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Thermostability Determination of Broad Spectrum Antibiotics at High Temperatures by Liquid Chromatography-Mass Spectrometry

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Abstract

Amoxicillin is a broad-spectrum semisynthetic antibiotic that is used to treat common bacterial infections in humans. Amoxicillin is a part of the beta-lactam antibiotic group and is commonly used to treat bacterial infections such as ear infections, bladder infections and pneumonia. It is the antibiotic of choice from the beta-lactam group due to its broad spectrum of uses to fight bacterial infections. When prescribed, patients are advised to store the antibiotics under certain conditions. According to drug regulations, amoxicillin should be stored at temperatures between 35.6°F to 46.4°F (2°C to 8°C). This regulation is to prevent the amoxicillin from degrading, which in turn reduces its effectiveness in treating the bacterial infection for which it was prescribed. If stored improperly, exposing it to high temperatures, the antibiotic may degrade and render itself ineffective. Such conditions may occur during the summer months by leaving the medication in a vehicle, which reaches very high temperatures in the sun. Due to this potential circumstance, there is a great need for the determination of the thermostability of this antibiotic when it is exposed to moderately high temperatures. The analytical method developed utilizes liquid chromatography with mass spectrometric detection to monitor changes in concentration of the antibiotics after exposure to elevated temperatures. When thermal degradation occurs, a decrease in analyte signal is detected on the LC-MS. Results have shown that the level of thermal degradation increases as temperature level and exposure time increase. Thermostability data will be presented for temperatures ranging from 90°F to 200°F (32°C to 93°C). The data collected will then be used to determine if the prescribed storage conditions for the antibiotics are valid.

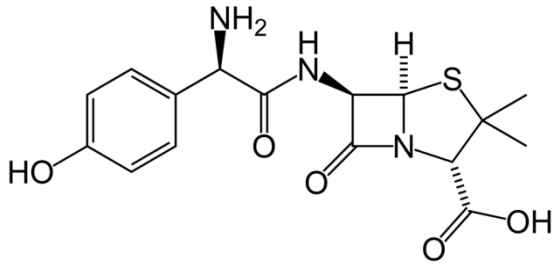
Keywords: Thermostability, Antibiotics, Liquid Chromatography-Mass Spectrometry

1. Introduction

Amoxicillin has a molecular formula of $C_{16}H_{19}N_3O_5S$ and a molecular weight of 365.4 g/mol[1]. It is considered a beta-lactam antibiotic because the structure contains a four-membered cyclic amide (see Figure 1)[2]. This particular antibiotic uses a bactericidal mechanism of action, meaning the compound inhibits the synthesis of the bacteria cell wall. A bacterial cell contains specific enzymes that are involved in both assembling and reshaping the cell wall; these enzymes are known as penicillin-binding proteins. Amoxicillin binds to these penicillin-binding proteins, specifically penicillin-binding protein 1A and 1B, causing them to become inactivated; when these enzymes are inactivated, the bacterial cell wall weakens and undergoes lysis[2].

Amoxicillin, an analog of Ampicillin, is a broad-spectrum semisynthetic antibiotic that is used to treat many infections in both children and adults. Some common ailments that are treated using amoxicillin include: strep throat, ear and sinus infections, bacterial pneumonia, bronchitis, tonsillitis, urinary tract infections, and Lyme disease[1]. Amoxicillin is the most prescribed antibiotic for children and the second most prescribed antibiotic for adults[3]. It is considered a broad-spectrum antibiotic because it treats infections caused by a range of different bacteria including both gram-positive microorganisms and gram-negative microorganisms.

A recommended storage temperature is provided with a prescription for amoxicillin; the recommended temperature is 2-8°C[3]. Amoxicillin is considered an aminopenicillin; an aminopenicillin is a chemical analog of penicillin. The two compounds are structurally similar; however, an aminopenicillin contains an amino group within the structure. Aminopenicillins have a wider range of activity and are not deactivated by acid hydrolysis, meaning it is possible to administer them orally. Because amoxicillin is an aminopenicillin, its stability is dependent upon the environment pH and temperature. Amoxicillin's stability will begin to degrade in a highly humid environment or at a temperature over 37°C. Starting at 90°C, the drug begins to brown and then progressively dehydrates, decreasing the concentration[1]. During the summer months, a vehicle can easily reach 37°C on the inside; this poses a problem if the medication is left in the vehicle for a given amount of time.



Amoxicillin

Figure 1. Chemical structure of amoxicillin.

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that has been used to detect both the presence of a compound as well as the amount of a compound in a given sample[4-6]. This technique is widely used in pharmacology and toxicology to detect the amount as well as the degradation that occurs via the metabolism in an individual sample[6]. LC-MS is currently being used for multi-target screening for medications and compounds in a person's system using blood samples[6]. This analytical technique allows the detection of a minute amount of a given substance in a solution. Another broad-spectrum antibiotic, ciprofloxacin, has also been analyzed in extract samples from muscles, kidneys, and bovine milk using LC-MS[7-9].

2. Methodology

2.1. Preparation of solvent with internal standard

Tyrosine powder was obtained from Fisher Scientific (Fair Lawn, NJ, USA). A solvent was prepared by dissolving 0.025g of tyrosine powder in 1000mL of 80% deionized water, 19.9% HPLC grade acetonitrile, and 0.1% formic acid to create a 25 ppm internal standard solvent solution.

2.2. Preparation of laboratory standards

Amoxicillin powder was obtained from Fisher Scientific (Fair Lawn, NJ, USA). A stock solution of the antibiotic was prepared by dissolving the antibiotic at a concentration of 100 ppm in the prepared internal standard solvent. The stock solution was stored at 4° C in the dark. Fresh stock solution was prepared weekly. Laboratory standards (1, 10, 25, and 50 ppm) of the antibiotic were prepared by diluting the stock solution with 80/19.9/0.1 water/acetonitrile/formic acid.

2.3. Preparation of samples for thermostability experiments

Solid samples of amoxicillin were placed in ovens at various elevated temperatures for periods of 1, 4, and 16 hours. The temperatures (32°C, 49°C, 71°C, 93°C) were chosen to simulate possible conditions in which the antibiotic may

be stored, such as the interior of a hot vehicle. Immediately following exposure, 25 ppm solutions of each sample were prepared in the same manner described above for the laboratory standards. Samples were prepared for each temperature and time range tested.

2.4. Liquid chromatography

The LC system used was a Thermo Finnigan Surveyor system (San Jose, CA, USA) equipped with an autosampler. LC separations were obtained with a Whatman Partisil 10-ODS 3 C-18 Reversed Phase Column (Piscataway, MJ, USA, having dimensions of 4.6mm x 250mm and a particle size of 10µm.

The sample injection volume was 20 µL. An isocratic mobile phase consisting of 80% deionized water with 0.1% formic acid and 20% acetonitrile with 0.1% formic acid was used at a flow rate of 1200 µL/min. Total analysis time for each sample was 6 minutes. All laboratory standards and samples were run in triplicate.

2.5. Mass spectrometry

The MS system used was a Finnigan MAT LCQ Electrospray Ionization ion trap equipped with a heated capillary interface. The MS was operated in positive ion mode. Thermo Scientific Xcalibur software was used to control all experimental conditions as well as analyze the quantitative data.

3. Data

The method that was developed for this experiment utilizing the LC-MS took six minutes to fully detect both the tyrosine and amoxicillin peaks. Using electrospray ionization, tyrosine and amoxicillin were detected primarily as molecular ions. The specific peaks of interest on the mass spectra were m/z = 181 (tyrosine) and m/z =365 (amoxicillin). Because of the different peaks, the internal standard (tyrosine) could be distinguished from the antibiotic (amoxicillin) by monitoring the subsequent ion as a function of time. This approach is known as selected ion monitoring and has already been established as an effective analytical technique for the determination of antibiotics[9]. Using the Xcalibur software, the LC-MS was specifically tuned to only detect and record the mass spectra ranges of 180-182 (tyrosine) and 364-366 (amoxicillin); the data was then analyzed using the XCalibur software to calculate the area of each of the peaks. Figure 2 displays a selected ion mass chromatogram of a 25ppm standard solution of amoxicillin with tyrosine internal standard.

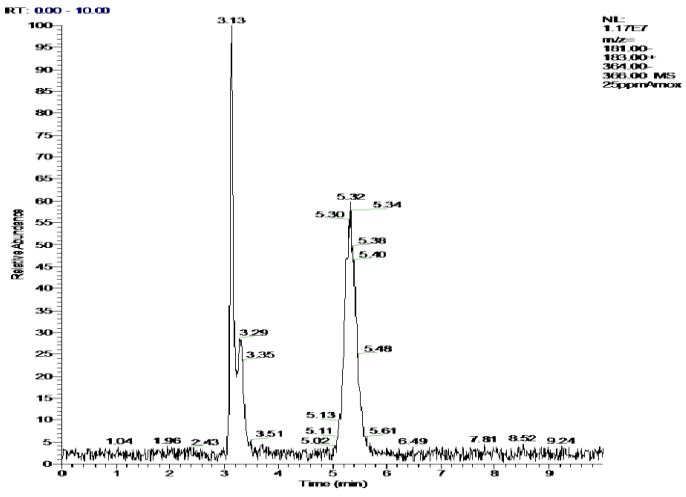


Figure 2. Extracted mass chromatogram for amoxicillin (second peak, 5.32 minutes) with tyrosine (first peak, 3.13 minutes) internal standard.

A standard curve for amoxicillin was constructed over a range of 1 to 40 ppm, specifically 1, 10, 25, and 40 ppm. By tuning the instrument specifically for amoxicillin and tyrosine, the amount of background signal reduced significantly.

The amoxicillin peak area from the extracted ion chromatogram was divided by the tyrosine peak area and then plotted versus concentration. Each solution used in creating the standard curve was run in triplicate. A linear response was obtained over the range for amoxicillin with a correlation coefficient R of 0.9972. An internal standard was used in this experiment to account for the fluctuations that occur within the instrument itself while processing samples. This improves the reliability of the data by adding precision to the calculations and analysis. Figure 3 displays the standard curve for amoxicillin with an internal standard of tyrosine.

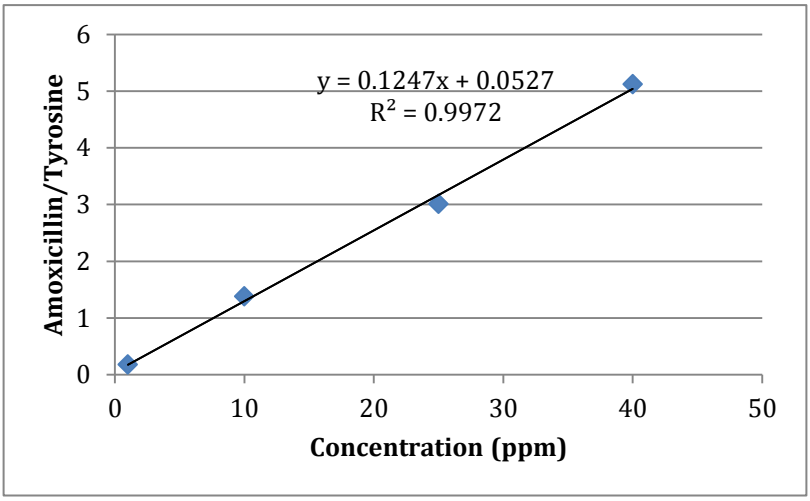


Figure 3. Standard curve for amoxicillin with tyrosine internal standard.

After establishing the standard curves for the analyte, amoxicillin samples that had been exposed to elevated temperatures were analyzed using the same method. Solutions of 25 ppm for each heat treated sample were prepared and run using the LC-MS. Each sample was run in triplicate. Both the standards and treated samples were run sequentially in the same session. The standard linear regression was used to calculate the respective concentrations of amoxicillin. A decrease in the overall concentrations of the antibiotic was present as predicted; the amount of decrease in concentration was greater as the temperature increased and as the length of exposure time increased. Table 1 summarizes the calculated concentrations of amoxicillin after exposure to different levels of increased temperature for selected amounts of time.

Table 1. Observed concentrations of 25 ppm solutions of amoxicillin following exposure to elevated temperatures.

Exposure Time	Temperature (^{0}C)			
	32^0	49^0	71^0	93^0
1 hour	8.80 ppm	8.74 ppm	8.65 ppm	8.27 ppm
4 hours	8.14 ppm	8.65 ppm	8.40 ppm	7.55 ppm
16 hours	8.12 ppm	7.89 ppm	7.88 ppm	6.29 ppm

4. Conclusion

Thermal degradation in concentration of amoxicillin has been observed following exposure to elevated temperatures. The amount of degradation depends on the length of exposure to the elevated temperature as well as the level of elevated temperature. A linear standard curve was obtained for amoxicillin at concentrations ranging from 1 to 50 ppm. Deviations from the standard 25 ppm solution were calculated and monitored using the equation

from the standard curve. Because the LC-MS method created has the ability to detect and monitor specific molecular ions generated by the antibiotic, other antibiotics could also be analyzed, expanding this study. This method also allows for a high volume of samples to be processed as a time because of the six minute processing time.

5. Future Work

The next step in this research is to utilize the methodology formed and used in this experiment and apply it to other broad-spectrum antibiotics. In addition to the thermostability tested in this experiment, another study is planned to measure the stability of broad-spectrum antibiotics when exposed to different humidity environments; this study would utilize the same method of monitoring concentrations by using the LC-MS internal standard method. It is anticipated that the methodology created for this experiment will without problems translate to the new studies.

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7. References

1. *DailyMed*. Bethesda, MD: U.S. National Library of Medicine, 2010. s.v. "AMOXICILLIN tablet, for suspension [Aurobindo Pharma Limiter]." http://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=4d68df6a-6e96-47a4-abeb-6afc7cc84bbc.
2. Bolton E, Wang Y, Thiessen PA, Bryant SH. PubChem: Integrated Platform of Small Molecules and Biological Activities. Chapter 12 IN *Annual Reports in Computational Chemistry*, Volume 4, American Chemical Society, Washington, DC, 2008 Apr. [free author manuscript]
3. Kofke-Egger, H, and M Udow-Phillips. *Antibiotic Prescribing and Use*. *Price of Care Issue Brief*. Ann Arbor, MI: Center for Healthcare Research & Transformation, 2011.
4. Graham L. Patrick, An Introduction to Medicinal Chemistry. Oxford: Oxford University Press, (2003) :379–435.
5. G. Anquetin, J. Greiner, N. Mahmoud, H.M. Santillana, R. Gozalbes, and K. Farhati "Design, Synthesis and Activity Against *Toxoplasma gondii*, *Plasmodium* spp., and *Mycobacterium tuberculosis* of new 6-Fluoroquinolones," European Journal of Medicinal Chmistry 41 (2006): 1478–93.
6. K. Granelli and C. Branzell, "Rapid Multi-Residue Screening of Antibiotics in Muscle and Kidney by Liquid Chromatography-Electrospray Ionization–Tandem Mass Spectrometry," Analytica Chimica Acta (2007): 289-295.
7. Pitt, JJ. "Principles and Applications of Liquid Chromatography-Mass Spectrometry in Clinical Biochemistry." *Clinical Biochemistry Review*. no. 1 (2009): 19-34.
8. Sara Bogialli, Giuseppe D'Ascenzo, Antonio Di Corcia, Aldo Laganà, and Simone Nicolardi, "A Simple and Rapid Assay Based on Hot Water Extraction and Liquid Chromatography–Tandem Mass Spectrometry for Monitoring Quinolone Residues in Bovine Milk," Food Chemistry 108 (2008): 354-360.
9. Marcela Seifrtova, Lucie Novakova, Celeste Lino, Angelina Pena, and Petr Solich, "An Overview of Analytical Methodologies for the Determination of Antibiotics in Environmental Waters," Analytica Chimica Acta 649 (2009): 158-179.