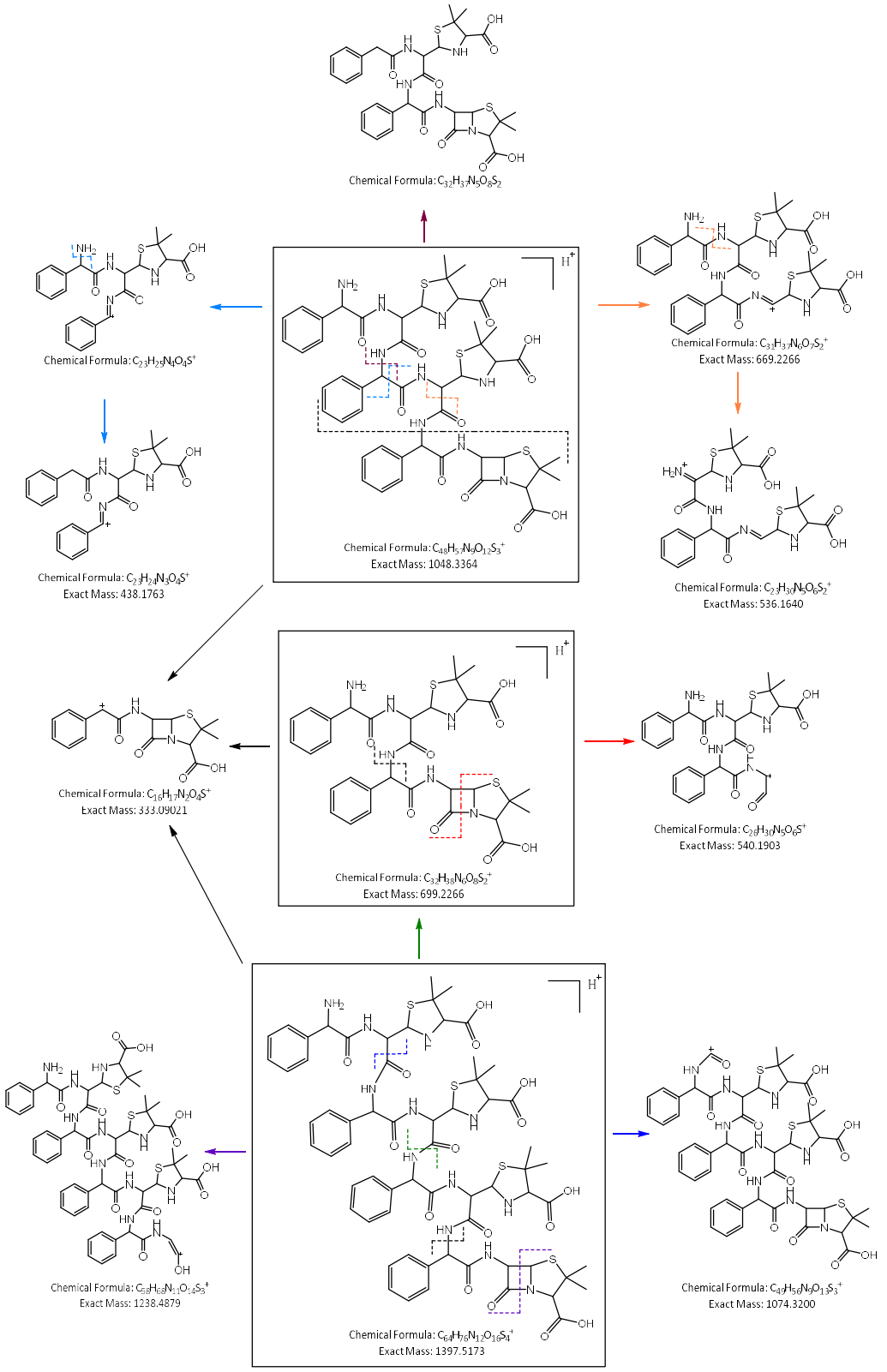


Figure 36: Fragmentation mechanism of ampicillin trimer, dimer and tetramer. Fragments previously observed not included. Different colours represent different fragmentation pathways that occurred.

Compound 12. was identified as ampicillin trimer (*m/z* 1048). The MS of the trimer (Figure 37 a.) contained a large peak at *m/z* 536. This suggested that the compound readily fragmented into the product. The fragmentation pathway from ampicillin trimer can be seen in Figure 36. The MS/MS spectrum (Figure 37 b.) had a significant number of peaks. Many of these peaks were not identified as related to the compound. The peaks which were identified included *m/z* 333, 160 and 106.

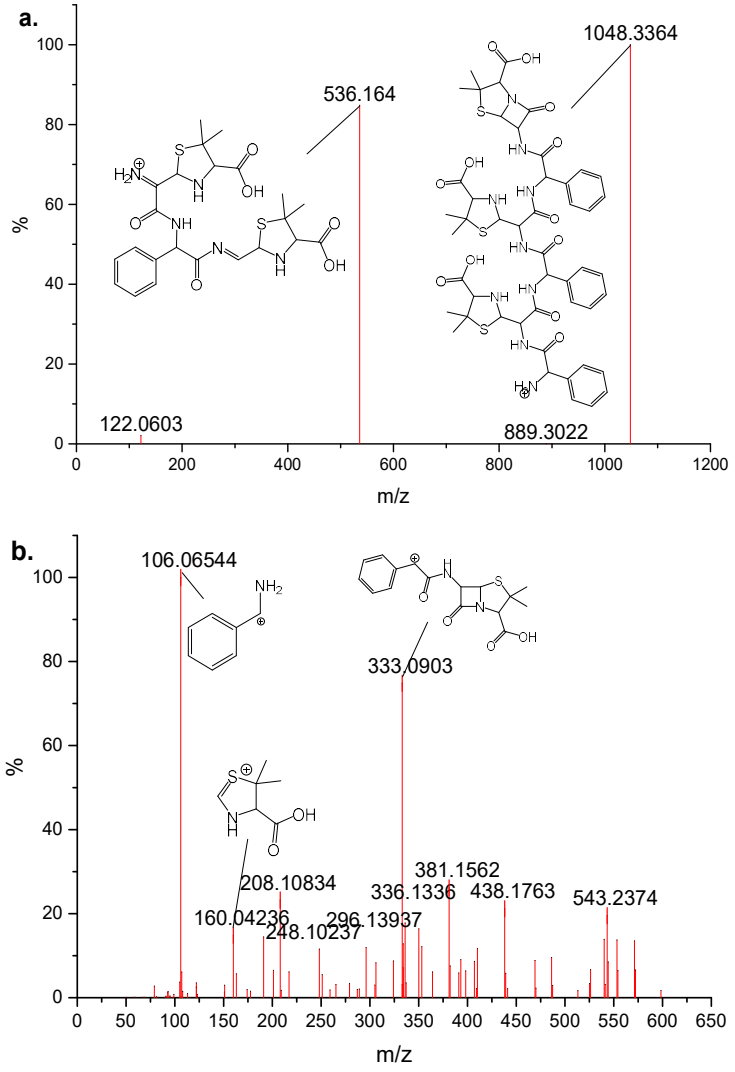


Figure 37: a. MS and b. MS/MS of compound 12. identified as ampicillin trimer in a Thermo Scientific™ Orbitrap LCMS system with corresponding fragment structures.

The tetramer formation of ampicillin sodium was only detected by the sample analysed by the Waters LCMS. This was likely due to the variation in concentration and degradation periods of the samples. Due to the small size of the peak, there was a large amount of background artifacts present in the spectrum. However, peaks at *m/z* 1397 (molecular weight of tetramer ion) and at *m/z* 699 (dimer ion) were clearly

visible. These two peaks are representitive of the tetramer. The spectrum, again, also had additional peaks which are common with fragmenation seen in the mass spectrum of ampicillin and the previously identified homo-oligomers; *m/z* 333, 540, 191 and 160. More fragments of the tetramer included $C_{58}H_{68}N_{11}O_{14}S_3^+$ (*m/z* 1238) and $C_{49}H_{63}N_{10}O_{12}S_3^+$ (*m/z* 1079). For structures of these fragments, see Figure 36.

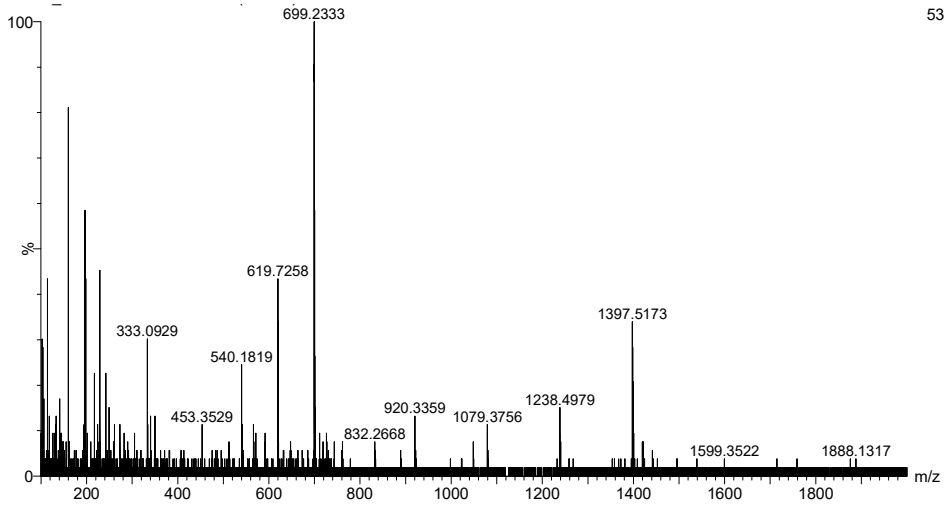


Figure 38: LCMS/MS of degradation product peak 13. identified as ampicillin tetramer in a Waters Synapt LCMS system. Peak detected using positive ion mode.

5.2.1.5 Compounds 8. and 9.: Hetero-Oligomeric Formations of Ampicillin Sodium and Ampicilloic Acid

In addition to the ampicillin dimer, trimer and tetramer formations, oligomers of ampicillin and its degradation products, classified here as hetero-oligomers, were also identified by LCMS. Compound 9. was found to be an isomer of the oligomeric formation of ampicillin and ampicilloic acid (*m/z* 717). However, compound 8. had two peaks in the MS spectrum, at *m/z* 717 and *m/z* 673 (Figure 39 a.). These two peaks could correspond to oligomeric formation of ampicillin and ampicilloic acid (*m/z* 717), or of ampicillin and ampilloic acid (*m/z* 673).

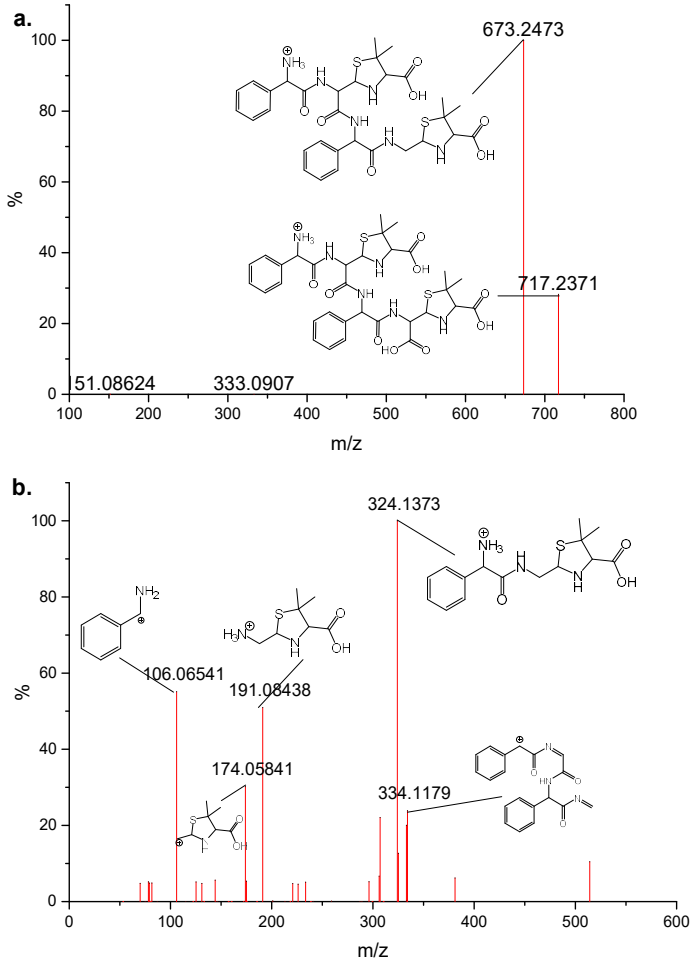


Figure 39: a. MS and b. MS/MS of peak 8. identified as ampicillin trimer in a Thermo Scientific™ Orbitrap LCMS system with corresponding fragment structures.

As ampicilloic acid (1., 2. and 2.a) had a simliar fragmentation pattern as ampilloic acid (2.b), it was surmised that a simliar occurance would happen with the hetero-oligomers. This made it difficult to determine if the spectra obtained by the Orbitrap LCMS system was due to the compound easily fragmenting, or due to co-elution of peaks (Figure 26). However, the Synapt LCMS system provided better separation of these peaks (Figure 25), and was used to determine the structure of the product. In this positive electron mode spectrum (Figure 40 a.), both *m/z* 717 and *m/z* 673 peaks were present. However, spectra obtained by the negatively charge electron spray gave a large peak at *m/z* 715 (Figure 40 b.). This meant that the compound was likely to be an ampicillin and ampicilloic acid oligomer.

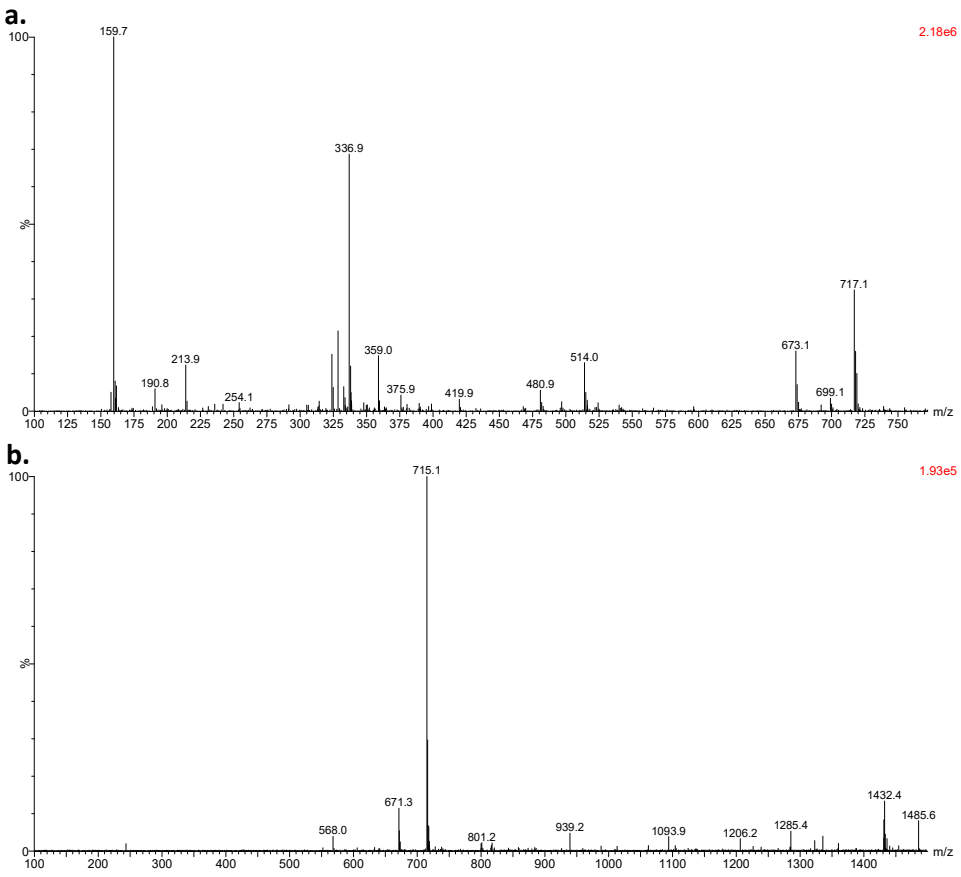


Figure 40: MS/MS of degradation product peak 8. identified as ampicillin tetramer in a Waters Synapt LCMS system, positive (a.) and negative (b.) ion mode.

A fragmentation mechanism of ampicillin and ampicilloic acid oligomer was created (Figure 41). Fragments identified in in both positive ion mass spectra of the compound (Figure 39 and Figure 40 a.). Some of the fragments from the compound had been previously identified, e.g. *m/z* 106, 160 and 174 fragments. The mechanism shows that the compound fragments into *m/z* 673 (oligomer of ampicillin and ampilloic acid), which could also fragment into many of the low *m/z* fragments.

Two fragments were identified to be unique to the hetero-oligomeric formations. In Figure 39 b., a peak at *m/z* 334 was observed. Multiple steps were required to break apart to form this fragment (Figure 41). The intermediate fragments in the mechanism did not produce their own MS/MS peaks.

The fragment at *m/z* 514 could form for both oligomers of ampicillin and ampilloic acid (*m/z* 673), and ampicillin and ampicilloic acid (*m/z* 717). Loss of the carboxyl group (CO_2) group is required to occur before or simultaneously with the loss of the five-membered ring, as seen in Figure 41 (blue).

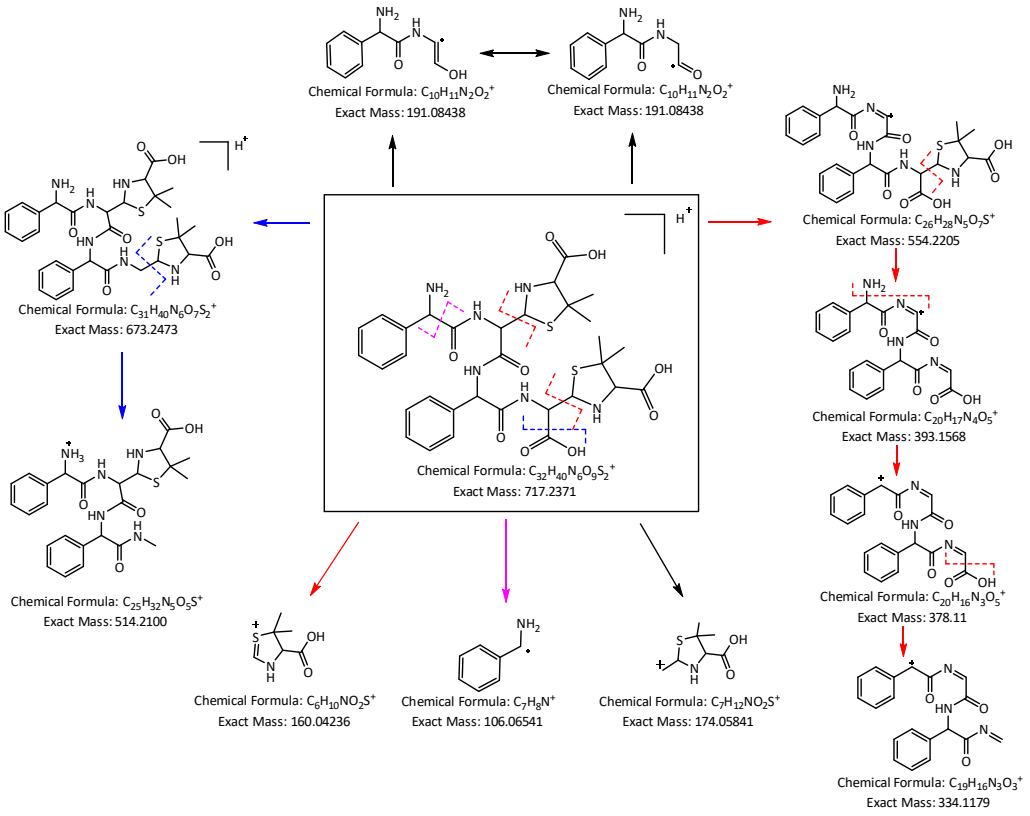


Figure 41: Fragmentation mechanism of the oligomer ampicillin and ampicilloic acid.

Some fragments could not be identified. For example, there was a large fragment peak at *m/z* 337 in the mass spectra (Figure 40 a.). This was also present in the mass spectra obtained for compound 9. in the Waters Synapt system, and therefore likely to be a fragment of the compound.

5.2.2 Degradation Mechanism

Twelve degradation products were identified using LCMS/MS. The identification of these major degradation products of ampicillin sodium in water, allowed for the creation of a possible degradation mechanism (Figure 42). The degradation products in the figure have all been previously identified in literature[34, 40-43, 74, 109]. However, the mechanism of how many of these degradation products form is not as known. From this project, two main degradation pathways were proposed for the degradation of ampicillin sodium: an intramolecular formation and an oligomeric formation. Using the TIC and UV-Vis absorbance detection it was determined that the

ampicillin dimer formation (11.) and ampicilloic acid (2.a) were the most abundant degradation products formed.

The formation of ampicilloic (2. and 2.a) and ampilloic (2.b) acids has been depicted in a number of articles. Commonly, the ampicilloic acid has been reported to readily form opening of the amide bond of the β-lactam ring structure in both acidic and alkaline solutions[22]. Once this product has formed, it has been reported to rapidly undergo a decarboxylation to for the ampilloic acid[22] (Figure 42).

The dimer has been previously identified to form in high concentrations of ampicillin[37]. Like a large component of the degradation of ampicillin sodium, this compound forms due to a nucleophilic attack of the amino side-chain to the carboxyl group of the β-lactam ring[37]. This interaction is called an auto aminolysis[37, 39, 111]. It was likely that all of the homo-oligomeric degradation products formed using this mechanism. However, it is unclear whether this was the case for the hetero-oligomeric products. There were two main directions in which these products were thought to take. For example, for compounds 8. and 9. (stereoisomers of oligomers of ampicillin and ampicilloic acid), the product could form by either ring opening of a dimer molecule, and further decarboxylation into an oligomer of ampicillin and ampilloic acid. Or, a molecule of ampicillin undergoes an intermolecular auto aminolysis with an ampicilloic acid degradation product. Some of the degradation products produced in the mechanism were not identified in the LCMS/MS. Due to the two possible pathways of hetero-oligomeric formation, and that they have been previously identified in literature[41], it was assumed that these compounds would be present in ampicillin degradation.

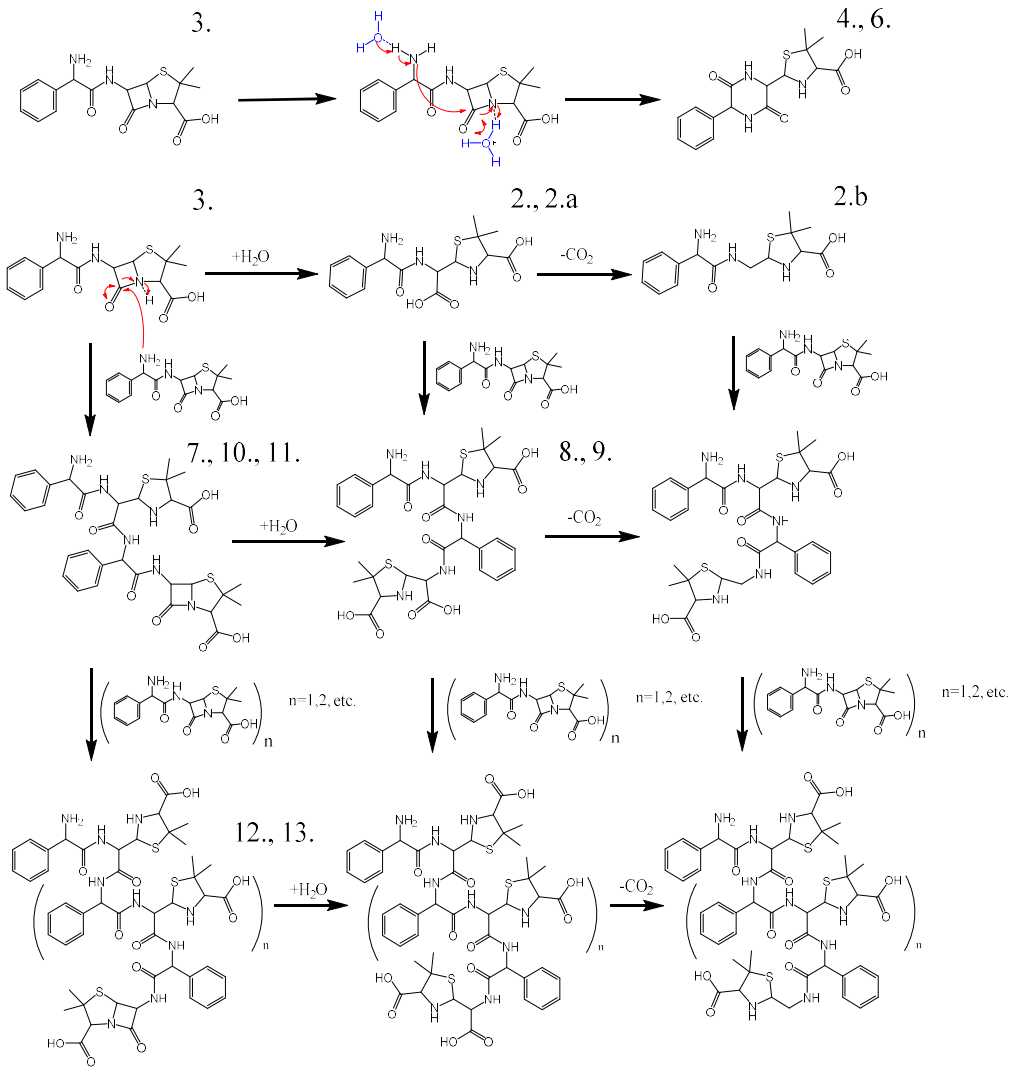


Figure 42: Proposed degradation mechanism of ampicillin sodium, from identified degradation products. Compounds labelled with identified degradation product peaks from LCMS chromatographs from Figure 25 and Figure 26.

Diketopiperazines are known to form in alkaline solutions[39]. Formation of this compound could have been initiated by the attachment of water to the amino side-chain (Figure 42). From this, the nitrogen and to the carbon of the carboxyl group on the β-lactam ring formed a bond, causing transformation of the β-lactam ring into a six-membered ring. Another possible mechanism is that the drug may have degraded into the diketopiperazine using an intramolecular auto aminolysis (Figure 43), where the same amino group attached directly to the carboxyl group without the initiation by water molecules.

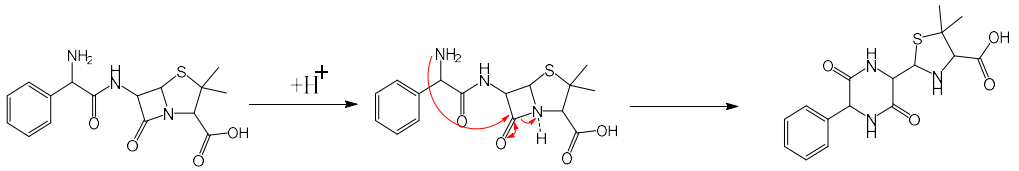


Figure 43: Degradation of ampicillin sodium into diketopiperazine *via* intramolecular auto aminolysis.

Aqueous solutions of ampicillin sodium had an initial pH of approximately 9.5 and 10 (Chapter 4.2), for samples of 16 mg.mL^{-1} and 56 mg.mL^{-1}, respectively. By the end of each kinetic analysis of ampicillin sodium degradation, the solutions showed to have a decrease in pH, becoming more neutral. The diketopiperazine has previously been said to form more readily in an alkaline pH below 9, and the rate decreases significantly above this point[38]. However, the product was observed to form in the early stages of ampicillin sodium degradation. This indicated that the degradation product can form in higher pH.

As further characterisation was required for compound 5. (2-Z-{[(Z)-amino-2-phenylethyl]imino}ethanal) this product was not included in the mechanism. It was unclear whether the degradation product 2-Z-{[(Z)-amino-2-phenylethyl]imino}ethanal formed for ampicillin directly or if it formed from another product.

The kinetic analysis of ampicillin sodium concentrated on the physical degradation of the drug. The identification of degradation products helped indicated whether these products would have some contributing factor to the potency of active compound. The main degradation products identified were mostly forms of intermolecular and oligomeric formations.

For effective dosage, the drug requires both the four-membered ring structure; for use against Gram-positive bacteria, and the amino group; for Gram-negative bacteria, to be unhindered[28, 110]. This is due to the process in which penicillins disrupt the bacterial cell wall. The carboxyl group on the β-lactam ring is a crucial component of all penicillins in effecting Gram-positive bacteria, by binding the bacteria's cell membrane, *via* penicillin binding proteins. The amino acid group on ampicillin sodium helps in the penetration of the cell membrane of Gram-negative bacteria. These processes cause lysis of the cell wall, rupturing and destroying the bacterial cell[28].

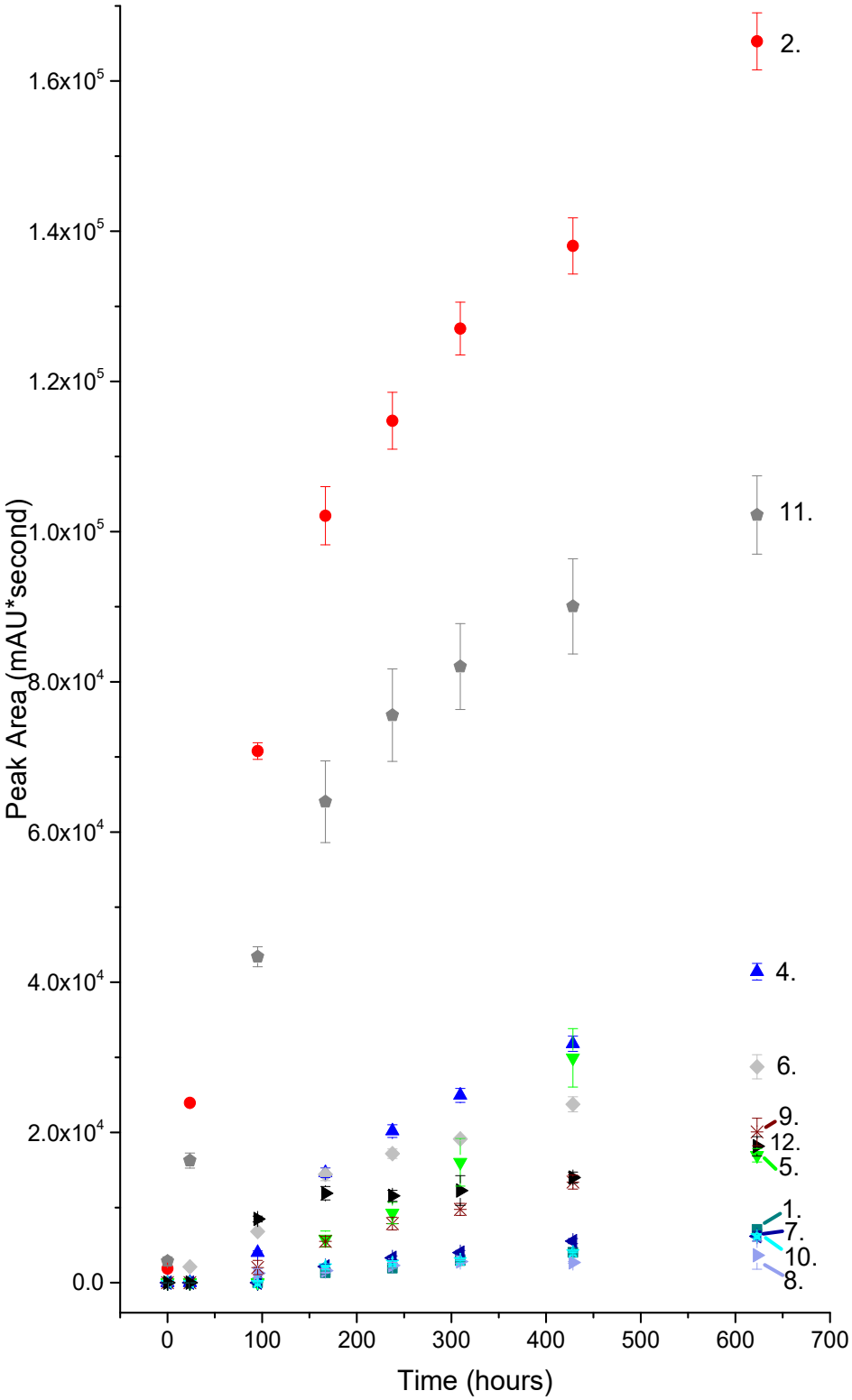
To combat lysis caused by the drug, bacteria often contain enzymes (β-lactamases), which forces the β-lactam ring open[110]. As most of the degradation products have lost these functional groups of ampicillin sodium, it was surmised that these degradation products would not be effective against the bacterial cells. In the case of the ampicillin dimer, trimer and tetramer, due to the size of the structures, the ability to bind to the penicillin binding proteins would likely be chemically hindered. This, combined with a reduced amount of ampicillin molecules, would likely cause a decrease in potency.

5.2.3 Kinetic trends of Ampicillin Sodium Degradation Products

The kinetic trends of each main degradation product were observed as a function of the peak area of the products over time, using UHPLC chromatograms obtained in water (Chapter 4.2). As 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal was only detectable in the 254 nm region of the UHPLC, the kinetic trends of the compounds were observed at this wavelength. Without the concentrations of the degradation products in the samples as ampicillin degraded, the data could not be treated in the same method as ampicillin sodium, therefore remained as peak area. As compounds had different molar absorptivity the peak area is not relative between each compound, at these specific wavelengths.

As previously stated the TIC from the mass spectrometry in combination of peak areas gave an indication on which products in these conditions were most dominant. Coupling this together with the absorbance, ampicilloic acid (2.) and ampicillin dimer (11.) degradation peaks are the largest produced by ampicillin sodium degradation in water.

Figure 44 showed the different trends of each main degradation product from the sample of ampicillin sodium at 16 $mg.mL^{-1}$ at 20 ^{o}C. The figure showed that some compounds, e.g. 2-Z-{[(Z)-amino-2-phenylethyl]imino}ethanal (5.) and the tetramer (14.), indicated a slower formation, suggesting that particular conditions or products may be required before forming. It was surmised that some of the products may be intermediates, however this was not clearly observed in all degradation products for both concentrations and temperatures tested.



1. Isomer of Ampicilloic Acid 2. Isomer of Ampicilloic Acid 4. Isomer of Diketopiperazine
5. 2-Z-{[(Z)-2-amino-2-phenylethyl]imino}ethanal 6. Isomer of Diketopiperazine
7. Isomer of Ampicillin Dimer 8. Oligomer of Ampicillin and Ampilloic Acid
9. Oligomer of Ampicillin and Ampicilloic Acid 10. Isomer of Ampicillin Dimer
11. Ampicillin Dimer 12. Ampicillin Trimer

Figure 44: Kinetic trends of degradation products formed from 16 mg.mL^{-1} of ampicillin sodium in 20 ^{o}C (254nm), in water. Errors calculated as ± 1 S.D.

The relationship between the peak area (at 254 nm) of the degradation products and the analysed temperatures and initial concentrations of ampicillin sodium, was investigated. Most degradation compounds were driven by both temperature and concentration (Chapter 4.2). However, some were observed to be specifically dependant on temperature and considered concentration independent, and others were driven by concentration, and independent of temperature. The degradation of products which were influenced by both concentration and temperature conditions, showed no clear trends when plotted as peak area over time (Figure 45).

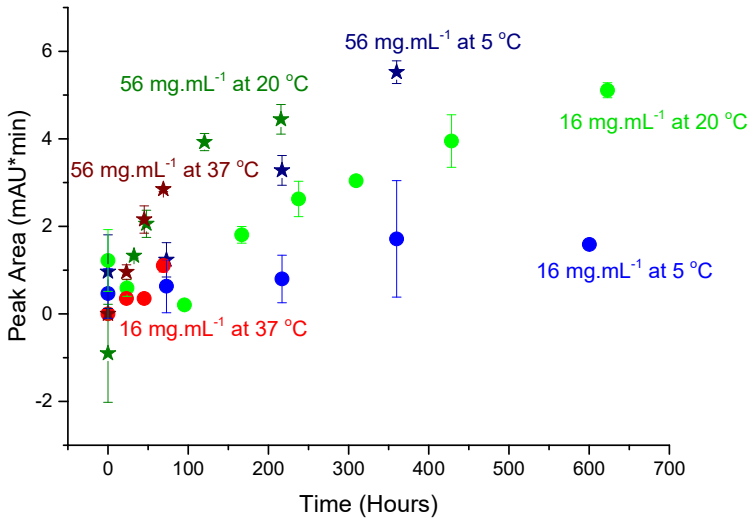


Figure 45: Kinetic trends of the hetero-oligomer of ampicillin and ampicilloic acid (8.) when initial concentrations of ampicillin are of 16 mg.mL^{-1} and 56 mg.mL^{-1}, and at temperatures of 5 °C, 20 °C, and 37 °C. Errors calculated as ± 1 S.D.

Some degradation products showed clear trends that the formation of these products was independent of concentration or temperature. The ampicilloic acid (2.) peak was analysed over the three different temperatures and two concentrations (Figure 46 a.) and three main trends were identified. The two initial concentration of 16 mg.mL^{-1} and 56 mg.mL^{-1}, at 37 °C, produced an overlapped exponential increase in the peak area over time. This was also seen for the samples at 20 °C and 5 °C. As previously mentioned, the ampicilloic acid (2.) peak consisted of multiple degradation co-eluting products. However, when plotted the degradation trends still had a similar degradation trend between the different temperatures. This indicated that the ampilloic acid degradation product may also be independent of concentration.

Another degradation product that produced a similar trend was the diketopiperazines (4. And 6.). Again, three main trendlines were visible (Figure 46 b.).

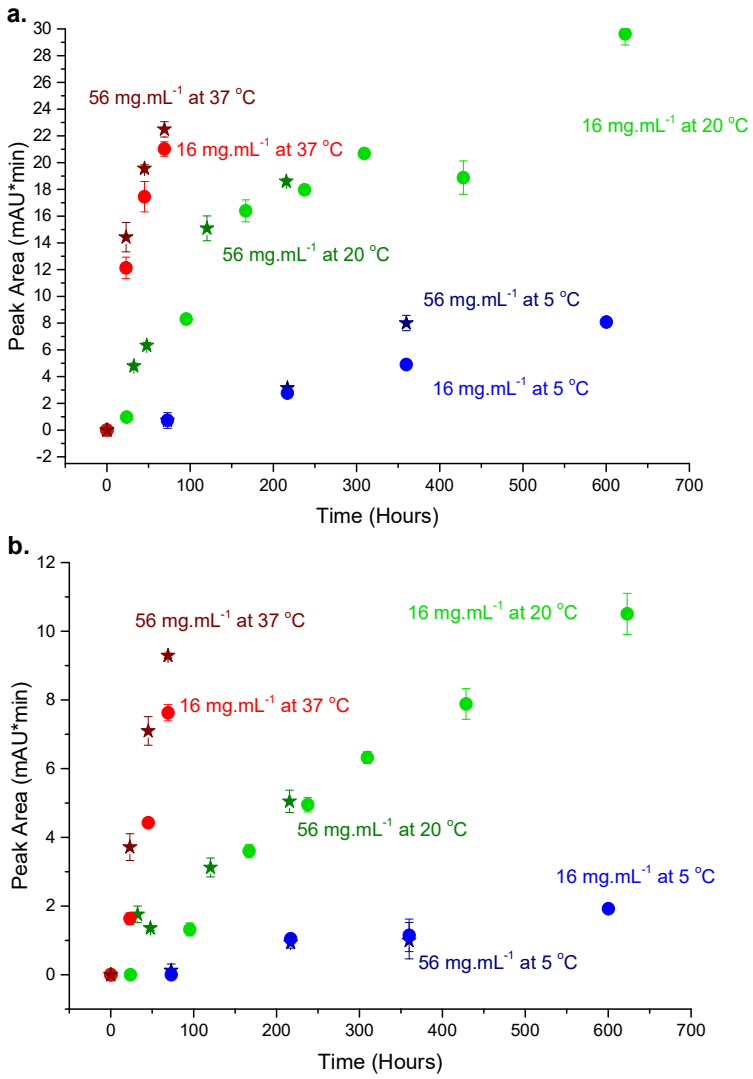


Figure 46: Kinetic trends of a. ampicilloic acid (2.) and b. diketopiperazine (4.) for initial concentrations of ampicillin sodium at 16 mg.mL^{-1} and 56 mg.mL^{-1}, and at temperatures of 5 °C, 20 °C, and 37 °C. Errors calculated as ± 1 S.D.

Other degradation products produced trends which indicated that they were independent of temperature and dependent on concentration of the product. These degradation products included isomers of the dimer (11.) (Figure 47 a.), and the tetramer (13.) (Figure 47 b.) of ampicillin sodium. The three different temperatures had a similar exponential increase of the peak area over time, for the samples of ampicillin sodium at initial concentrations and 16 mg.mL^{-1}, and 56 mg.mL^{-1}. This result in which the homo-oligomeric products are dependent on concentration has been previously reported in literature[35, 37].

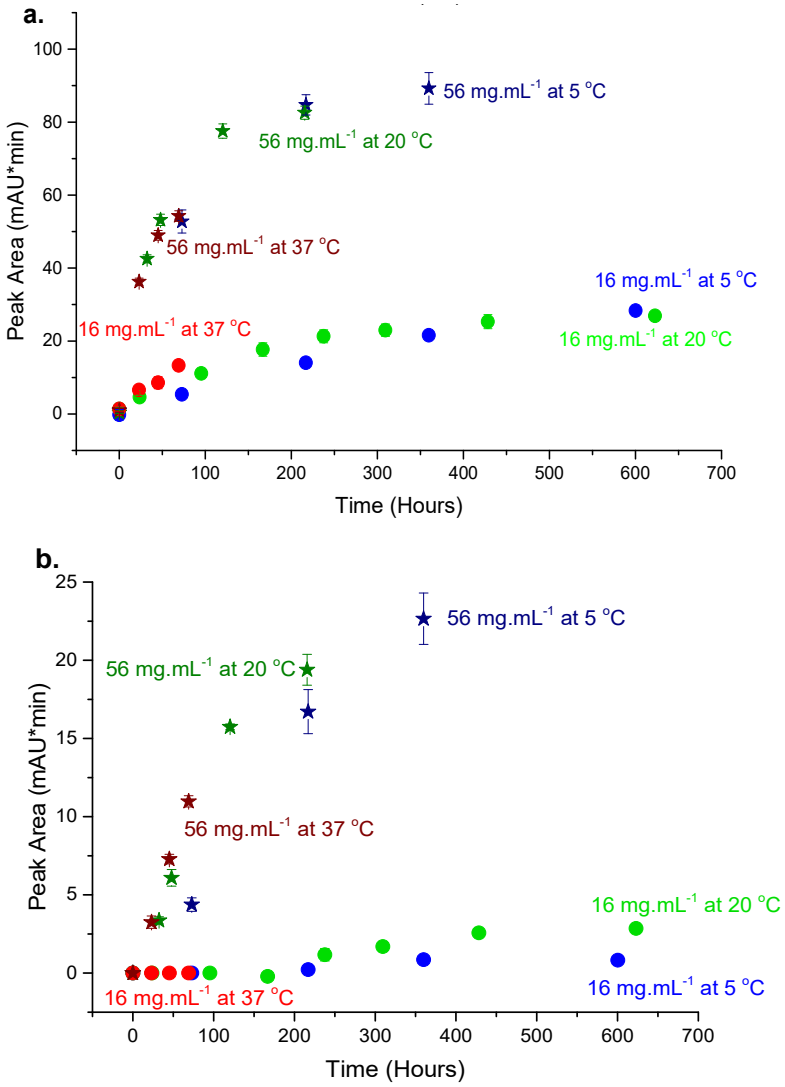


Figure 47: Kinetic trends of degradation products, a. dimer (11.) and b. trimer (12.) when initial concentrations of ampicillin sodium are of 16 mg.mL^{-1} and 56 mg.mL^{-1}, and at temperatures of 5 °C, 20 °C, and 37 °C. Errors calculated as ± 1 S.D.

For samples using water as the IV fluid, a different injection volume was used between the 16 mg.mL^{-1} and 56 mg.mL^{-1} samples. This difference would only affect the trends of the degradation products which kinetic rates are influenced by both temperature and concentration. If the injection volume were the same, e.g. 0.5 µL, for both ampicillin sodium concentrations (16 mg.mL^{-1} and 56 mg.mL^{-1}), this change would not affect the trends displayed by the concentration independent products. Degradation products that were temperature independent, the peak area of those formed by ampicillin sodium at 56 mg.mL^{-1}, would increase the peak area values; shifting the trend up.

In Chapter 4.2.1.1, the kinetic stability of ampicillin sodium was measured at a temperature of -20 ^{o}C. The results of this analysis indicated that the 56 mg.mL^{-1} ampicillin sodium samples had an increase in the stability over samples analysed at 5 ^{o}C. However, the 16 mg.mL^{-1} samples had an opposite effect, where the frozen samples had a decrease in the stability of the drug than the 5 ^{o}C samples. It has been determined that some degradation products are dependent on the temperature. This result was evident in the chromatograms (Figure 17), where isomers of ampicilloic and ampilloic acids (2., 2.a., and 2.b.), and diketopiperazines (4., and 6.) reduced formation in both frozen samples. It is likely that the reduction in temperature caused an effect on the activation energy required to form these degradation products. With the reduction in these compounds, the chromatograms showed a possible increase in the formation of the homo-oligomer products. This was likely due to the sample freezing increasing the concentration of ampicillin sodium (Chapter 4.2.1.1), which causes an increase in the probability of ampicillin molecules interacting with each other and by reducing number of competitive degradation pathways (Figure 42).

5.2.4 Analysis of Degradation Products in IV Solutions

The composition of the solvent, i.e. water, had a significant effect on the degradation kinetics of ampicillin sodium. The identification of the degradation products allowed further insight into these effects. It was previously theorised when comparing the samples saline solution with water (Chapter 4.2.2.1), that ionic strength of the samples was influencing the rate of the product formation. Due to the change in ionic strength, the charge on the amine group could change, causing a change in the stability of the drug. In the case of the oligomers e.g. Compound 11., and diketopiperazine (4. and 6.) products, the rate-limiting step of the formation, is likely the attachment of the amino group to the carboxyl group (Figure 42). In Chapter 4.2.2, 1 mg.mL^{-1} ampicillin sodium in various IV fluids samples were compared. In the chromatograms (Figure 19), the peaks identified as diketopiperazine and dimer products, have a reduced peak area in the saline solutions, compared to those degradation of ampicillin sodium in water. In the case of ampicillin sodium at the higher concentrations in saline, this could not be established, as there were no comparable chromatograms to determine if the degradation pathway was different to that of ampicillin in water for these concentrations.

Chromatograms of ampicillin sodium (16 mg.mL^{-1}, at 37 ^{o}C) in water, 0.3 M citrate buffer and 0.1 M phosphate buffer, at similar ampicillin sodium concentration percentages were compared in Figure 48. The citrate buffer was observed to not influence the degradation products when compared to the water sample. The phosphate buffer, however, had ta large effect on the kinetics of the degradation product formation. At 0.01 M phosphate buffer, ampicillin sodium had a similar degradation rate to samples of the drug in water, at the same concentration and temperature parameters. However, the increase in buffer concentration from 0.01 M to 0.2 M (pH 7) showed that the buffer caused a decrease in the concentration of the drug. In Figure 48, increasing buffer concentration clearly showed a change in which products were dominant, but no new degradation peaks formed. With an increase of buffer concentration to 0.1 M, the products showed a degradation pathway which favoured formation of two products; diketopiperazine (8.) and dimer (11.), and decreased the peak areas of the ampicilloic and ampilloic acids (2, 2.a. and 2.b.) degradation products (Figure 48).

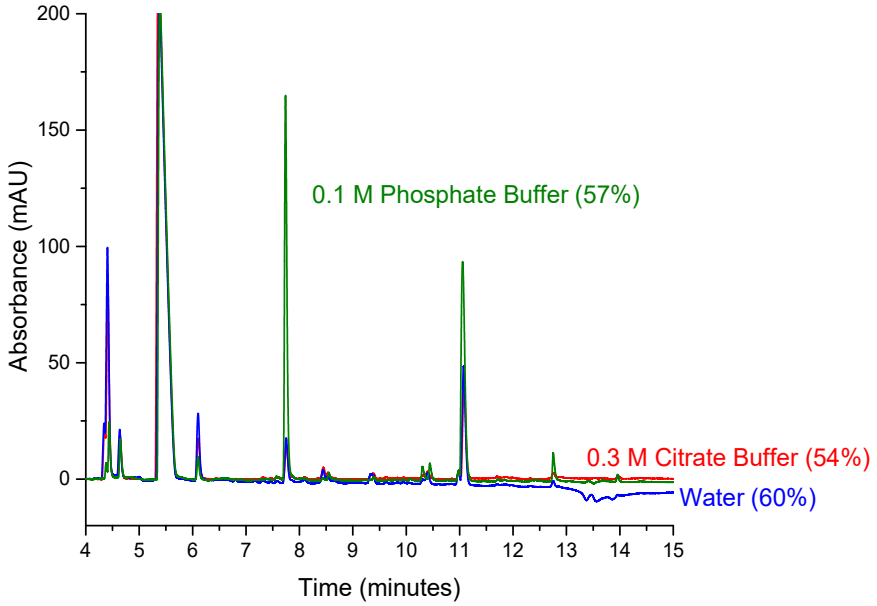


Figure 48: Chromatograms of ampicillin sodium, between 54 and 60 % ampicillin sodium remaining, of water, 0.3 M citrate and 0.1 M phosphate buffers.

There are two main possibilities in which the increase in the phosphate buffer concentration may be influencing the degradation rate. The higher buffer concentrations had a lower initial pH than those of the 0.01 M buffer made to a pH of 6.82 (Table 18). The lower pH could have influenced the degradation products that

would form. For example, it has been previously recorded that diketopiperazines form in basic solutions[40]. However, an intramolecular aminolysis degradation mechanism (Figure 43) was also possible. This did not necessarily explain why other stereoisomers of the diketopiperazine would not increase as significantly as the diketopiperazine (8.) isomer. The samples may have undergone a change in the ionic strength, where the higher concentration of phosphate salts changed the ionic strength of the solution. As the ionic strength would be different to the water, saline and citrate buffer solutions, this could have caused the limitation of the drug to change the rate at which the products form; reducing some and allowing the increase in formation of others. Multiple stereoisomers of degradation products were observed to not degrade at the same abundancy. The formation might rely on the isomer of the starting material, and the energies required for the formation. If the activation energy of one isomer was larger than that of another, the product with the larger activation energy would be unfavourable.

5.2.5 Application of Ampicillin Degradation Products and ^{1}H NMR Kinetics

The ^{1}H NMR comparison to the UHPLC method (Chapter 3.2.1.2) showed a slower degradation rate of ampicillin sodium than the UHPLC method. In Chapter 3.2.1.2, it was theorised that the degradation products may produce similar NMR spectrum, where the peak signals overlap with the drug. The molecular structures of the main degradation products (Figure 42) were similar to the structure of ampicillin sodium. For a comparison of the ^{1}H NMR spectra of ampicillin and the main degradation products identified, theoretical spectra using (ChemDraw Professional 15.0©) was calculated. Figure 49 showed the difference between a. the theoretical ^{1}H NMR spectrum and b. a measured NMR spectrum of ampicillin sodium. The theoretical spectrum had some difference, to the obtained spectra, in the chemical shifts of a number of the peaks. Most notable was the single peak representing the methyl hydrogens. Instead of two distinct peaks in the obtained data, the theoretical spectrum displayed one peak. Additionally, the theoretical spectrum calculated two peaks separate peaks for the β-lactam hydrogens instead of one. Furthermore, the protons of the amine and carboxyl groups were not detected in the obtained spectra. There were slight chemical shifts of all other peaks, however the theoretical spectrum

was calculated with the assumption that the solvent is DMSO and not D_2O, which could account for this shift[112].

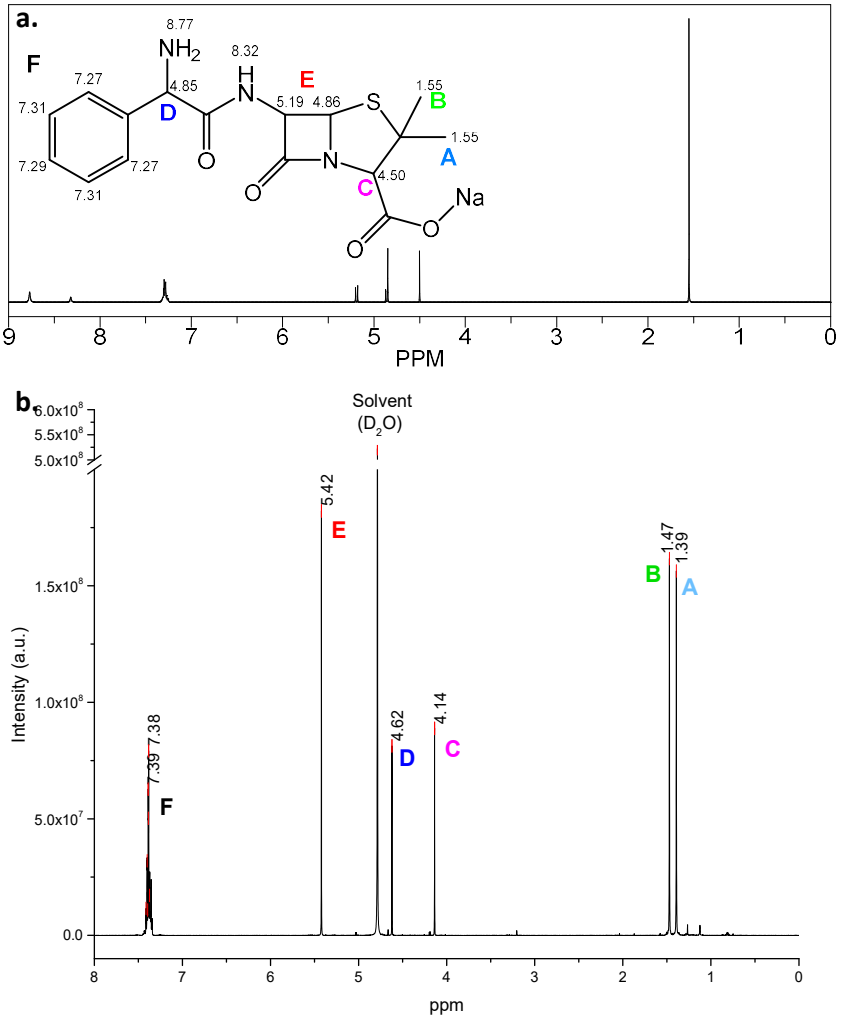


Figure 49:Proton NMR spectra of ampicillin a. of predicted by ChemDraw Prime 15® software, and b. collected spectrum of 16 mg.mL^{-1} sample at initial time, in D_2O.

The identification of the degradation products produced evidence to support the theory. The structures of the dimer, trimer and tetramer compounds had an intact β-lactam ring structure, that would have had very little effect on the chemical environment of these the peaks, A, B, C and F. The diketopiperazine and the ampilloic and ampicilloic acids would also have a similar effect on these compounds, as little change occurs for the hydrogens. All of the main degradation products were determined to have the aromatic ring attached. Figure 50 a. is a colour co-ordinated theoretical overlap of the identified degradation products and ampicillin. From this figure, a number of products had similar peak shifts for the aromatics, providing supporting evidence of the theory to the reason why there was no decrease in the

amplitude of the integration in NMR (multiplet peak F). Some degradation products were also observed to have had an overlayed on the peaks A/B and F.

A large amount of the ^{1}H peaks (Figure 49 a.) of the degradation products clustered between 4 and 5.5 ppm. A number of distinguishable peaks were observed. In the theoretical spectrum (Figure 50 a.), the ampilloic acid (green) degradation product had two distinct peaks at 3.52 and 3.77 ppm. When compared to that of the experimental spectrum (Figure 49 c.), some small peaks were observed at 3.2 to 3.6 ppm, which may have corresponded to this product. For the diketopiperazine (light blue) and ampicilloic acid (red), the theoretical spectrum showed isolated protons at 4.76 ppm and 5.82 ppm, and 4.61 and 4.68 ppm, respectively. It was difficult to compare these to the obtained spectrum (Figure 50 c.) as the D_2O solvent peak is present. However, a number of degradation peaks were observed to be protruding from the base of the solvent peak, which might be related to these compounds, also supported by 2D carbon-hydrogen NMR (See Figure 59 in Appendix 7.3). The dimer of ampicillin (dark blue) had all proton peaks overlayed with either ampicillin or another degradation product, therefore could not be seen in the figure. No peaks of 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal were present in Figure 50 c., however this could be due to a low concentration of the product in the sample.

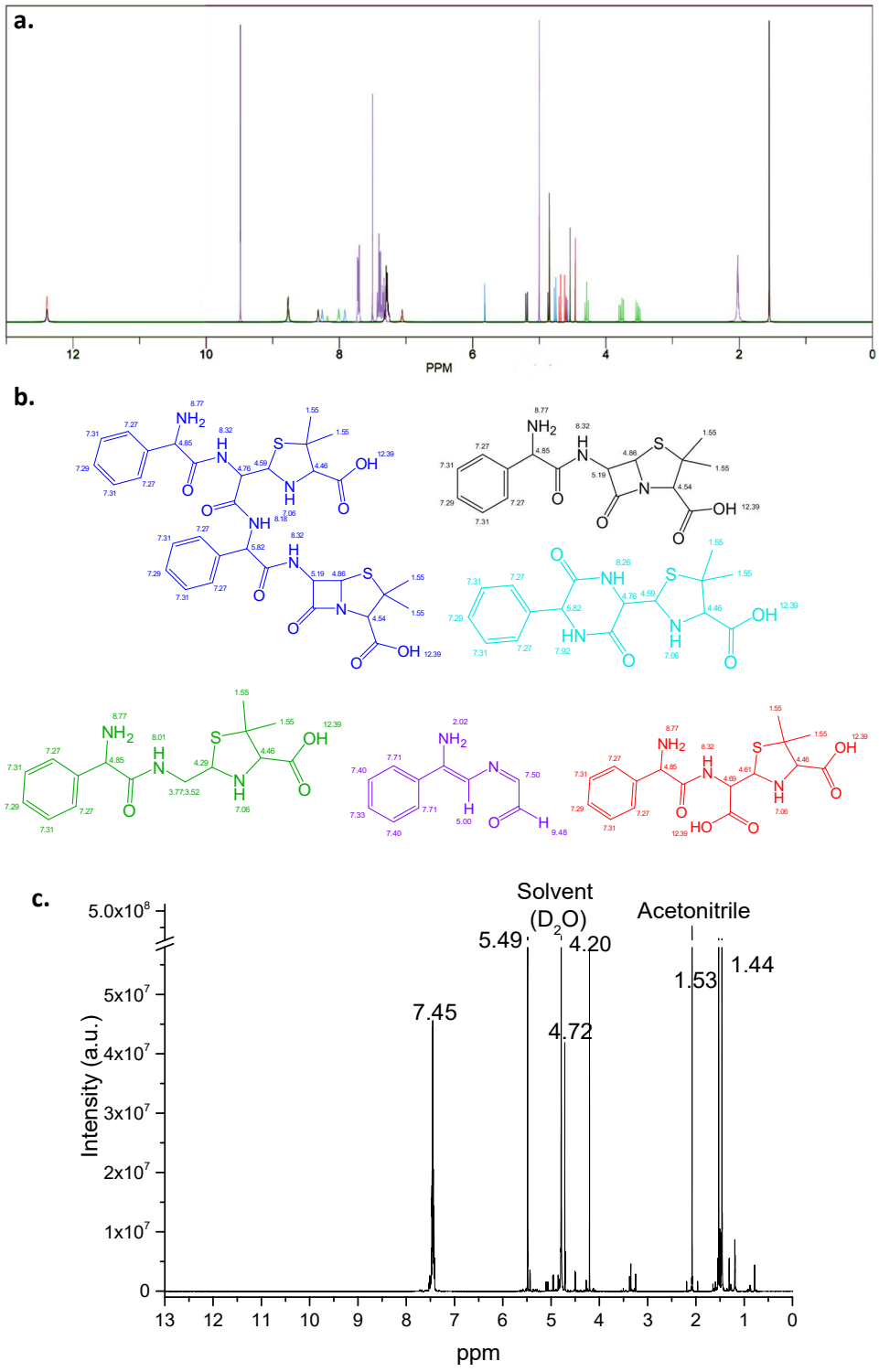


Figure 50:Proton NMR spectra of ampicillin and the main degradation products a. of predicted by ChemDraw Prime 15® software, b. colour co-ordinated structures to the theoretical predicted spectrum (a.) of ampicillin sodium (black), ampicilloic acid (red) , ampilloic acid (green), diketopiperazine (light blue), dimer (dark blue) and 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal (purple), and c) collected spectrum of a degraded sample of ampicillin sodium (16 mg.mL^{-1}) sample at initial time, in D_2O (and acetonitrile internal standard at (2.1 ppm)).

5.3 Conclusions

From the identification of the degradation products formed by the instability of ampicillin sodium, it was evident that these products could pose an issue in providing the drug to the patient at the concentrations. As the homo-oligomeric formations have been shown to cause potential anaphylaxis in some people[22], understanding the degradation of ampicillin sodium, into these products, is critical for the HITH program. The identification is a step in understanding the cause of the formation, could help in the understanding of how the drug degrades and how to reduce the amount of these products.

Identification and kinetic analysis of ampicillin sodium degradation products provided further insight to the stability of ampicillin sodium. The kinetic analysis showed that some degradation products were solely dependent on concentration e.g. ampicillin dimer, or temperature dependent e.g. diketopiperazines. Identification of the degradation products provided further information about how IV fluids could influence the degradation mechanism of the drug. From the identification of the degradation products, it was apparent that different IV fluids, or combinations of IV fluids e.g. phosphate saline buffer, could be used to control which degradation products are produced. This could reduce the formation of anaphylactic causing degradation products, or reduce ampicillin sodium degradation.

Identification of the degradation products also provided an explanation for the results observed in the NMR kinetic analysis of ampicillin sodium. The identified products all formed with an intact benzene ring, which could have explained why the aromatic proton peaks (F) were stable throughout the degradation. The theoretical spectrum derived from the products determined by LCMS/MS showed some insight into the potential overlap in the proton peaks, causing the rate to appear slower in the ^{1}H NMR compared to the UHPLC analysis. However, to confirm this, further separation and characterisation of these peaks using NMR spectroscopy is required.

6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The degradation and stability of ampicillin sodium was investigated. Using and comparing two methods, developed for proton NMR and UHPLC instruments, the kinetic trends of ampicillin sodium were analysed. An exponential decay fit was used to determine the degradation rate of ampicillin sodium. The ^{1}H NMR kinetics method did not appropriately represent the degradation of the drug. When compared to the UHPLC method, a slower degradation rate was calculated for each proton peak signal. It was surmised that this was due to peak overlap of ampicillin and at least one of its main degradation products, likely to be the oligomeric formations of ampicillin sodium. Therefore, the proton NMR method developed was not a viable method for kinetic analysis of ampicillin sodium.

The main products of formed by degradation of ampicillin sodium in aqueous solution were identified *via* LCMS. The degradation products were determined to be stereoisomers of diketopiperazines, homo- and hetero-oligomers of ampicillin, ampicilloic acids and ampilloic acids. From this, a degradation mechanism was developed. The UV absorbencies and total ion count obtained by LCMS indicated that the most abundant of these degradation products were an isomer of ampicillin dimer and an isomer of ampicilloic acid.

Ampicillin sodium at the high concentrations and temperatures, and in water, was found to be highly unstable. The increase in temperature and concentration significantly influenced the degradation rate of the drug. At refrigerated temperatures ampicillin sodium at both initial concentrations (16 mg.mL^{-1} and 56 mg.mL^{-1}) could be used for a long infusion period (over 24 hours). At higher temperatures, 56 mg.mL^{-1} degraded rapidly and would not be suitable for long infusion periods. Samples of 16 mg.mL^{-1} could be stable at 20 ^{o}C, but not at 37 ^{o}C. Freezing samples influenced the rate of degradation of the drug, where the difference in concentration influenced whether the stability of the drug improved or decreased.

The kinetic trends of the formation of the degradation products formed from ampicillin sodium, in water, showed that the change in concentration and temperature influenced the degradation mechanism. Some of degradation products were independent of concentration, where others were independent of

temperature. The homo-oligomeric formations (dimer, trimer, etc.) were observed to form largely in the higher concentration (56 $mg.mL^{-1}$) samples than the lower concentration (16 $mg.mL^{-1}$). The diketopiperazine isomers were observed to be influenced by the temperature of the samples, not concentration. Other degradation products (hetero-oligomeric products) were influenced by both concentration and temperature. Analysis on frozen samples of ampicillin sodium showed that a reduction in temperature dependant products, forced the concentration dependant products to become more dominant in the drug's degradation.

In an attempt to reduce the degradation rate, several solvents: saline, citrate buffer and phosphate buffer, were analysed. However, these solvents did not improve the stability of ampicillin sodium at these concentrations. The pH was tested to determine whether the buffers would control the pH of the solutions. The buffers reduced the initial pH of the samples, compared to ampicillin sodium in water. However, the results indicated that the concentration of ampicillin sodium may have been too high for the buffers to effectively control and reduce the instability of the drug. As the solvents seemed to have an effect on the degradation rate of ampicillin, ionic strength may have had an influence on the stability. Ionic strength is dependent on pH.

It was found that a change in solvent could influence the degradation mechanism, by changing the rate and/or the formation of products. Phosphate buffer was observed to influence the mechanism by reducing the formation of the ampicilloic acid, and favour a specific isomer of diketopiperazine. Samples of ampicillin at 1 $mg.mL^{-1}$ displayed a difference in degradation product formation between IV solvents of saline, citrate buffer and 5 % glucose. The samples in 5 % glucose solution was observed to likely produce different degradation products.

The stability of the drug was analysed in two IV containers; Freeflex® and EVA. Stability of the IV containers was compared to the stability of ampicillin sodium in glass containers. The results indicated that the IV containers had no effect on the stability of ampicillin sodium

6.2 Future Directions

The research performed and analysed in this project was a starting point and small fraction of the investigation into the stability of drugs for the purpose of the HITH program. With ampicillin sodium alone, further investigation is required. The samples analysed were kept in controlled temperatures. In the home, the drug would not be exposed to restricted temperatures. To further simulate the temperatures in the home, investigation of samples in a fluctuating temperature environment could be tested to observe variation in the drug's stability.

Additional testing of commonly used IV infusion buffers could see an improvement of ampicillin sodium stability. For example, a combination of phosphate-citrate buffer, giving a larger effective pH range incorporating both pH 5 and 7. This combination could cause a better outcome in degradation of ampicillin by potentially reducing the negative effects seen of each buffer. Additional parameters, such as organic or mixtures of aqueous and organic solvents, that are safe for injection in the human body, could reduce instability. Introducing biomedical friendly materials, such as cyclodextrins or nanoparticles, and analysing the effects on the stability of the drug.

Initial testing showed that there are potential issues and influences of freezing samples of ampicillin sodium. Further investigation is required into the effects observed. Ampicillin sodium, at the two concentrations in water solutions, had similar degradation trends. Further analysis of the samples is required to determine if the solutions did freeze in a non-homogeneous block of water and drug. The frozen samples were analysed in samples of 2 mL volumes. The effects of the drug stability increasing the volume of the IV solution would also need to be investigated. The method and length of time for defrosting these frozen samples would vary with increase in volume.

Isolation and further investigation of the main degradation products could be used to better understand the degradation mechanism. Isolation of the degradation products could allow for the kinetic rate of each individual product to be analysed and calculated. The isolation of the degradation products would confirm peak overlap in the ^{1}H NMR was the cause to the slow kinetic rate observed. Separation of the products would allow further analysis using different techniques to characterise

these products. For example, confirming the structure of the previously unidentified compound: 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal. Further analysis could identify all products produced by the degradation of ampicillin sodium at temperatures and concentrations experienced in the home.

7. APPENDICES

7.1 Structures of common β-lactam antibiotics: Penicillins and Cephalosporins

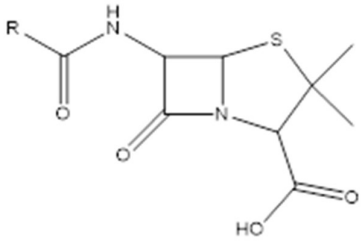


Figure 51: Generic molecular structure of penicillins

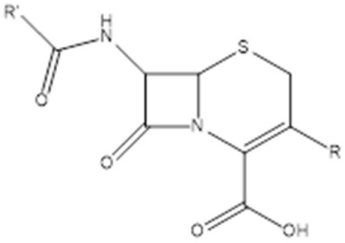


Figure 52: Generic molecular structure of cephalosporins

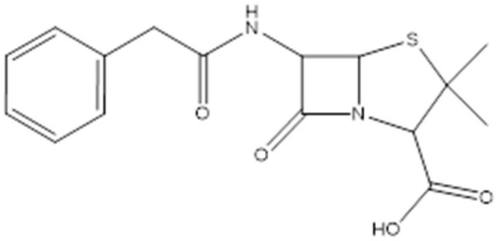


Figure 53: Structure of the penicillin, Penicillin G.

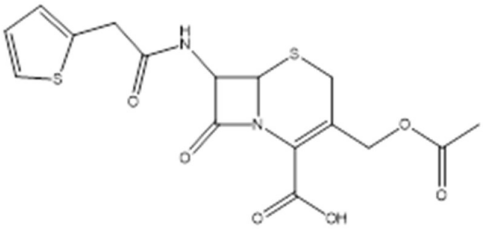


Figure 54: Structure of Cephalothin

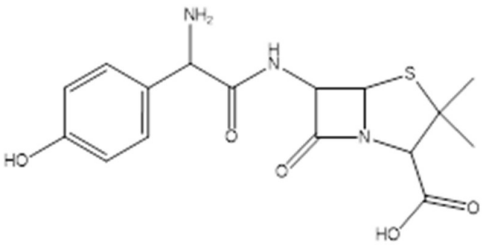


Figure 55: Structure of Amoxicillin (penicillin).

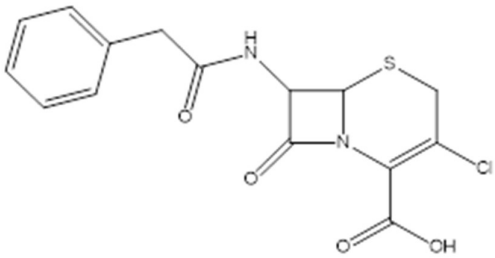


Figure 56: Structure of Cefaclor

7.2 Additional Information on Kinetics and NMR Theory

7.2.1 First Order Kinetics

Kinetics is the measurement of the change in a substance's concentration over time. For example, the decrease in concentration of the reactant ampicillin over time. [48][88]

$$\text{rate} \propto [\text{Amp}]^{\text{n}}$$

$$\text{rate} = \text{k}[\text{Amp}]^{\text{n}}$$

Where, k is the rate constant, [Amp] is the concentration of the reactant and n is the order of the reaction in respect to the reactant ([Amp]). The rate constant value is relative to the concentration of the reactants as it changes over time e.g. ampicillin sodium as reactant. If n = 1, the overall rate order of the reactant (ampicillin sodium) reaction is first order reactions[48]. First order kinetics is when the reaction undergoes a spontaneous irreversible decomposition of a reactant[49, 50].

For first order kinetics:

$$rate = -\frac{d\,[Amp]}{dt} = k[Amp]$$

And therefore, can mathematically be expressed as:

Equation 2:

$$ln[Amp] = ln[Amp_{int}] - kt$$

The first order reaction equation uses the data collected to produce a graph as a function of the natural logarithm ($\ln[Amp]$) versus time (t), constructing a linear plot, where the slope (k) is negative[48]. This negative slope equates to the rate constant, from Equation 2:. Previous studies stated that ampicillin sodium, like most drugs, degrades following pseudo-first order kinetics the Equation 2:[26, 38, 44, 79, 113-115]. Pseudo-first order refers to when more than one reactant is present, but the concentrations of the other reactant/s e.g. the aqueous solvent, are so large than the reactant, e.g. ampicillin sodium, the change of concentration is insignificant in comparison[49].

From rearranging the first order equation, the 90 % shelf life can be determined:

Equation 3:

$$t = -\frac{ln[0.9]}{k}$$

7.2.2 NMR Chemical Shift and Spin-Spin Coupling

The position of the peaks in the spectrum is dependent on the environment of the nucleus being observed, as it characteristically influences the resonances. When a compound is strongly shielded, by neighbouring atoms, it means that the protons of interest resonate at low fields and therefore do not have a high chemical shift. For example, the substitution of a proton from a methyl group causes deshielding; causing the peak to increase in shift, and the remaining protons are subject to resonate at lower fields. Hydrogen bonding and an increasing electronegativity of adjacent atoms also increases the chemical shift of the peaks[89]. A change in chemical environment can cause a chemical shift in the spectrum, and is measured by parts per million (ppm). The chemical shifts of the peaks are influenced by the solvent used, concentration and temperature[116]. These are usually small affects, but can be significant.

Spin-spin coupling is a process in which the spinning nuclei of two or more atoms, which are in different chemical environments, interact. The effects of the coupling are detected in the spectrum by the splitting of a peak at a given chemical shift to give a multiplet peak. The protons in one functional group undergo spin-spin coupling with the protons of an adjacent functional group. Splitting of peaks requires that the protons reside in different chemical environments; peaks will not split if two or more nuclei reside in equivalent chemical environments[73]. The aromatic hydrogen peaks are often observed to produce one peak with multiple splitting. This is due to the hydrogens being in similar chemical environments, caused by coupling occurring with neighbouring hydrogens and across the aromatic ring.

7.3 Supportive data for NMR kinetics

Analysis of ampicillin degradation by proton NMR, showed significant loss of intensity and peak shifts caused by degradation of the drug over 166.4 hours.

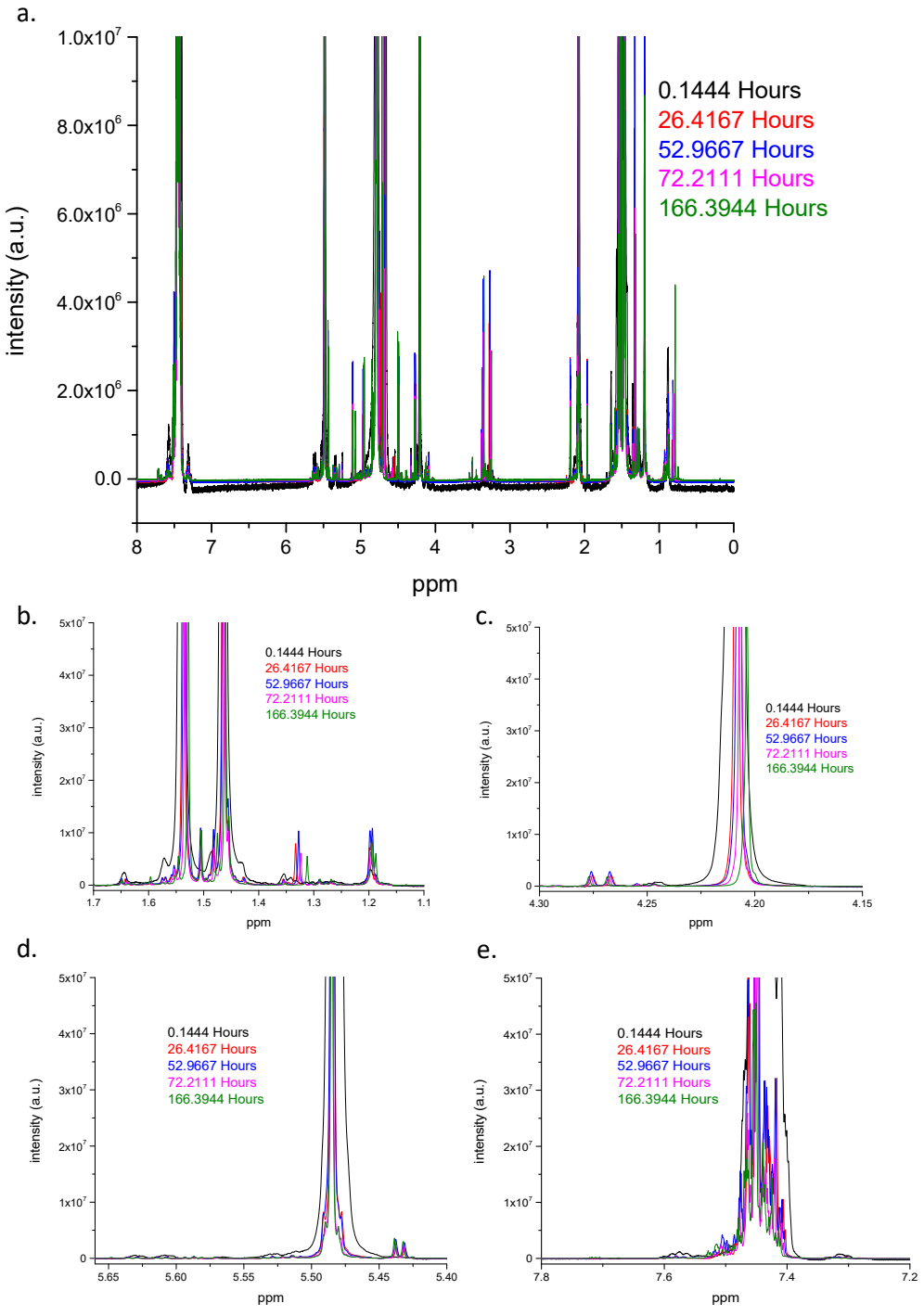


Figure 57: Proton NMR spectra of degraded ampicillin samples a. full spectrum, b. Peaks A and B, c. Peak C region, d. Peak E region, and e. Peak F region.

Analysis of a sample when fresh (Figure 58) and degraded (Figure 59) ampicillin sodium (56 mg.mL^{-1}), using 2D HMQC (Proton detected heteronuclear multi-quantum coherence) NMR. For the purpose this research, the use of multidimensional NMR allowed for the analysis of the connectivity of atoms in the molecule between ^{13}C and ^{1}H atoms[96]. The 2D HMQC NMR analysed the interactions between ^{13}C (y-axis) and the corresponding ^{1}H (x-axis), that are connected to these carbon atoms. As such, the carbon-hydrogens interactions of these atoms can determine which protons are effected by the degradation of ampicillin into other compounds. The analysis was performed to determine if peak overlap was contributing to the slower degradation rate observed in NMR when compared to the UHPLC. Due to the length of time required to produce a 2D HMQC plot, the fresh sample did degrade to an extent during the analysis.

The degraded sample for the comparison of the two figures (Figure 59) showed an increase in the number of peaks in the spectrum than the non-degraded sample (Figure 58). However, the data indicated that there was peak overlap of protons. As a number chemical shifts were observed in the carbon spectrum to occur, the corresponding proton spectrum showed little change. This was observed as small points were observed to move down the y-axis but remained with the same ppm shift in the x-axis.

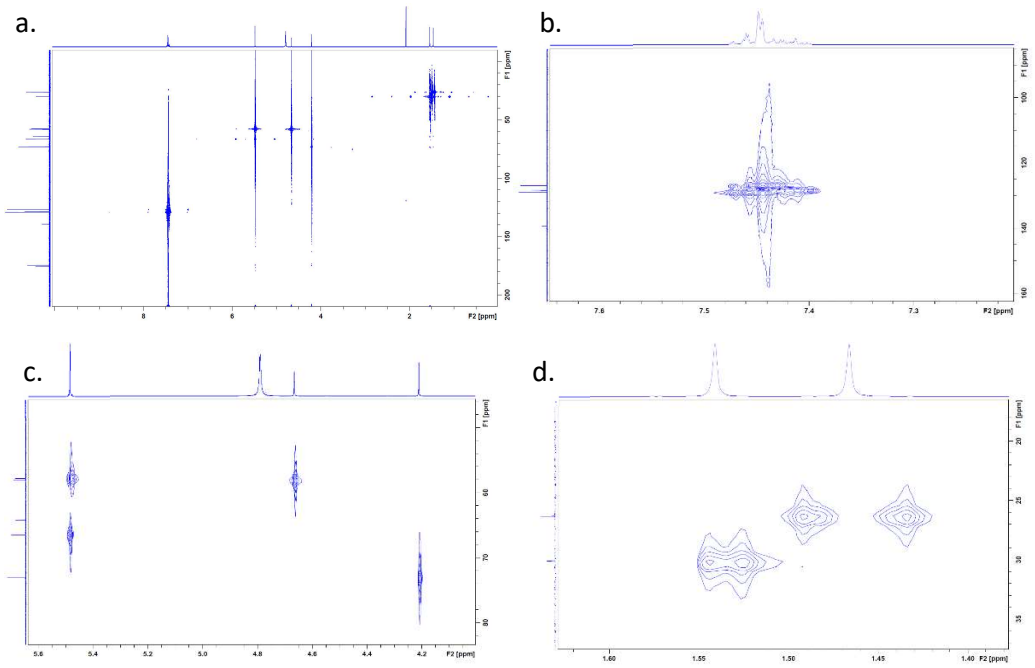


Figure 58: 2D Carbon-Hydrogen NMR of ampicillin sodium (56 mg.ml^{-1}) with acetonitrile internal standard a. full size, b. aromatic carbon-hydrogen (F), c. proton peak signals C, D and E, and d. peaks A and B.

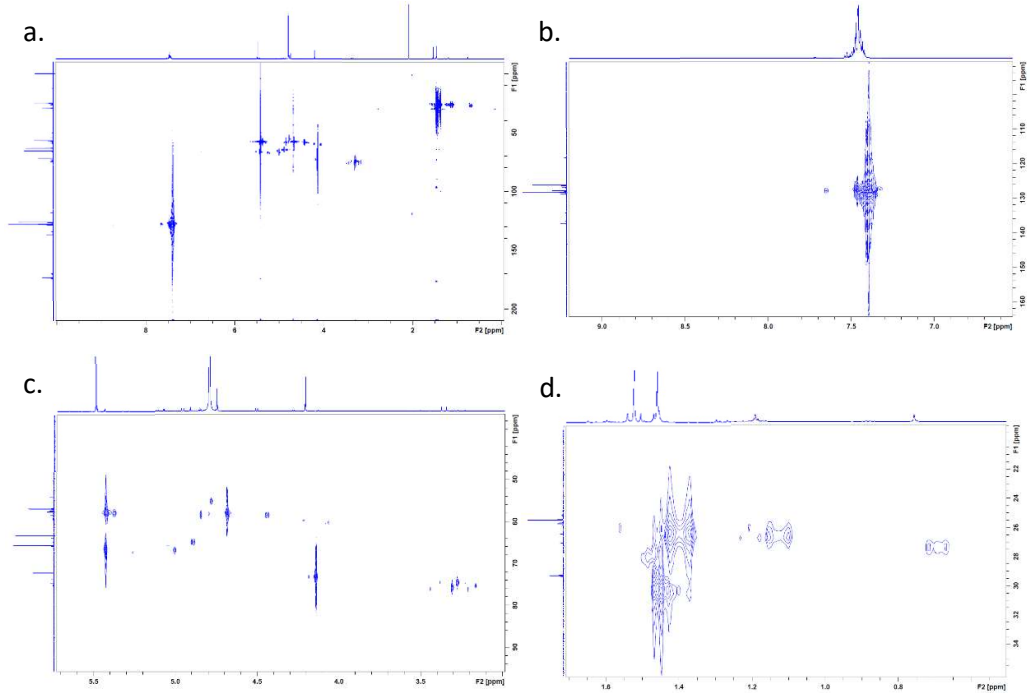


Figure 59: Ampicillin sodium (initial concentration 56 mg.ml^{-1}) degraded over 10 days, with acetonitrile internal standard a. full size, b. aromatic carbon-hydrogen signals (F), c. proton peak signals C, D and E, and d. peaks A and B.

7.4 Mass Spectra of all Identified Degradation products

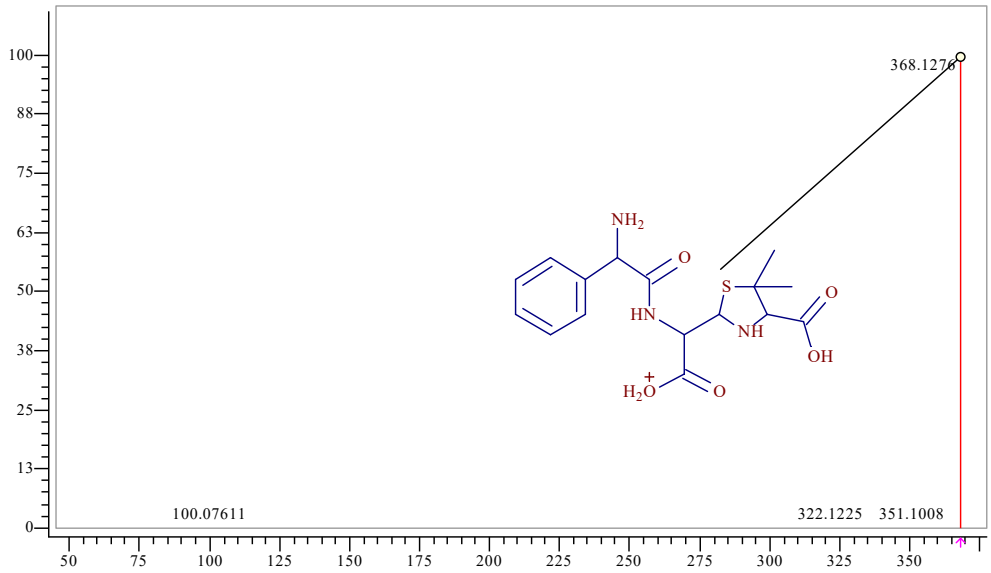
Peak Retention Times (mins)

Thermo Scientific™ Orbitrap LCMS: 4.29

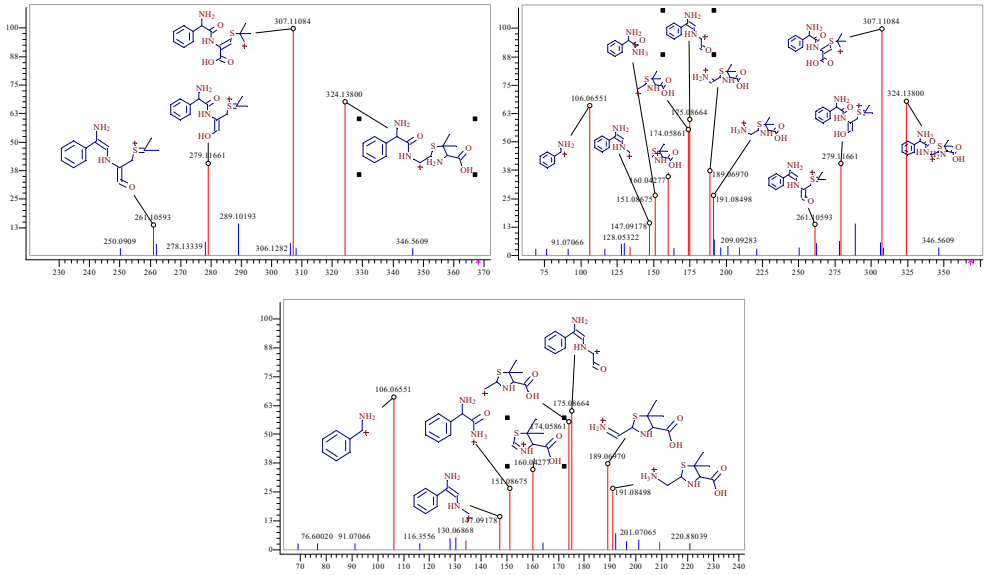
Waters LCMS: 5.75

Compound 1: Stereoisomer of Ampicilloic Acid (*m/z* 368)*

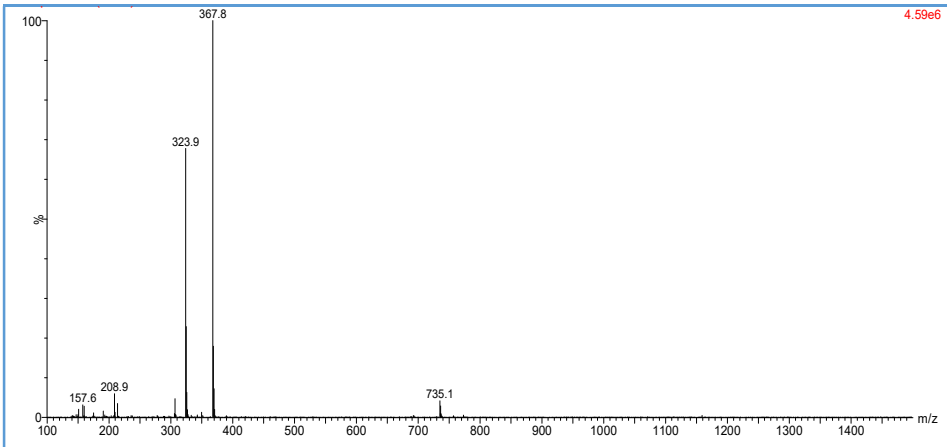
Thermo Scientific™ Mass Spectrum:



MS/MS NODES:



Waters LC-MS/MS Mass Spectrum Positive ES:



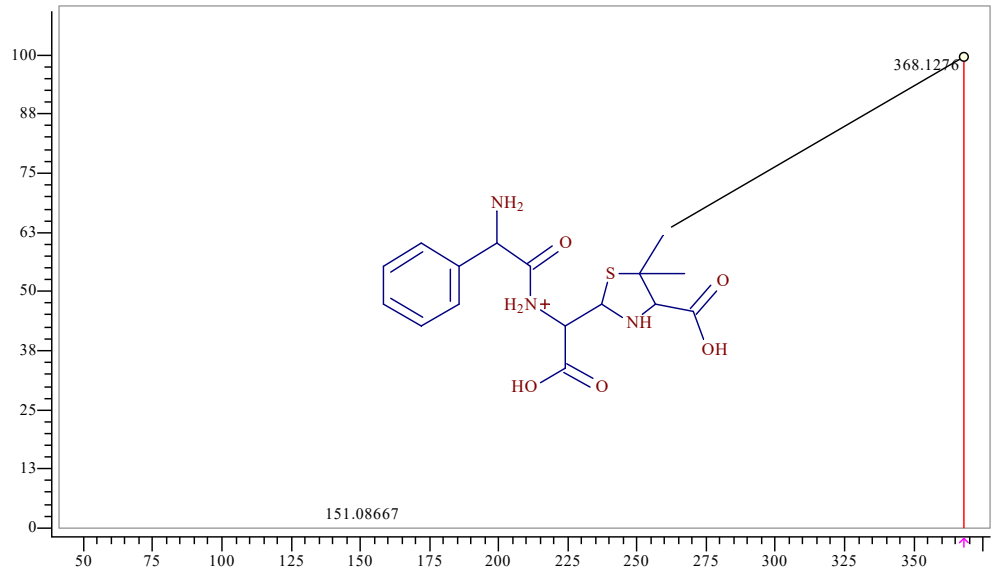
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 5.28

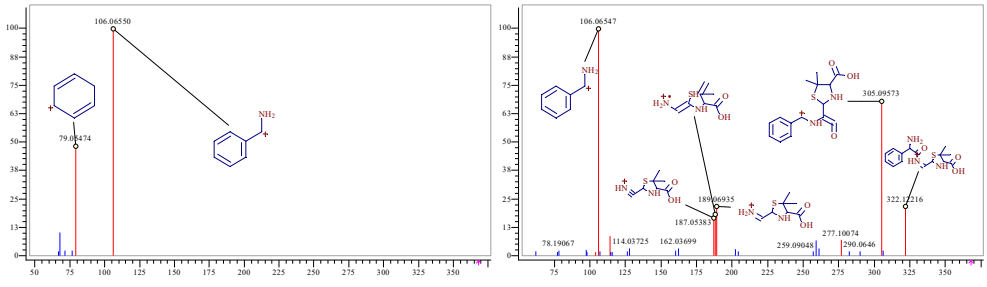
Waters LCMS: 6.46

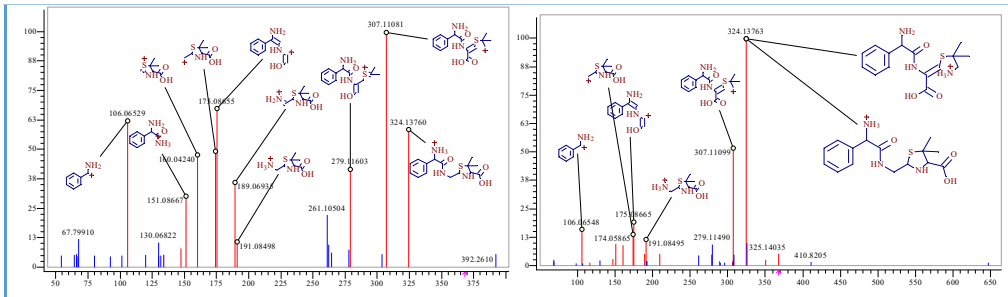
Compound 2: Stereoisomer of Ampicilloic Acid (*m/z* 368) *

Thermo Scientific™ Mass Spectrum:

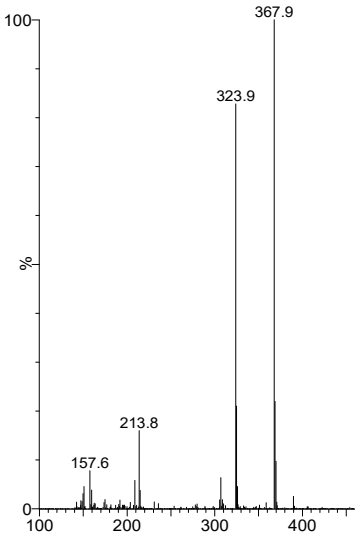


MS/MS NODES:

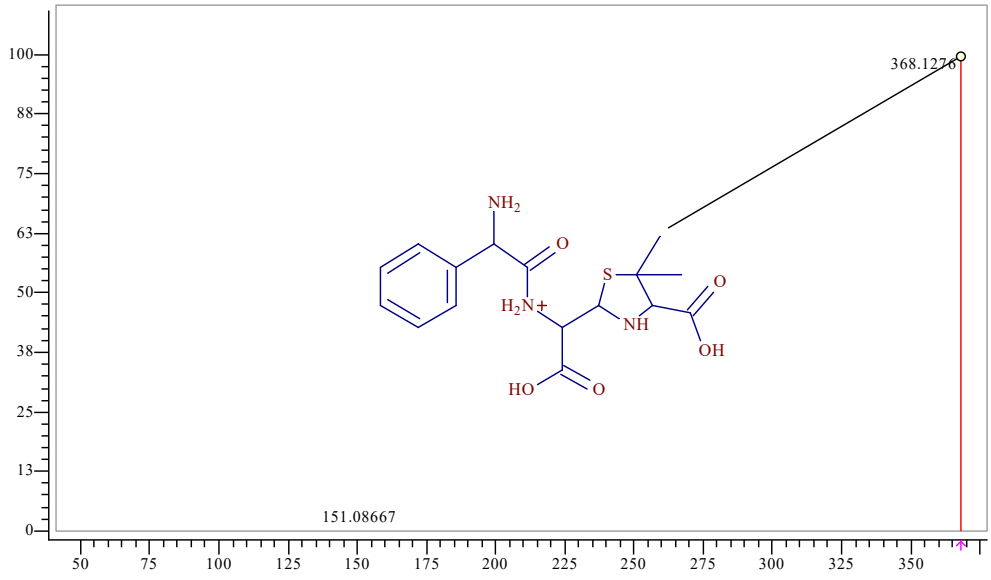




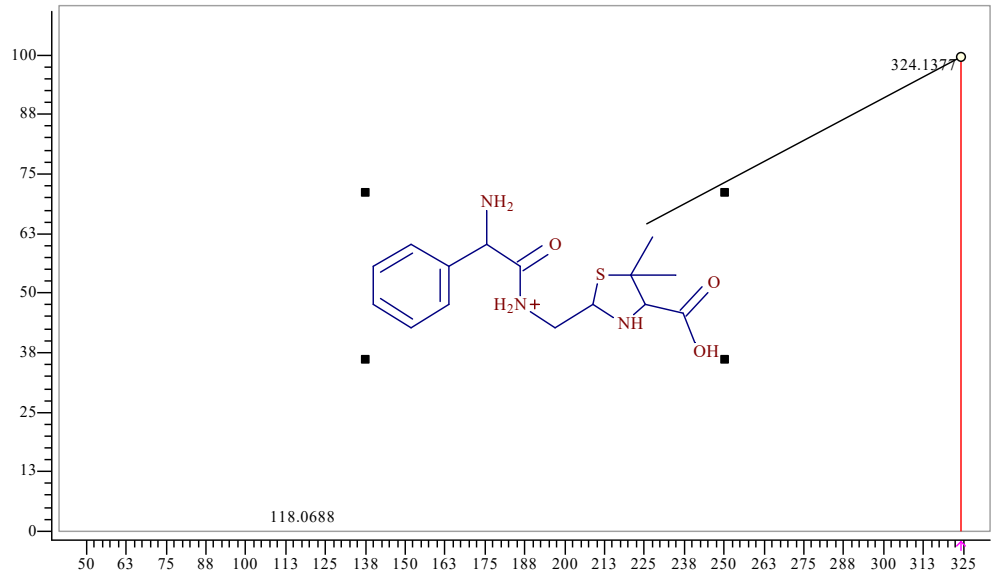
Waters LC-MS/MS Mass Spectrum:



2.a. 5.53 mins:



2.b. 5.96 mins:



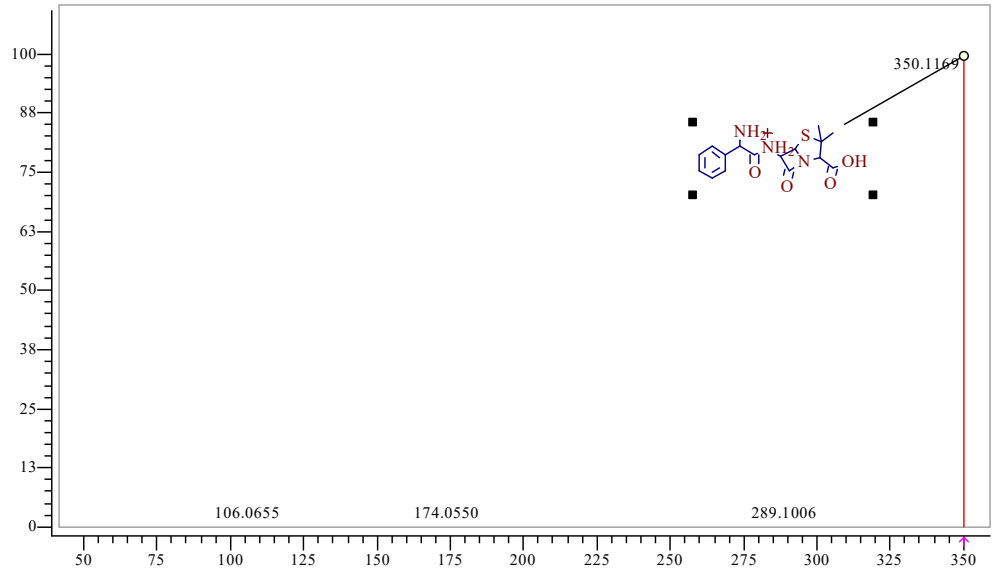
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 6.55

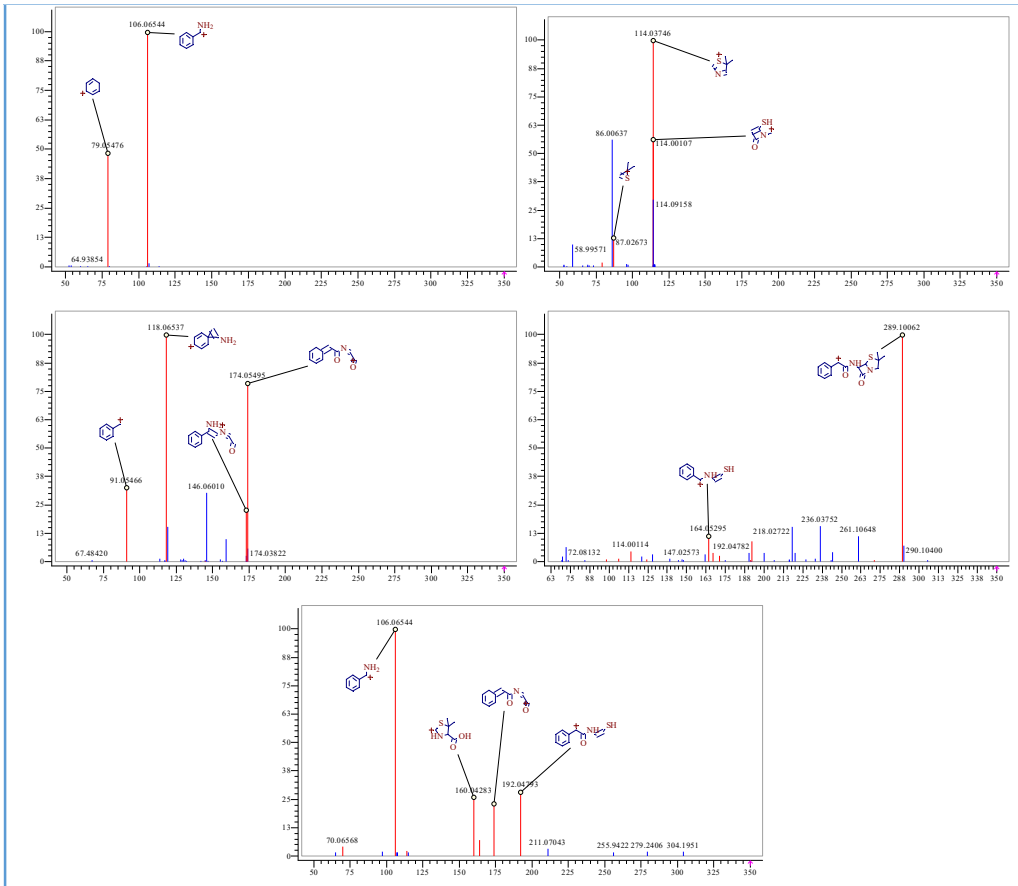
Waters LCMS: 7.09

Compound 3: Ampicillin (*m/z* 350) *

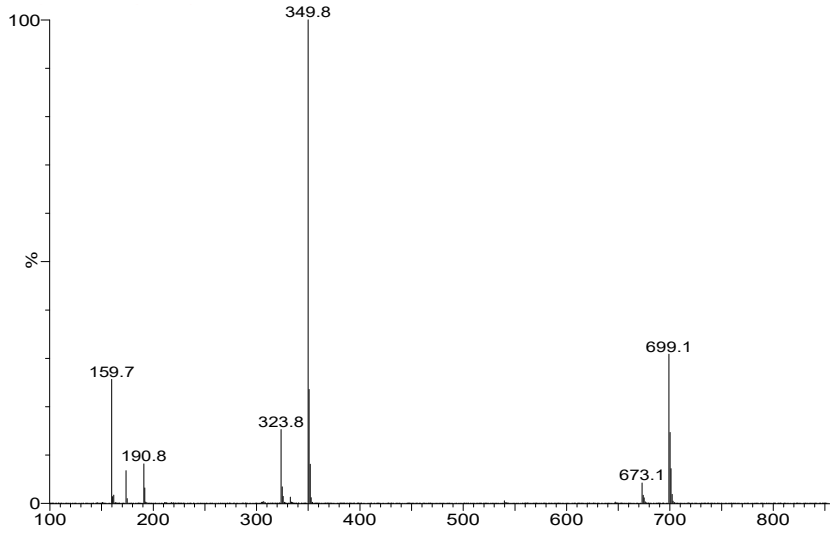
Thermo Scientific™ Mass Spectrum:



MS/MS NODES:



Waters LC-MS/MS Mass Spectrum:



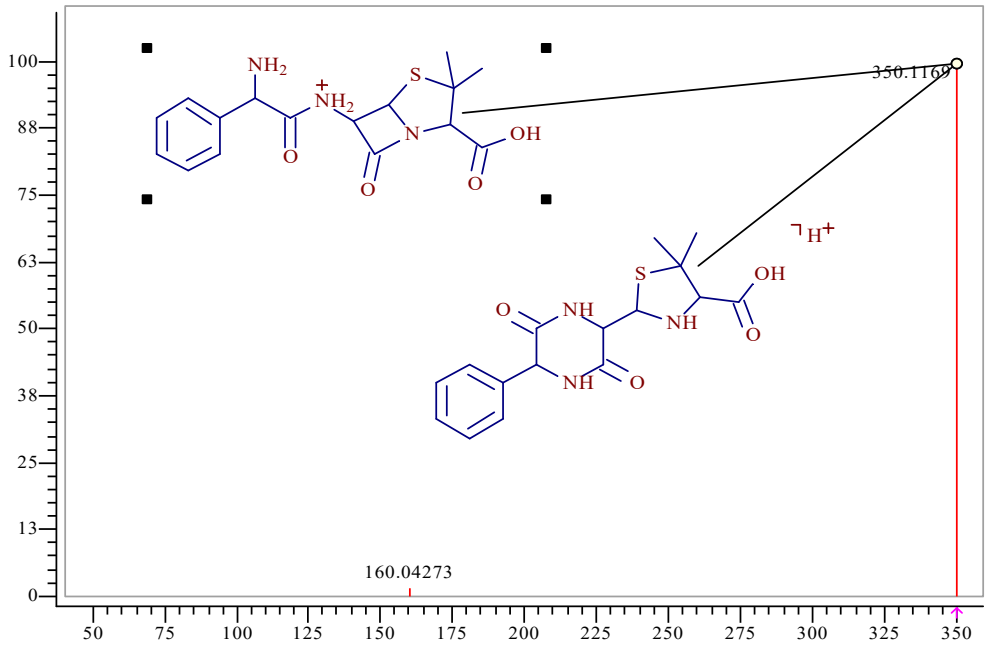
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 7.15

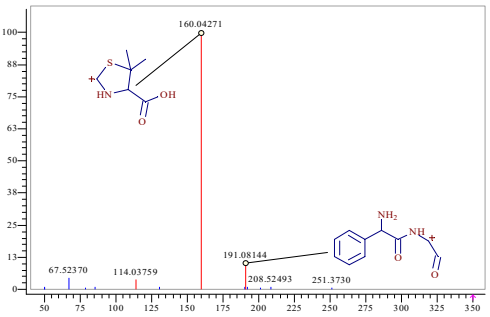
Waters LCMS: 8.45

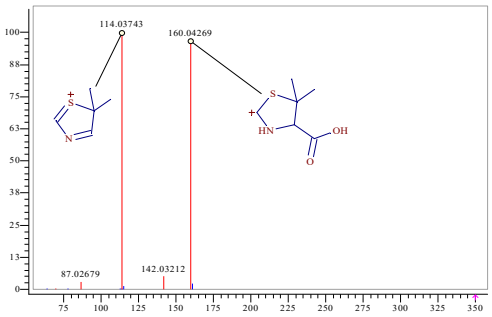
Compound 4: Stereoisomer of Diketopiperazine (*m/z* 350) *

Thermo Scientific™ Mass Spectrum:

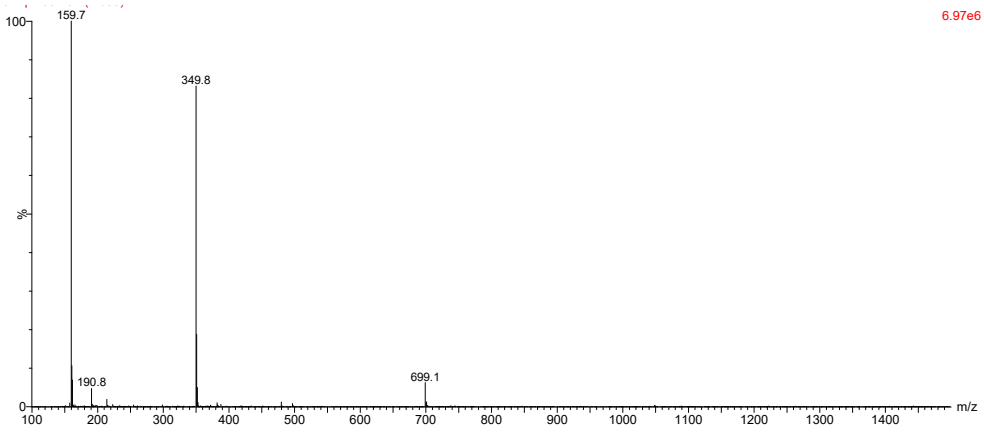


MS/MS NODES:





Waters LC-MS/MS Mass Spectrum:



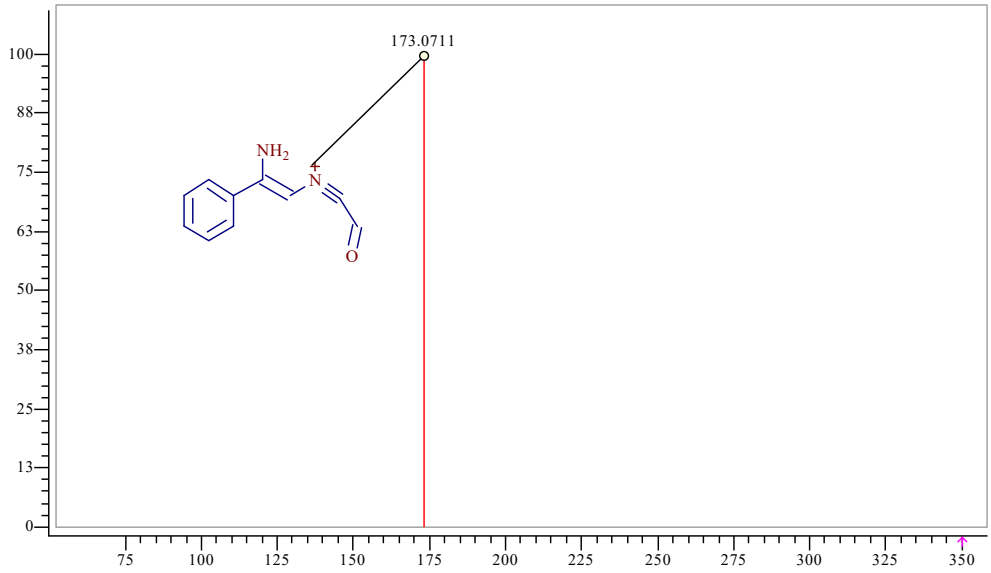
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 7.86

Waters LCMS: 9.77

Compound 5: 2-Z–{[(Z)-2-amino-2-phenylethenyl]imino}ethanal (*m/z* 173)

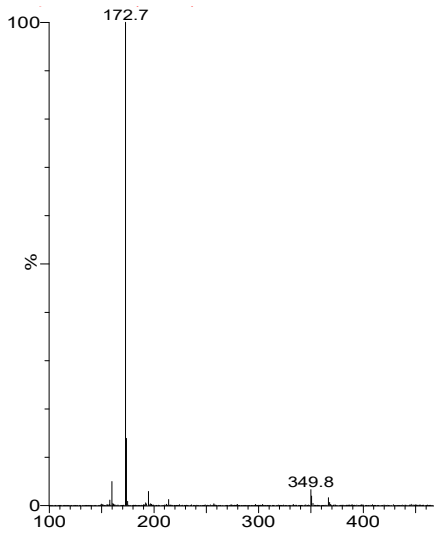
Thermo Scientific™ Mass Spectrum:



MS/MS NODES:

No fragmentation

Waters LC-MS/MS Mass Spectrum:



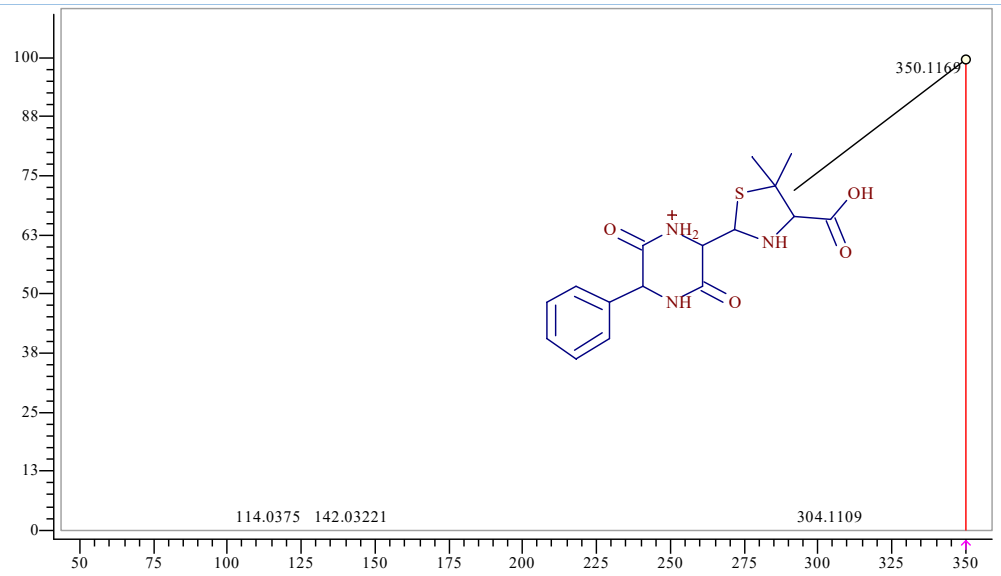
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 8.93

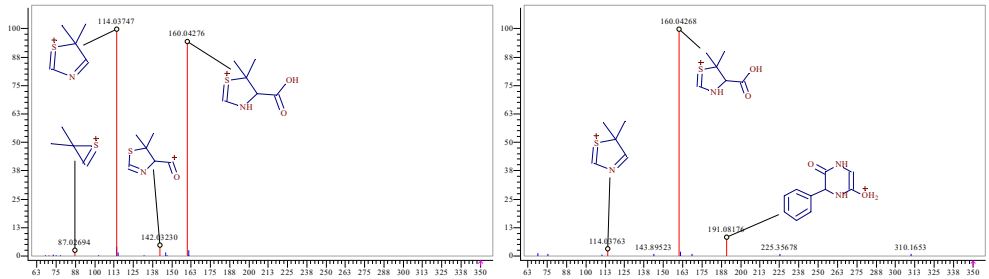
Waters LCMS 6: 10.27

Compound: Stereoisomer of Diketopiperazine (*m/z* 350) *

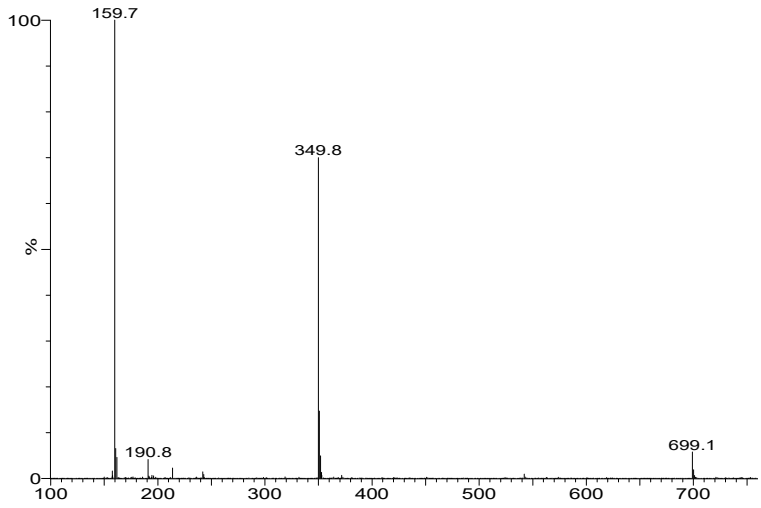
Thermo Scientific™ Mass Spectrum:



MS/MS NODES:



Waters LC-MS/MS Mass Spectrum:



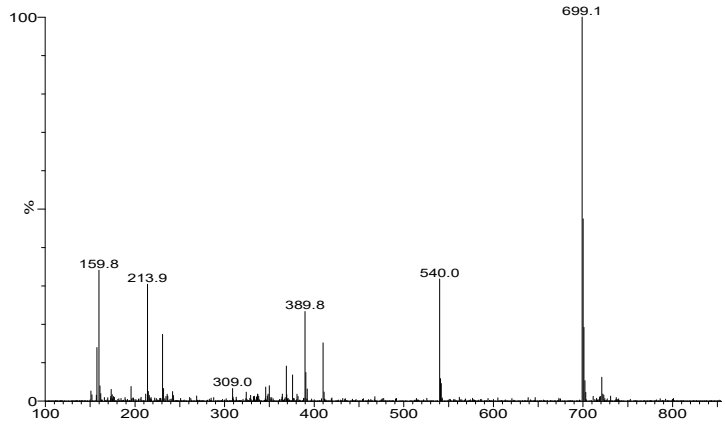
Peak Retention Times (mins)

Thermo Scientific™ LCMS:

Waters LCMS : 11.20

Compound 7: Stereoisomer of Dimer (*m/z* 669) *

Waters LC-MS/MS Mass Spectrum:



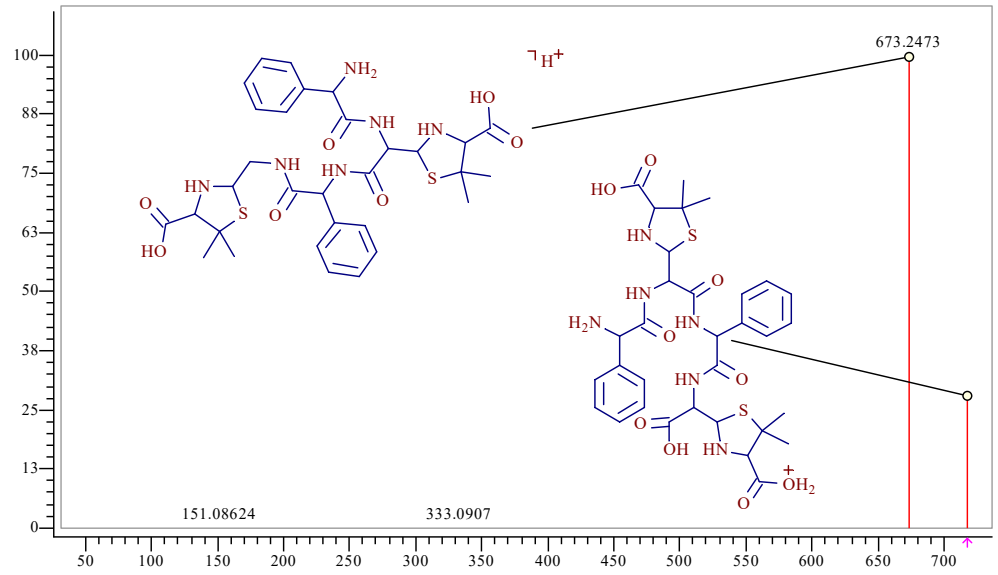
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 11.54

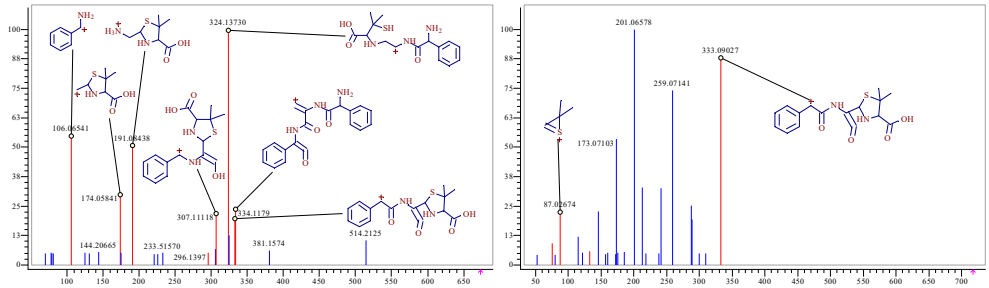
Waters LCMS: 12.35

Compound 8: Oligomer of Ampicilloic Acid and Ampicillin (*m/z* 717) *

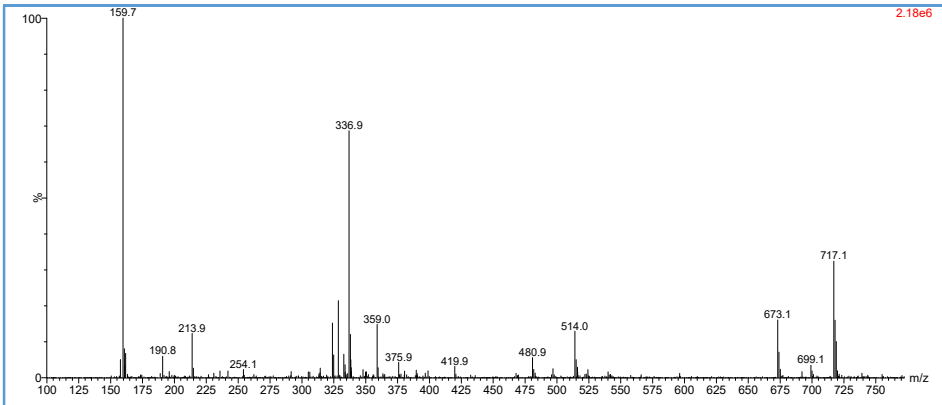
Thermo Scientific™ LCMS:



MS/MS NODES:



Waters LC-MS/MS:



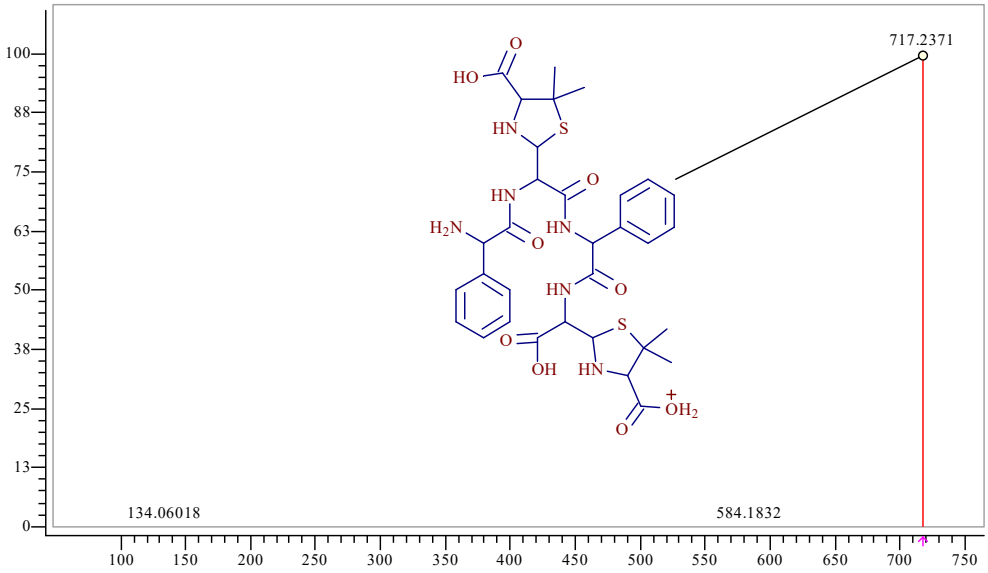
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 11.92

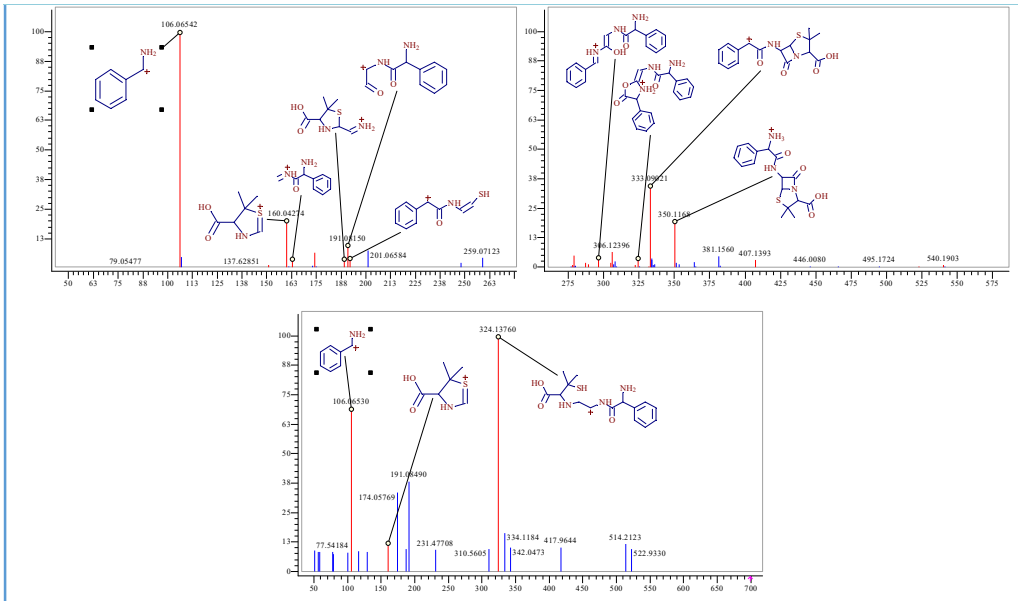
Waters LCMS: 12.48

Compound 9: Oligomer of Ampilloic Acid and Ampicillin (*m/z* 717) *

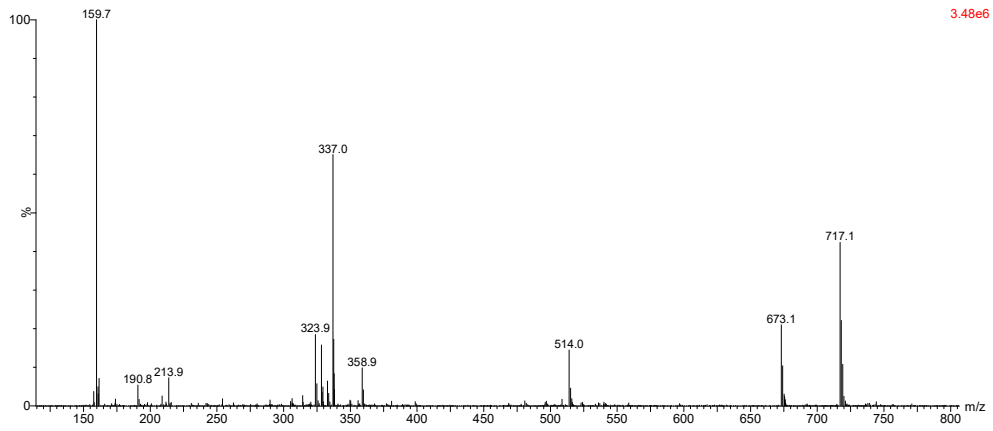
Thermo Scientific™ LCMS:



MS/MS NODES:



Waters LC-MS/MS:



Peak Retention Times (mins)

Thermo Scientific™ LCMS:

Waters LCMS: 12.71

Compound 10: Stereoisomer of Dimer (*m/z* 699) *

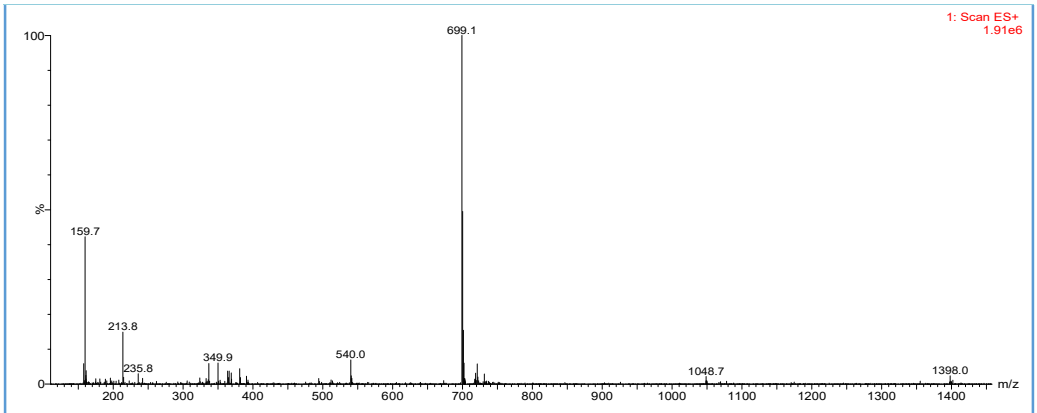
Thermo Scientific™ LCMS:

N/A

MS/MS NODES:

N/A

Waters LC-MS/MS:



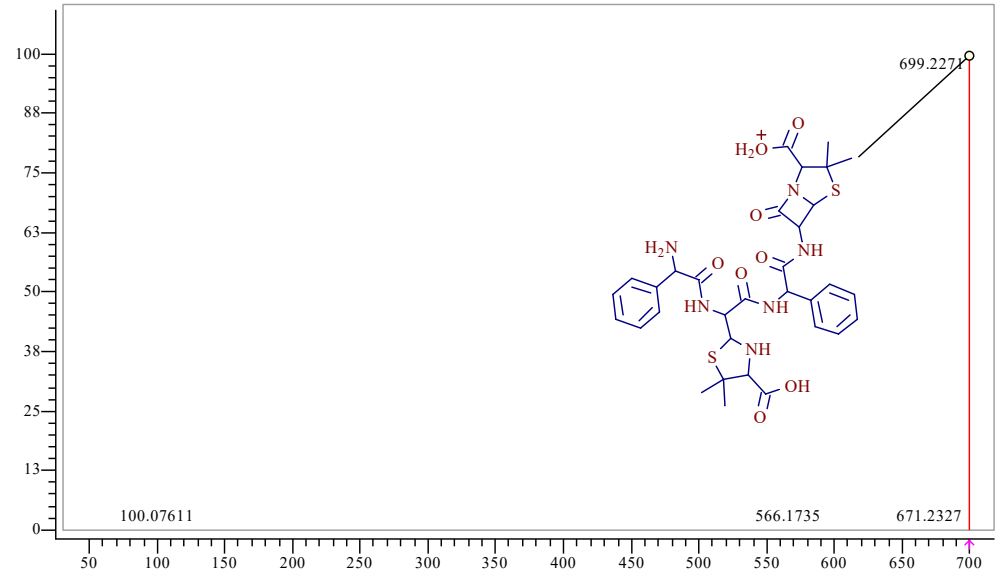
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 12.50

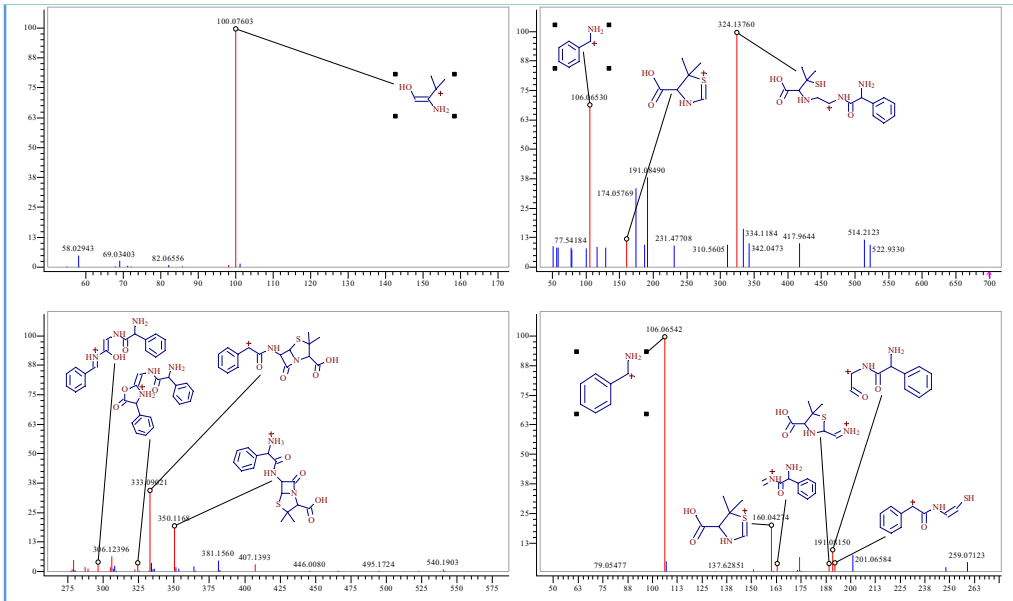
Waters LCMS: 12.82

Compound 11: Stereoisomer of Dimer (*m/z* 699) *

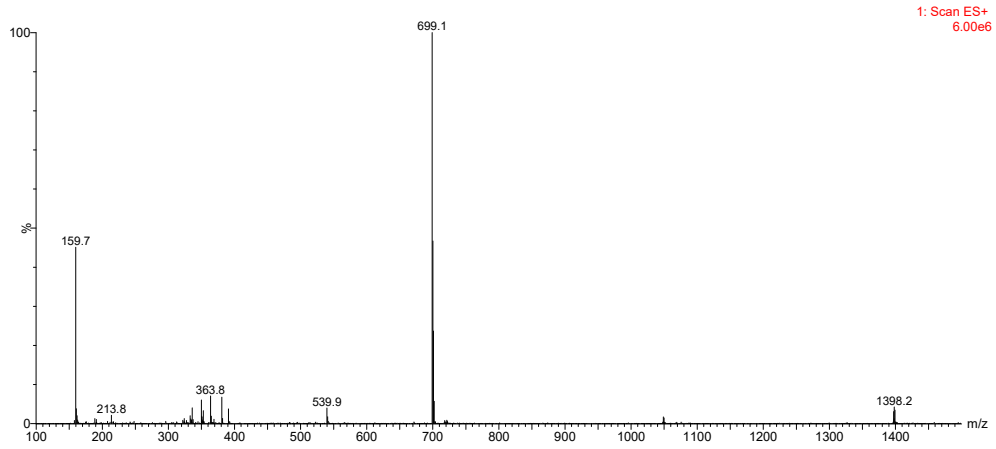
Thermo Scientific™ LCMS:



MS/MS NODES:



Waters LC-MS/MS Mass Spectrum:



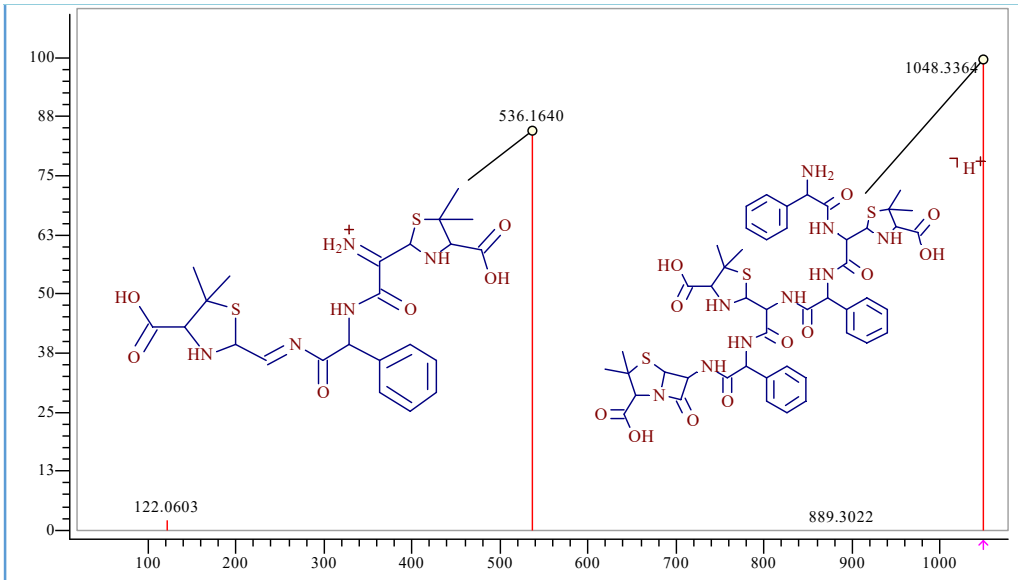
Peak Retention Times (mins)

Thermo Scientific™ LCMS: 14.17

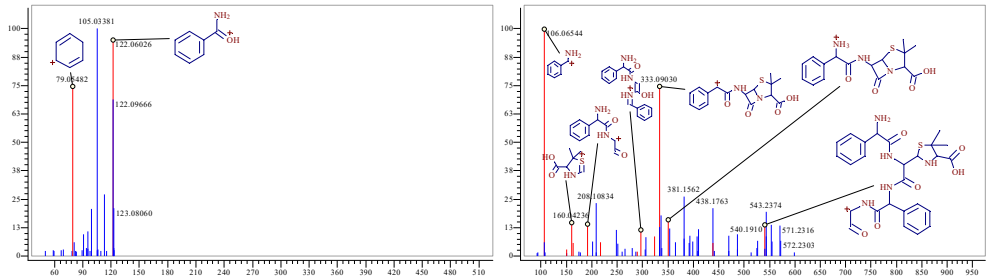
Waters LCMS: 14.52

Compound 12: Trimer (*m/z* 1048) *

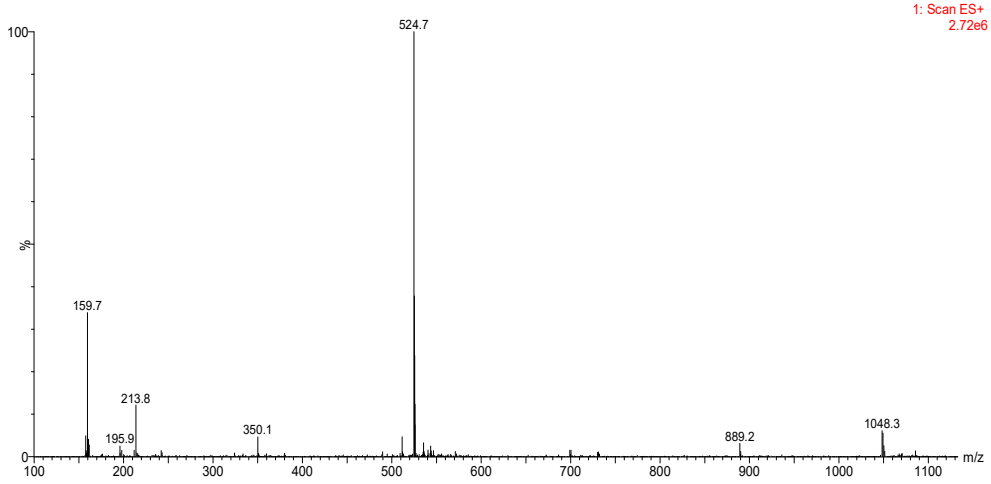
Thermo Scientific™ LCMS:



MS/MS NODES:



Waters LC-MS/MS:



Peak Retention Times (mins)

Thermo Scientific™ LCMS: N/A

Waters LCMS: 15.63

Compound 13: Tetramer (*m/z* 1397) *

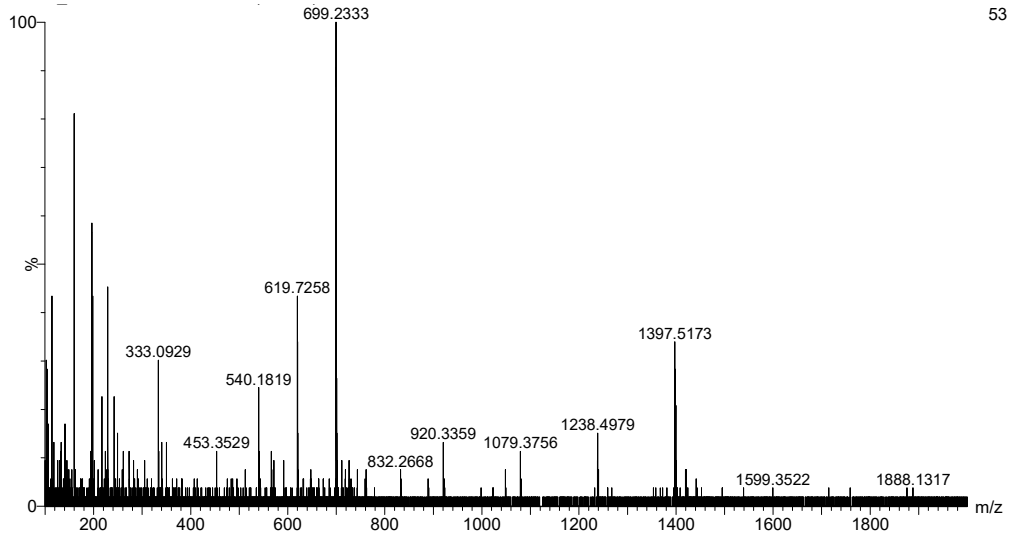
Thermo Scientific™ LCMS:

N/A

MS/MS NODES:

N/A

Waters LC-MS/MS:



7.5 Supportive Data for UHPLC Method Development

7.5.1 Physical Observation of Ampicillin Sodium

Reconstituted ampicillin sodium solutions have a clear, colourless appearance (Figure 60). The 56 mg.mL^{-1} solution took longer to fully solvate, suggesting the solution was reaching the solubility threshold (approximately 50 mg.mL^{-1})[99]. However, samples displayed no light scattering when tested; the solutions were transparent, with no evidence of suspended particles or colloids.

Upon storage solutions of the drug turned yellow (Figure 60), indicating degradation had taken place, which has been previously reported[59]. The time frame of the colour change was dependant on the conditions of storage (temperature, concentration, buffer, pH). Samples that were stored for extended periods of time (months to years) were observed to precipitate.

Figure 60: Physical appearance of ampicillin sodium as a (from left to right) powder, freshly reconstituted drug in solvent, degraded solution (weeks) and highly degraded (years) solution.

7.5.2 Example Calibration Curve of Ampicillin Sodium

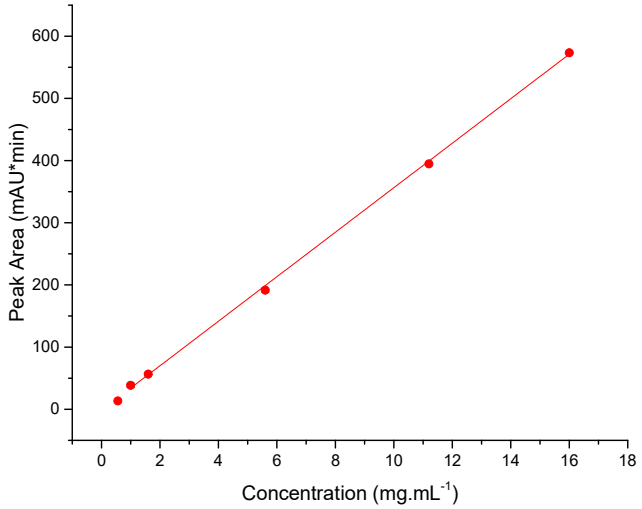


Figure 61: A calibration curve of ampicillin sodium using Thermo Scientific™ Vanquish UHPLC system, at wavelength of 225 nm.Errors calculated as ± 1 S.D.

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