Quantification of Ampicillin and Cloxacillin in the Antibiotic Ampiclox using Reversed-Phase High-Performance Liquid Chromatography

By

Rachel Caitlin Hurrell

Honors Project Thesis Submitted in Partial Fulfillment
Of the Requirements for a Baccalaureate Degree "With Distinction"
In the Ron and Laura Strain Honors College of
THE UNIVERSITY OF INDIANAPOLIS
May 2020

Faculty Advisor: Levi H. Mielke, Ph.D.

Executive Director of Honors: James B. Williams, Ph.D.

Abstract

Substandard and falsified medical products are increasingly prevalent in developing nations. These products pose a significant threat to human health and place a strain on many resources in the medical industry. Included are pharmaceuticals that contain a different amount or identity of the active ingredient. Such a discrepancy may result in ineffective treatment of illness and inflict harm on the patient. Antibiotics are among the most widely reported classes of substandard and falsified drugs. It is important to the well-being of society to address this growing issue. One effort currently employed to identify these pharmaceuticals is reversed-phase high-performance liquid chromatography (RP-HPLC). Initially, this investigation focused on the development of a method using RP-HPLC to quantify the ampicillin and cloxacillin contained in the antibiotic Ampiclox. Strict guidelines, regulations, and quality assurance procedures specified by the United States Pharmacopeia (USP) must be followed during pharmaceutical analyses. The method was tested to ensure that the separation produces parameters that satisfy USP standards. Following validation, the method was used for the analysis of 21 Ampiclox tablets collected in Liberia. These analyses indicate promising application of the method to future analysis of Ampiclox samples.

Table of Contents

Acknowledgements

Without the generous support of the Shaheen College of Arts and Sciences through their Undergraduate/Scholarly Creative Activity research grant, this investigation would not have been possible. These funds were used to purchase supplies crucial to the investigation. Additionally, the funds were used to support an oral presentation accepted to the National Conference on Undergraduate Research March 2020.

Additionally, the support and guidance from the Distributed Pharmaceutical Analysis Laboratory (DPAL) and the University of Notre Dame played a pivotal role throughout the course of the investigation. Specifically, Dr. Marya Lieberman who heads DPAL, as well as Sarah Bliese, offered individual support throughout the method development and pill analysis procedure. Furthermore, Dr. Robyn Goacher from Niagara University also provided support and advice throughout the method development process.

Dr. Levi Mielke is an outstanding advisor who offers an expertise in analytical chemistry. Without his guidance this investigation would not have been possible. His extensive knowledge of chromatography techniques fostered my growth and success throughout the process.

Finally, the Department of Chemistry at the University of Indianapolis offered extensive support including research space, equipment, reagents, and advice throughout this investigation. Specifically, Dr. Joe Burnell provided guidance throughout the method development process with his experience in HPLC. Dr. Styers-Barnett also provided important input and helped with the writing process.

List of Tables

List of Figures

Statement of Purpose

The purpose of this investigation was two-fold. The primary focus of this project was the investigation of drug purity for pharmaceuticals, specifically Ampiclox, sold in developing nations. Pharmaceuticals that contain less than the specified quantity of active ingredient are considered substandard and are increasingly common in developing nations. Such products pose significant risk to the health and well-being of these communities. A secondary focus of the project was to develop a reverse-phase high-performance liquid chromatography (RP-HPLC) method to simultaneously quantify ampicillin and cloxacillin contained within the antibiotic Ampiclox. First, the method was approved by the Distributed Pharmaceutical Analysis Laboratory (DPAL) to confirm that it yields accurate and reproduceable results. Subsequently, the method was used for analysis of a subset of 21 Ampiclox samples obtained from a developing nation. Such an analysis provides insight into the quality of the samples and facilitates a conclusion regarding the purity of the pharmaceuticals.

Introduction

Antibiotics have played a critical role in society especially since their rising availability between 1940 and 1970.[1] This class of drugs alone has doubled the human life expectancy in the 20th century.[1] Antibiotics are diverse medications with a widely accepted definition being any "chemical substance produced by micro-organisms, which has the capacity to inhibit growth of and even to destroy bacteria and other micro-organisms. The action of an antibiotic against micro-organisms is selective in nature, some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific antimicrobial spectrum."[1] The "specific antimicrobial spectrum" of each antibiotic is an important feature of this drug class and allows for targeted and effective therapy development. One very important and widely used class of antibiotics is the β-lactam class, which will be the focus of this investigation. The β-lactam class is defined by the presence of a highly strained and reactive 4-membered, cyclic amide β-lactam ring. This ring plays an important role in the mechanism by which antibiotics inhibit the growth of bacteria and other micro-organisms.[2] Specifically, this ring structure binds to the active site of the penicillin binding protein to inhibit the synthesis of the cell wall leading to "cell-lysis and death" of the bacterium.[3]

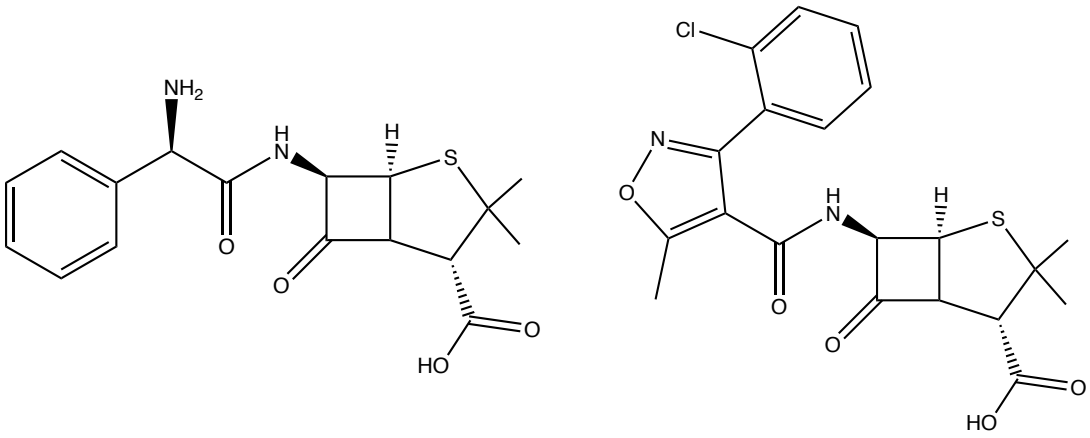


Figure 1: Structures of the ß-lactam antibiotics ampicillin (left) and cloxacillin (right)

Antibiotics, especially those in the β-lactam family, have proven to be incredibly effective in their treatment of bacterial infections and ultimately in the improvement in the quality of human life. β-lactam antibiotics are also among the most commonly reported substandard antibiotics.[4] A substandard medical product, according to the World Health Organization (WHO), is one that "fail[s] to meet either [its] quality standards or specifications or both".[5] In addition, medical products can also be falsified meaning that they "deliberately/fraudulently mispresent their identity, composition, or source".[5] The distinction between substandard and falsified is important in multiple regards. Primarily, while both substandard and falsified medical products pose a significant risk to society, a substandard drug contains some quantity of active drug, but less than the recommended therapeutic dosage. In contrast, a falsified drug may contain no active ingredient, and may even have an alternative substance replacing the active drug which, in some cases, has been a known human toxin making these falsified products particularly dangerous.[6]

β-lactam antibiotics are not the only medication with reports of substandard samples being distributed to the public. Anti-malarial drugs are most commonly reported

as substandard, however, many medicines, vaccines, and other "therapeutic categories"[5] have failed to meet quality standards. Low-quality medical products have become increasing prevalent in society, particularly in those nations faced with widespread poverty. The WHO states that approximately 10% of medical products produced in these nations are either substandard or falisified.[5] These products are not restricted to these regions, however. Falsified and substandard medical products impact every region of the world.[5]

Both substandard and falsified pharmaceutical and therapeutic products pose a serious threat to society and have wide-reaching impacts. Most generally, they increase morbidity (the state of being diseased) and mortality.[6] Several specific factors contribute to such drastic and impactful consequences. One such contributing factor is the spread of anti-bacterial resistance.[7] When a patient is prescribed a substandard or falsified antibiotic to treat a bacterial infection, not all of the bacteria will be killed due to the lower concentrations of active ingredient. Those bacteria not destroyed by the low dosage of the antibiotic contain mutations that make the bacteria resistant to the drug.[7] These remaining bacteria will reproduce and rapidly multiply increasing the population of resistant bacteria.[7] The impacts of resistant bacteria do not end with this patient, however. As the patient with resistant bacteria travels and exposes other members of society to the bacteria, the resistance will spread on a large scale.[7] In addition, the medication will fail to cure the patient's ailment, reducing quality of life and leaving the patient susceptible to complication. Subsequently, substandard drugs also have an economic impact. As

doctors search to determine why treatments are ineffective, there is a great deal of money and time lost to superfluous research for alternative treatments.[7]

It is also important to understand how and why these falsified and substandard products are made available for human consumption. According to the WHO, these medical products make it into the market most commonly when "they fill a vacuum".[7] In impoverished nations, there is high demand for medicines and diagnostics tests with an extreme lack of resources available to meet those needs.[7] As a result, falsified medical products are used in place of their safe and effective counterparts. Manufacturers of these falsified products are driven by "an unsavory combination of the ill-informed, the careless, the unprincipled and the criminal" allowing them to "thrive in places where the technical capacity is poor and the risk of detection is low".[7] Healthcare institutions will do whatever it takes to obtain medicines in the face of a rampant outbreak, including sourcing their products from unreliable producers.[7]

It is clear that the repercussions of substandard and falsified drugs are immense. Unfortunately, this problem is not being adequately addressed. "Only 5-15% of the 191 member states of the WHO report cases of counterfeit drugs".[6] The WHO suggests that there are various reasons for the staggeringly low numbers of reported cases. One is that reporting processes in affected nations are incredibly complex or, in some cases, nonexistent.[7] Additionally, there is a general fear among officials that reporting cases of substandard or falsified products will have grave repercussions.[7] For example, prescribers of falsified medicines fear that public exposure of the use of a low-quality product will leave them susceptible to retribution by supervisors, leading to loss of employment, or, in

severe cases, “prosecution or civil action”.[7] The WHO is aware of these limitations and is implementing several systems by which such substandard and falsified drugs can be more easily reported. The WHO, for instance, has developed a Global Surveillance and Monitoring System (GSMS). This system is an organized attempt amongst WHO member states to reduce and eliminate the prevalence of falsified and substandard medical products circulating on the market.[7] There are several main objectives of GSMS to mitigate the impacts of falsified products on society including, but not limited to, measures to make reporting easier and more accurate, designating “focal points” throughout the world to allow communication between national and global authorities, and the creation of a “global database” to coordinate and track reports of falsified products.[7] Another simple, yet critical component of these mitigation efforts, is to spread awareness, especially to those directly impacted.[7]

While there are many areas in which improvements can be made to reduce the harm that these medical products are causing to the public, one area of particular interest is the use of various detection methods both in the field and in the lab. There are several methods currently being employed to take such measurements. Most commonly, falsified and substandard drugs are detected through the use of “inspection, colorimetric methods, chromatography, and spectrometry”.[4] This list is not exhaustive, and each of the aforementioned techniques has several variations that can be used to detect quantities of active drug. Both qualitative and quantitative measurements are paramount to understanding the repercussions of these medical products throughout society. It is important to distinguish between a qualitative and quantitative analysis of a medical

product. A qualitative measurement can be used to indicate the presence of an active ingredient.[13] As a result, such an analysis is used to quickly distinguish between those drugs with and without the active ingredient.[13] A quantitative analysis is used to determine the exact quantity of the active ingredient (or other substances contained in the product) and conclusions using such data can be extended to understand how the drug may impact a patient.[13] Both types of analyses are required to complete a comprehensive conclusion regarding drug quality.[13]

Of current interest to many groups investigating the prevalence of substandard medical products is the availability of rapid testing that can be used in the field to yield immediate conclusions regarding the active drug content of the pharmaceutical. One very important initial screening that can be conducted in any location is a visual inspection. The WHO created a checklist to aid in the identification of potential fraudulent medications using only a preliminary visual inspection. The checklist suggests special attention should be given to the packaging and labeling, as well as the physical characteristics of the drug tablet or capsule itself.[8] Such an inspection requires little to no training and does not require access to expensive instrumentation, making it a valuable technique. Although visual inspection can provide a good first indication of the presence of counterfeit products, a more accurate analysis that can offer quantitative and qualitative data is necessary to confirm fraudulent practices. Some other rapid testing methods include portable technologies such as Raman and Near-infrared Spectroscopy (NIR).[9] Both NIR and Raman spectroscopy are used to determine a “molecular fingerprint” based on the vibration of the chemical bonds of the molecule being

analyzed.[9] In order to yield useful conclusions from the spectra produced by these instruments, a comparison to spectra for known compounds must be completed.[10] This is a limiting factor as research must be conducted before a conclusion can be made. Furthermore, these handheld instruments can cost up to $50,000 and thus place an economic restraint on their use.[10]

On the leading edge of portable technologies for the analysis of pharmaceutical samples is the development of various paper assays that use chromatographic (separation of constituents in the sample) and colorimetric (color change to indicate presence of the component of interest) techniques to indicate the presence of active drugs.[10] Such an assay provides a qualitative assessment of the product. When a liquid sample of the medication is placed on the card, capillary action carries the drug up several separate channels, each containing a different reagent used to indicate the presence of the drug.[11,12.] As the drug comes into contact with the reagent, a reaction, often one that results in a color change, occurs to indicate the presence of the active drug.[11,12] Several studies regarding these paper based analyses indicated that "overall, these tests show high sensitivity and specificity for analytes and provide a useful chemical profile of active pharmaceutical ingredients (APIs) and excipients within a few minutes".[12] One limitation of these paper assays is their inability to distinguish between samples with little active ingredient and no active ingredient.[11]

It is clear that while portable methods for detection of substandard medical products is important, there are also several limitations on the quantitative data these techniques provide. Often, only techniques carried out in a laboratory by a trained

technician allow for quantification of the active ingredient contained in a pharmaceutical sample. One commonly used technique for a quantitative analysis of the sample is high-performance liquid chromatography (HPLC).[10] HPLC "is generally regarded as the definitive technique for drug content analysis".[13] HPLC is the focus of this investigation.

While there are many different types of chromatography, they all operate on the same principle: using the different physical properties of each component of a mixture (in this case, the drug) to separate the mixture into its individual components.[14] More specifically, chromatographic techniques involve two main components, a stationary phase and a mobile phase, both usually contained in a column.[14] The stationary phase ("the one that stays in place inside the column") is often a "viscous liquid chemically bonded… onto the surface of solid particles packed into the column".[14] The mobile phase, however, moves through the column freely and "is either a liquid or a gas".[14] One of the two phases will be polar and the other is nonpolar.[14] As a result, the components of the mixture that are polar will be attracted to the polar phase while those that are nonpolar will remain attracted to the nonpolar phase.[14] A chromatogram that relates detector response to elution time (the time required for the compound of interest to pass through the column) allows for identification of each component of the mixture.[14]

Chromatograms produced from chromatography instruments resemble a graph with a number of peaks corresponding to the number of separated analytes. Each peak corresponds to an analyte. Peaks can be identified by their retention time, or the time required for the solute to reach the detector following injection.[14] Two important characteristics of chromatograms include the peak height and peak area of each eluted

peak. The peak area is found by integrating the peak of interest (to determine the area beneath the curve) while the peak height is the distance from the base of the peak to the top of the peak.[14] Each of these quantities is proportional to the concentration or amount of the analyte which reached the detector. Thus, the peak height and area allow for the construction of a calibration curve. A calibration curve relates the instrument response (peak height or area) to the concentration of the analyte. Thus, this plot may be used to determine the concentration of an analyte in an unknown solution.

Chromatography is particularly applicable to the identification of pharmaceutical samples as it uses principles which "match perfectly with the physicochemical properties of drugs".[15] This investigation will specifically use reverse-phase high-performance liquid chromatography (RP-HPLC). Reverse-phase liquid chromatography indicates that a polar mobile phase and a nonpolar stationary phase are used.[15] Liquid chromatography must be used in the analysis of pharmaceutical compounds because the sample cannot be volatilized for analysis by gas chromatography.[14] In HPLC, "high pressure [is used] to force solvent through closed columns containing fine particles that give high-resolution separations."[14] The solvent consists of two components: an organic solvent, and a buffered solution. The buffer is particularly important when the analyte is ionizable (can gain or lose a proton due to changes in pH). A buffered solution resists changes in pH so that the analyte remains in only one form. A variety of detectors may be used to identify compounds separated using HPLC. The HPLC used in this investigation utilizes a spectrophotometer. A spectrophotometer is an instrument that determines the absorption of a selected wavelength of light (specific to the compound of interest).[14]

In this investigation, the technique of reverse-phase high-performance liquid chromatography was used to analyze the quantity of active drug contained in Ampiclox. Ampiclox is a combination of the two β-lactam antibiotics ampicillin and cloxacillin (Figure 1).[16] Individually, both antibiotics are effective in their treatment of bacterial infections. Both drugs, due to their classification as members of the β-lactam family, inhibit bacterial growth using the mechanism described earlier. More specifically, ampicillin is a "broad-spectrum" antibiotic that is not "resistant to beta-lactamases", the enzymes responsible for resistance to β-lactam antibiotics.[17] Cloxacillin is a more "narrow-spectrum" antibiotic that is resistant to beta-lactamases.[17,18] Cloxacillin, however, is limited in its use as it is "incompletely absorbed from the gastrointestinal tract…".[18] The lack of beta-lactamase resistance of ampicillin is satisfied by the resistance of cloxacillin to these enzymes. Furthermore, the malabsorption of cloxacillin by the body is fulfilled by the high bioavailability of ampicillin making the combination of the two drugs a very powerful antibiotic treatment for many bacterial infections.[16,17]

The focus of this investigation was to develop a RP-HPLC method to simultaneously analyze the presence of both ampicillin and cloxacillin in Ampiclox tablets obtained from third world nations. Specifically, the method development process determined parameters such as solvent, mobile phase, detector wavelength, injection volume, and run time required for the successful separation of the active ingredients contained in pill samples. Samples of this antibiotic are obtained from the Distributed Pharmaceutical Analysis Laboratory (DPAL) led by the University of Notre Dame. DPAL consists of a collection of several academic institutions who are "determin[ing]

analytically the quality of medicines collected from low- and middle-income countries (LMICs)".[19] Beginning in the Fall of 2018, the University of Indianapolis became a member of DPAL, granting the university access to pharmaceutical samples for HPLC analysis. Participation in DPAL requires strict "system suitability requirements" and thus ensures that high quality accurate data are produced.[19] These measures of system suitability are concerned with monitoring the accuracy and reproducibility of methods used to analyze pharmaceutical samples. There is strict regulation throughout the method development process, sample analysis, and data processing. The specific metrics that are indicative of the precision of the method are detailed in the methods section. Despite the high level of integrity through which laboratory and data analysis are conducted, "DPAL is not a certified pharmaceutical analysis laboratory" and any "suspicious samples" are reported to the WHO and sent to the "regulatory agencies that are equipped to perform the compendial testing".[19]

Not only does working in conjunction with DPAL ensure highly regulated and accurate results, it provides access to pharmaceutical samples for analysis. Samples of pharmaceuticals are collected from "small medicine shops" across Africa.[19] The samples are collected at a central location, the Moi Teaching and Referral Hospital (MTRH), where defining characteristics that can be used to track the drug are recorded before distribution to DPAL.[19] It is important to note that antibiotics are not highly regulated in many developing nations. In fact, antibiotics are widely available "over the counter, without prescription and through unregulated supply chains" making access for analysis by groups such as DPAL easily attainable.[20]

Several successful analyses of multidrug antibiotics have been conducted previously. One such example is the successful separation of Novaclox into its amoxicillin and dicloxacillin constituents by Patil et al. (2014) in their "Development and Validation of RP-HPLC Method for Simultaneous Estimation of Amoxicillin and Dicloxacillin in Bulk Drug and Capsules".[21] Another successful separation of a multi-drug antibiotic was completed by Battula and Shivaraj in their "RP-HPLC Method for Simultaneous Estimation of Ampicillin and Cloxacillin in Capsule Dosage Form".[22] This paper was also focused on the analysis of Ampiclox. The completion of the simultaneous analysis of the active drugs in Ampiclox previously indicates that method development to detect the active drugs present is attainable. The parameters used in this investigation will be different than those used by Battula and Shivaraj as the method is sensitive and specific to the instrument used. Several adjustments were made to optimize the method for the instruments used in this investigation.

Upon initiation of the method development process, it was determined that the method developed in the paper by Battula and Shivaraj was not entirely compatible with the HPLC belonging to the University of Indianapolis (UIndy) Department of Chemistry. Thus, to successfully develop a reliable and accurate method for the analysis and quantification of ampicillin and cloxacillin in Ampiclox, several additional published methods were referenced. Specifically, a method used previously to successfully analyze another drug, Metformin, with the HPLC at UIndy was referenced to estimate a more optimal buffer concentration than that suggested in the method developed by Battula and Shivaraj. This method for analysis of Metformin was published in the HPLC

Methodology Manual which is distributed by DPAL. Additionally, a procedure developed by Ashinagar and Naseri to analyze three penicillin antibiotics using HPLC indicated that the wavelength of the detector should be set to 225 nm as opposed to the 254 nm as suggested by Battula and Shivaraj.[27]

To solidify and further optimize the method, United States Pharmacopeia guidelines for analysis of ampicillin and cloxacillin were referenced. The methods used by the USP suggested that an isocratic analysis, one that maintains a composition of mobile phase, is preferable as compared to a gradient analysis in which the composition of the mobile phase changes over time.[24,28] The USP guides also confirmed that a potassium phosphate buffer should be used and that the detector should be set to a wavelength of 225 nm.[24,28] The guides were also crucial in the method validation process as they specified the USP requirements for analytical metrics including tailing factors (an indication of the symmetry of the peak), resolution (the degree of separation between peaks), and theoretical plates (the efficiency of the separation).

USP guidelines detail stringent requirements for the shape of each eluted peak on the chromatogram. As a result, several resources were used to build a comprehensive understanding of factors which impact peak shape. Primarily, information from Agilent Technologies regarding peak shape in HPLC was used. Most notably, poor peak shape in HPLC is often due to unwanted interactions between the stationary phase and analyte.[29] Thus, it is crucial for the production high resolution peaks to minimize these interactions. An HPLC column is most commonly packed with silica which contains an ionizable silanol side chain; this meaning that under certain levels of pH, the groups may become

negatively charged.[30] Most often, these interactions are the result of working with a buffer that is basic, promoting the transfer of a hydrogen ion from the silanol group on the column to the buffer. This negatively charged group within the column can then interact with the analyte moving through the column, especially if the analyte is positively charged (a common characteristic of many β-lactam antibiotics). Such an interaction results in poor peak shape due to tailing and altered retention times.[30] One solution to this problem involves the addition of an acidic mobile phase modifier such as trifluoroacetic acid.[29] This mobile phase modifier minimized interactions between the ionized stationary phase and the analyte by protonating the deprotonated silanol groups.[29]

A successful analysis of Ampiclox has important and wide-reaching implications. If the amount of active drug contained in the pharmaceutical sample obtained from DPAL and MTRH is unusual, the data will be reported to the WHO for further investigation. Reporting suspicious drug samples to the WHO is important in the mitigation of the negative impacts that substandard and falsified medical products present in developing nations. An increased awareness and detection of these products can ultimately be used to reduce the number of the products available for consumption, thus reducing their negative impacts on society.

Methods/Procedure

Sampling:

In this investigation, a method to separate ampicillin and cloxacillin contained in the antibiotic Ampiclox using reversed-phase high-performance liquid chromatography (RP-HPLC) was developed. Additionally, this method was used to quantify the APIs present in Ampiclox tablets obtained from a developing nation. The Ampiclox tablets supplied by the Distributed Pharmaceutical Analysis Laboratory (DPAL) were obtained from drug stores in Liberia.[19] Pharmaceuticals in developing nations such as Liberia are easy to obtain as the drugs are commonly available "over the counter, without prescription and through unregulated supply chains".[20] Upon receipt, DPAL processed the pharmaceuticals, and assigned them identification numbers and identified defining characteristics such as packaging details.[19] These identification numbers were used throughout the analysis to ensure the identity of each pill was preserved. The specific brand and dosage (amount of active pharmaceutical ingredient [API]) of the pharmaceutical are unknown. Twenty-one pills were obtained for quantification of API.

Sample Preparation:

Safe Handling:

Throughout this investigation, chemicals that have the potential to be toxic or dangerous were used. Appropriate precautionary measures were taken to minimize the risk for any harm. The hazards posed by each chemical were thoroughly studied before handling the chemical (links to relevant Safety Data Sheets (SDS) included in Appendix

1). Proper laboratory attire (long pants, closed toed shoes) and protective equipment (goggles and nitrile gloves) were worn at all times. Special care was given when handling the pharmaceutical products since they contain active pharmaceutical ingredients with known biological impacts.

System Suitability:

DPAL requires that all of its collaborators observe strict system suitability requirements to ensure accurate and precise data are collected. Before proceeding with the quantification of the API present in Ampiclox pills, several metrics demonstrating the reliability of the method were met. First, several standard solutions of ampicillin and cloxacillin were prepared. Standard solutions were used to create a calibration curve which was used to quantify the API present in the Ampiclox pills obtained from DPAL. A calibration curve relates the area beneath the peak on the chromatogram to the concentration of that analyte. DPAL requires a calibration curve with a correlation coefficient of 0.98 or greater.[23] The high correlation coefficient indicates that the standards used were properly prepared and lie within the linear dynamic range of the instrument; the range of concentrations over which the instrument response and concentration vary according to a linear relationship.[14] This is a metric that ensures accurate quantification of API.

All standard solutions were prepared in calibrated glassware. Glassware is designed to give accurate measurements at 25 ºC and assumes no imperfections. Laboratory conditions do not always match these ideal conditions. This correction

eliminated any error resulting from the expansion and contraction of borosilicate glass at temperatures other than 25ºC or any other imperfections in the glassware. The procedure for the calibration of all glassware used in the experiment is as follows. First, the ambient room temperature was measured in order to determine the corresponding density of water. Next, the selected piece of glassware was carefully filled to its appropriate capacity with de-ionized (DI) water. The mass of this water was then measured with a balance and recorded. The glassware was dried, and the procedure was repeated twice more to yield a total of three masses of water. Each of these measurements was corrected for buoyancy and the expansion of borosilicate glass.[14] Following this correction, using the density of water, the true volume of water held by each piece of glassware could be accurately determined. An average of the corrected volume for each of the three trials was used in any calculation in which the volume of the solution was relevant.

To prepare the standard solutions that were used to produce a calibration curve, five samples with concentrations ranging from approximately 5%-200% of the expected quantity of active ingredient was prepared. In this investigation, the Ampiclox pills are expected to contain 250 mg ampicillin and 250 mg cloxacillin. The specific range of calibration standards prepared were as follows: 500, 350, 250, 100, 50, and 25 parts per million(ppm) or mg/L. The standard solutions were made from secondary pharmaceutical standards of ampicillin and cloxacillin obtained from the chemical sales company Sigma Aldrich. A stock solution of ampicillin and cloxacillin of concentration 500 ppm was prepared in a calibrated 250 mL volumetric flask. To prepare this stock solution, a mass of 0.1250 g each of ampicillin and cloxacillin was obtained and transferred to the

volumetric flask. Approximately 125 mL of DI-water was added to the flask to allow for the API's to be dissolved and thoroughly mixed. Once the solution was homogenous, the remaining water was added to bring the solution to the 250 mL mark on the glassware. One must account for the purity of each standard to determine the exact concentration of the stock solution. Sigma Aldrich provides a certificate of analysis that lists the exact purity of each lot of standard (appendix 2). The purity (91.6% for ampicillin and 94.4% for cloxacillin), in combination with the corrected volume of the calibrated volumetric flask, was used to determine an exact concentration for prepared stock solutions.

To prepare the remaining calibration standards, the 500 ppm ampicillin/cloxacillin stock solution was diluted using appropriate volumetric pipets and 50 mL volumetric flasks. To prepare the 350 ppm standard, 35 mL of the 500 ppm stock solution was measured and transferred to the 50 mL volumetric flask. This solution was brought to 50 mL with DI-water and inverted to mix thoroughly. As above, the exact concentration of each dilution was determined by considering the exact volume of the glassware and the purity of the standards. This general procedure was repeated for all remaining concentrations of standard solutions; the calculated volumes necessary to prepare each dilution are summarized in Table 1.

Table 1: Summary of Preparation of Calibration Dilutions

Desired Concentration (ppm)	Volume of Stock Solution (mL)
350	35
250	25
100	10
50	5
25	2.5

The 0.01M potassium dihydrogen phosphate (KH_2PO_4) buffer at a pH of 3 with 0.1% reagent grade tri-fluoroacetic acid (TFA) was used for all analyses on the instrument. 1.0 L of the buffer was prepared at a time to minimize risk for algal growth. The buffer was prepared by dissolving 1.3619 g KH_2PO_4 in less than 1.0 L of DI water. Once all KH_2PO_4 was thoroughly dissolved, 1 mL of TFA was added and mixed. The pH was adjusted to 3 with the addition of H_3PO_4 and KOH. These components were added dropwise while monitoring the pH with a pH probe. Once the desired pH was achieved, the buffer was transferred to a 1.0 L volumetric flask and was brought to a volume of exactly 1.0 L.

All prepared solutions must be filtered using specialized HPLC filtration systems to prevent clogging and contamination of the HPLC column. The buffer was filtered using a vacuum filter and the standards were filtered using Whatman 0.45 micron PTFE disposable syringe filters. This allows for filtration of only small volumes as necessary.

Additionally, Sigma-Aldrich HPLC grade acetonitrile was purchased so that possible chemical interferences were not introduced into the instrument.

The HPLC used throughout this investigation is an Agilent InfinityLab LC Series 1220 Infinity II LC System fitted with an Agilent Eclipse C18 column. A gradient elution proceeding from 90% buffer to 36% buffer (the remaining percentage is acetonitrile) over 12 minutes maximized the separation and resolution of the API's (Figure 2). Between injections, a four-minute re-equilibration time was incorporated to prepare the instrument for the next separation. During this re-equilibration period, the instrument was returned to the initial 90% buffer 10% acetonitrile ratio. The 16-minute run begins at time 0.0 when the sample is injected. The flow rate of the instrument was 1.0 mL/min with an injection volume of 20 μL.[22] The detection wavelength was set to 225 nm.[22]

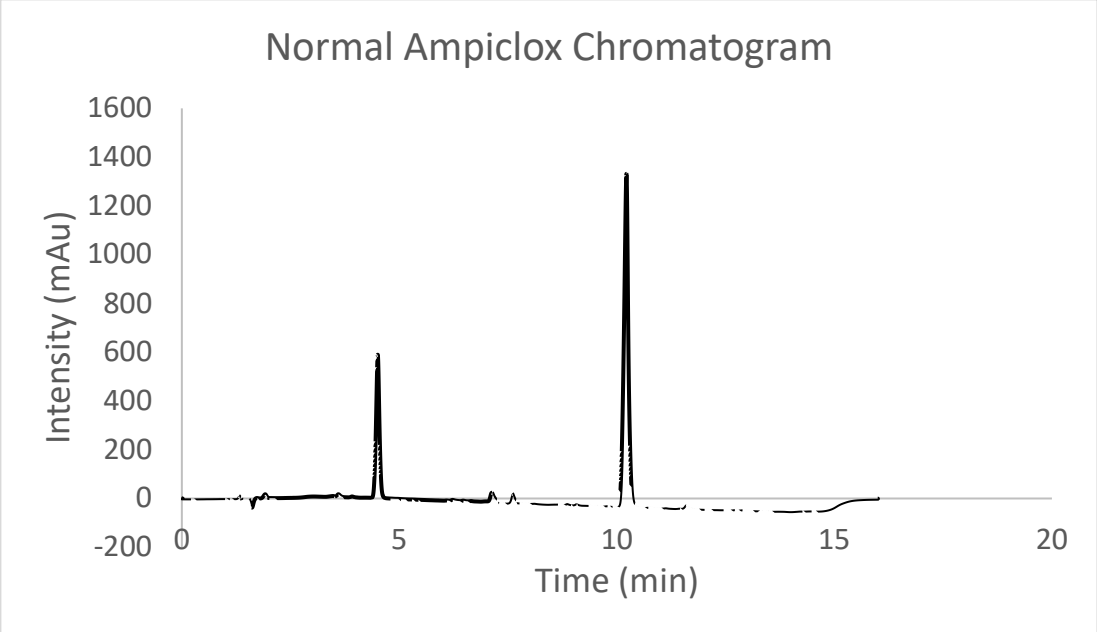


Figure 2: Representative Chromatogram (ampicillin left peak, cloxacillin right peak)

These parameters were used to produce the linear calibration curve. The calibration was constructing according to the following steps. A small aliquot of each calibration standard was filtered into an HPLC vial. These vials were labeled and inserted into the HPLC for analysis. Further system suitability requirements included demonstrating the precision of the instrument through the calculation of the relative standard deviation (RSD) of "6 consecutive injections of the known normal standard."[23] The RSD value for these replicates should be less than 2%.[23] The 250 ppm external standard (prepared as described above) in the middle of the linear dynamic range of the instrument was used to satisfy this requirement. This test ensured that the instrument yielded a consistent response over the course of several injections. Consistency is imperative because several consecutive injections into the instrument were made during the analysis of Ampiclox pills. Consistency throughout the injections is critical to the accuracy of API quantification.

In addition, quantification of API in an overdosed, normal, and deficient sample (a standard containing ~150%, ~100, and ~35% of the normal concentration of API respectively), was determined.[23] Three replicate injections of each sample were run with an external standard run every fourth measurement to ensure the instrument was yielding reproducible data.[4] To meet system suitability requirements, the RSD needed to be less than 2% for the external standard and each of the overdosed, normal, and deficient standards should be within ±2% of the true concentration.[23] The successful completion of this requirement demonstrated that if an Ampiclox pill contained an amount of API different than expected, the method accurately quantified the amount of API present.

Accuracy of the method using a spike recovery was also demonstrated. An Ampiclox tablet from a known source (one that contains a known concentration of API) obtained from DPAL was prepared by dissolving the pill in DI water and diluting it to a concentration within the linear range of the instrument. An aliquot of this pill solution was "spiked" with ~30% API from the external standards.[23] The concentration of API in this spiked pill solution was determined and had to fall within 90-110% of the true value.[23] This metric indicated that the instrument response was proportional to changes in concentration.

The specificity of the instrument was confirmed by the successful completion of a spike recovery from "a degraded dosage form matrix".[23] This solution was created by "baking the tablet for an hour at 60° C, then spiking the pill with an extra ~30% of the pure API and measure the spike recovery."[23] This metric measured the ability of the instrument to detect the API in the presence of a complex matrix, or one containing unexpected and unpredictable compounds. Such a matrix could potentially be present in pills that are substandard or falsified. It is important that even in the presence of such an obscure matrix, the API could still be accurately quantified and detected by the instrument.

Finally, the limit of detection (LOD) and lower limit of quantification of the instrument must be reported (LLOQ). This was determined by calculating the standard deviation of the peak area of a standard solution near the estimated LOD (from the calibration curve) and the slope of the calibration curve. The LLOQ is a measure of the smallest possible quantity of drug that can be accurately quantified by the HPLC

instrument. The LOD is the smallest amount of drug that is statistically different from the blank. These limitations are important to determine as pill solutions should not be prepared below these concentrations to ensure accurate quantification of API.

Throughout the sample analysis, an external standard was injected after every fifth run of the instrument. The peak height for this standard was compared to the six replicate injections. If the RSD was within 2%, the instrument was yielding precise, consistent data, and analysis could proceed. If the peak heights differed by more than 2%, the linearity of the instrument had to be reestablished with five external standard solutions as described above.

Sample Analysis:

System suitability requirements were met in May 2019, resulting in the authorization of UIndy to receive Ampiclox sample pills from DPAL. Quantification of ampicillin and cloxacillin contained in each pharmaceutical tablet were determined using the same RP-HPLC and method parameters used in the system suitability process as described above. Similar to the system suitability and method development process, there are strict requirements that must be followed throughout the analysis of Ampiclox pills. Most notably, tracking the identity of all pills is crucial to the integrity of the project. Each pill has a unique identification code, the Notre Dame Identification Number (NDID). This coding system is used to track each sample pill so that the results may be reported back to DPAL. Each NDID has three individual pills associated with it. All three pills have been obtained from the same manufacturing facility. Each pill in a set is

labeled pill A, B, and C upon receipt at UIndy. This designation is arbitrarily made by the analyst. Such a classification allows for additional tracking, as well as an additional level of quality control. Additionally, a photograph of the pills was captured and catalogued alongside the NDID and any other identifying information obtained upon visual inspection (Figure 3). The left and center images show the packaging and Ampiclox capsules. The rightmost image shows the contents of an Ampiclox capsule.

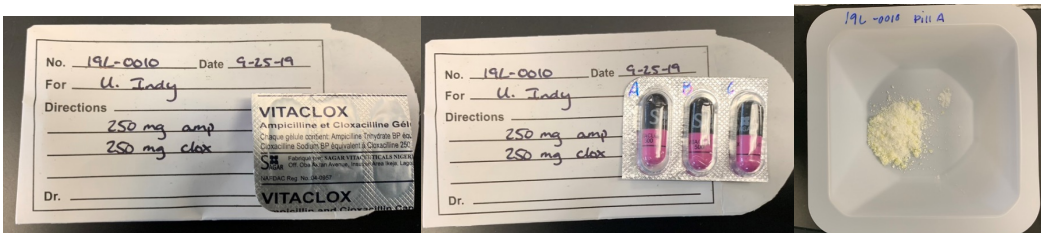


Figure 3: Example images used in pill documentation

Before analysis of any pills can be done, five injections of a 250 ppm external standard must yield a peak area within 2% relative standard deviation and the retention times of these peaks must be within 30 seconds.[23] Throughout the analysis, after every fifth injection of the pill, a 250 ppm external standard was run, and its intensity had to be within 2% of the five initial injections preceding initiation of analyses. Each injected pill was considered to pass analysis if the measured quantity of ampicillin and cloxacillin was within 90-110% of the advertised amount.[23] Pill A is the first pill to be analyzed. If this sample fails analysis, a second portion of pill A would be analyzed. If this pill fails a second time, a portion of pill B would analyzed. If the pill is still outside of the range of 90-110%, the sample is returned to DPAL for further analysis. If the sample fails at this level, a letter is sent to the WHO for possible action.

All Ampiclox pills received in this investigation were gel-capsules. After all procedures for documenting the pill were completed, the capsule was removed from the packaging and the mass of the whole pill was recorded. Subsequently, the gel-capsule was gently separated, and the contents were removed. The mass of the empty capsule was recorded, and the mass of the contents was determined by difference. The theoretical dosage of each pill was 250 mg ampicillin and 250 mg cloxacillin. Due to excipients found in pharmaceutical products (non-API additives to aid in the production and increase bioavailability of the drug to the patient[31]), the mass of the contents of the pill was greater than 500 mg. As a result, the mass to be weighed was determined using ratios. If the entire pill was to be dissolved in a 1.0 L flask, the concentration of the resulting solution would be 250 ppm of each API. Instead, only a tenth of the pill was prepared at a time. Thus one-tenth of the mass of the whole pill was dissolved in a 100 mL calibrated volumetric flask. The exact mass of the portion of pill used in the analysis was recorded for each sample. As the external standards were prepared, the contents of the pill were dissolved in DI-water. To ensure that the entire pill was thoroughly dissolved, sonication was used. The final step in the preparation for analysis was filtration of the solutions using syringe filters as described in the system suitability procedures above.

Sample Storage:

Once prepared, any solutions containing the API were stored in a chemical only refrigerator (2ºC) available in the Department of Chemistry to minimize the degradation

of the active ingredients over time. The prepared solutions were stored in the refrigerator for up to a month before fresh solutions were prepared to guarantee the integrity of the external standards and pill solutions were maintained. It was found that the ampicillin and cloxacillin standards degraded if stored for periods longer than one week. Thus, new external standard solutions were prepared before all pill analyses. Filtered buffers and mobile phases that did not degrade at room temperature were stored in covered 1.0 L bottles to prevent contamination.

Results

Calculations:

Before a determination regarding the quality of the pill can be made, the theoretical concentration of each capsule solution must be determined. This calculation considers the mass of the excipients in the pill as well as the calibrated volume of the glassware used to prepare the solution.

$$Theoretical\ Concentration\ Ampiclox\ (ppm) = \frac{Theoretical\ Mass\ Ampiclox\ (mg)}{Volume\ of\ Solution\ (L)} * \frac{Mass\ Weighed\ Portion\ (g)}{Total\ Mass\ Pill\ Contents\ (g)}$$

Despite the fact that there are two active ingredients in each capsule, the calculation for theoretical concentration is the same for both active pharmaceutical ingredients (APIs). This is attributed to the fact that the pills contain both ampicillin and cloxacillin as a part of a complex matrix of excipients. The theoretical mass of Ampiclox for all samples is 250 mg as indicated by the manufacturer. The volume of solution is known as a result of the calibration of all glassware, and the remaining masses were recorded during the preparation of the sample solutions.

Additionally, it was discovered that the dosage form of the standard ampicillin and cloxacillin needed to be taken into consideration. That is, both ampicillin and cloxacillin are manufactured and distributed in their salt form (ampicillin sodium and cloxacillin sodium). Thus, not only must the concentration of the standards be corrected

for their purities, but also for the difference in molar mass due to the presence of sodium. This correction is applied as shown below.

$$Correction\ Factor = Purity\ of\ Standard * \frac{Molar\ Mass\ pure\ Ampicillin}{Molar\ Mass\ Ampicillin\ Salt\ Standard}$$

$$Corrected\ Concentration\ (ppm) = Correction\ Factor * Theoretical\ Concentration(ppm)$$

Once the theoretical concentration has been determined, the actual concentration determined via analysis with high-performance liquid chromatography (HPLC) can be calculated. Figures 4 and 5 show a characteristic calibration curve for ampicillin and cloxacillin, respectively. These plots were constructed from six concentrations of standard ampicillin and cloxacillin within the linear dynamic range of the instrument.

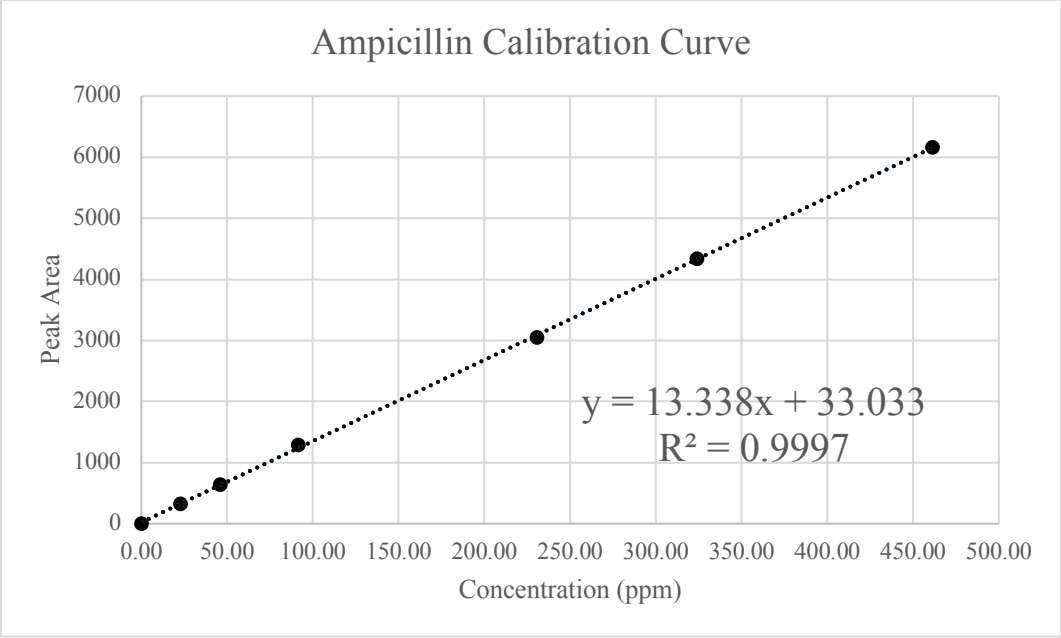


Figure 4: Representative calibration curve for ampicillin

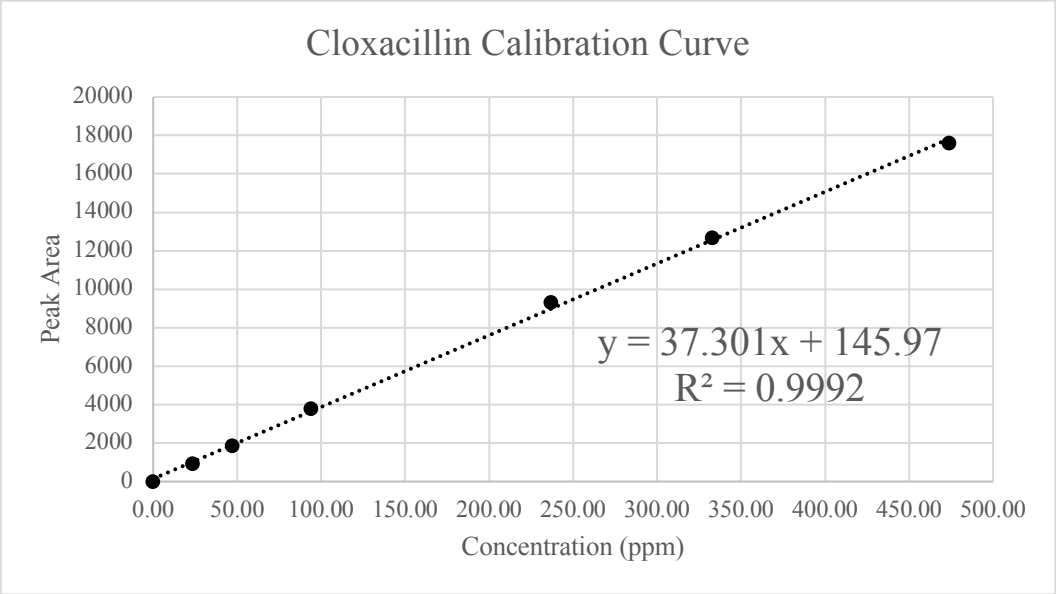


Figure 5: Representative calibration curve for cloxacillin

The linear regression lines for these plots yield an equation from which the concentration of the pill sample may be determined. The generic equation used to determine the concentration of ampicillin is shown below.

$$Measured\ Concentration\ (ppm) = \frac{peak\ area - (y\text{-}intercept)}{slope}$$

In this equation, the measured peak area was the y-value, and the equation was solved algebraically for x (the concentration in ppm). This calculation was repeated for all ampicillin and cloxacillin samples.

Once the theoretical and experimentally measured concentrations had been determined for all pill samples, the percent difference was calculated as shown below.

$$Percent\ Difference = \frac{measured\ concentration - theoretical\ concentration}{theoretical\ concentration} * 100\%$$

Using this equation, a positive percent difference indicates an overdosed pill while a negative percent difference indicates a deficient pill. Following Distributed Pharmaceutical Analysis Guidelines (DPAL) guidelines, a pill fails the analysis when the percent difference is greater than ±10%. Tables 2 and 3 provide a summary of the theoretical, experimentally determined, and percent difference in concentration for all 21 analyzed pill samples.

Data:

Table 2: Summary of Results for Ampicillin

Pill ID	Theoretical Concentration Ampicillin (ppm)	Measured Concentration Ampicillin (ppm)	Percent Difference (%)	Preliminary Assessment of Pill Quality
NDID-19L-0001	252.43	265.06	5.00	N/A
NDID-19L-0002	250.81	282.48	12.63	Overdosed
NDID-19L-0003	253.75	201.81	-20.47	Deficient
NDID-19L-0004	249.52	246.27	-1.30	Pass
NDID-19L-0005	250.99	267.71	6.66	Pass
NDID-19L-0006	250.54	264.39	5.53	N/A
NDID-19L-0007	248.07	256.00	3.20	Pass
NDID-19L-0008	251.78	342.09	35.87	Overdosed

Table 2, Continued

Pill ID	Theoretical Concentration Ampicillin (ppm)	Measured Concentration Ampicillin (ppm)	Percent Difference (%)	Preliminary Assessment of Pill Quality
NDID-19L-0009	214.37	254.49	18.71	Overdosed
NDID-19L-0010	246.39	201.30	-18.30	Deficient
NDID-19L-0011	252.05	256.81	1.89	Pass
NDID-19L-0012	251.20	263.19	4.77	N/A
NDID-19L-0013	215.98	260.02	20.39	Overdosed
NDID-19L-0014	213.33	266.30	24.83	Overdosed
NDID-19L-0015	249.95	270.78	8.34	N/A
NDID-19L-0016	249.58	259.77	4.08	Pass
NDID-19L-0017	249.24	266.91	7.09	Pass
NDID-19L-0018	253.99	247.18	-2.68	N/A
NDID-19L-0019	249.85	216.78	-13.24	N/A
NDID-19L-0020	248.55	265.76	6.93	Pass
NDID-19L-0021	216.24	260.68	20.55	Overdosed

Table 3: Summary of Results for Cloxacillin

Pill ID	Theoretical Concentration Ampicillin (ppm)	Measured Concentration Ampicillin (ppm)	Percent Difference (%)	Preliminary Assessment of Pill Quality
NDID-19L-0001	252.43	152.15	-39.73	N/A
NDID-19L-0002	250.81	266.25	6.16	Pass
NDID-19L-0003	253.75	26.66	-89.49	Deficient
NDID-19L-0004	249.52	253.23	1.49	Pass
NDID-19L-0005	250.99	233.10	-7.13	Pass
NDID-19L-0006	250.54	161.65	-35.48	N/A
NDID-19L-0007	248.07	201.41	-18.81	Deficient
NDID-19L-0008	251.78	289.73	15.07	Overdosed
NDID-19L-0009	214.37	138.36	-35.46	Deficient
NDID-19L-0010	246.39	26.79	-89.13	Deficient
NDID-19L-0011	252.05	223.44	-11.35	Deficient
NDID-19L-0012	251.20	240.86	-4.12	N/A
NDID-19L-0013	215.98	215.13	-0.40	Pass
NDID-19L-0014	213.33	229.75	7.70	Pass
NDID-19L-0015	249.95	205.27	-17.87	N/A
NDID-19L-0016	249.58	245.45	-1.66	Pass
NDID-19L-0017	249.24	274.34	10.07	Overdosed
NDID-19L-0018	253.99	265.47	4.52	N/A

Table 3, Continued

Pill ID	Theoretical Concentration Ampicillin (ppm)	Measured Concentration Ampicillin (ppm)	Percent Difference (%)	Preliminary Assessment of Pill Quality
NDID-19L-0019	249.85	239.16	-4.28	N/A
NDID-19L-0020	248.55	243.76	-1.93	Pass
NDID-19L-0021	216.24	230.44	6.57	Pass

When making the preliminary assessment of pill quality, any pill with a percent difference within the ±10% range passed analysis. Any pill with a percent difference less than -10% or greater that +10% was deemed deficient or overdosed, respectively. It was not possible to make any conclusions regarding pills that were analyzed when the relative standard deviation of the intensity of 250 ppm external standard was outside of the accepted ±2% range (indicated by N/A in Tables 2 and 3).

Analysis/Conclusion

The results obtained in this analysis are a reflection of a preliminary analysis of 21 Ampiclox pills obtained from the Distributed Pharmaceutical Analysis Laboratory (DPAL). Overall, this was a successful analysis yielding meaningful results. Not only can the results indicate the quality of the Ampiclox sample pills, but they are also a reflection of the successful development of a method to analyze these samples. Despite these significant successes, it was observed that a remarkably high number of samples were discovered to be outside of the accepted range of ±10% difference. Such a result suggests that there may be some weakness in the methods used and that revisions are necessary.

These surprising results prompted conversations with DPAL and a collaborating university (Niagara University) also developing a method for analysis of Ampiclox. After conversations with Dr. Robyn Goacher at Niagara University, and upon thorough analysis of data and procedures, a major concern developed regarding the stability of the prepared external standard solutions. A plot of the intensity of the peak area of the 250 ppm external Ampiclox standard over time revealed that there is a significant decrease in intensity corresponding to the degradation of the standard (Figures 6 and 7). The upper and lower limits for the intensity are denoted by the dashed lines.

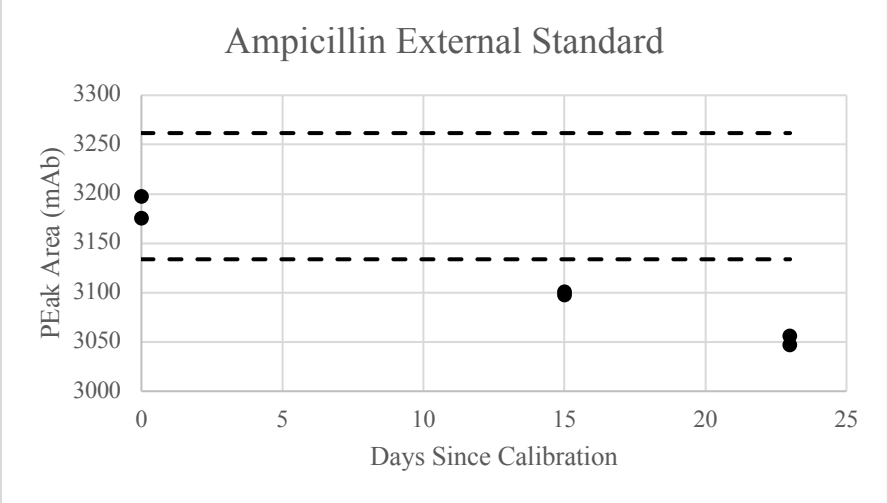


Figure 6: Intensity versus time plot for ampicillin

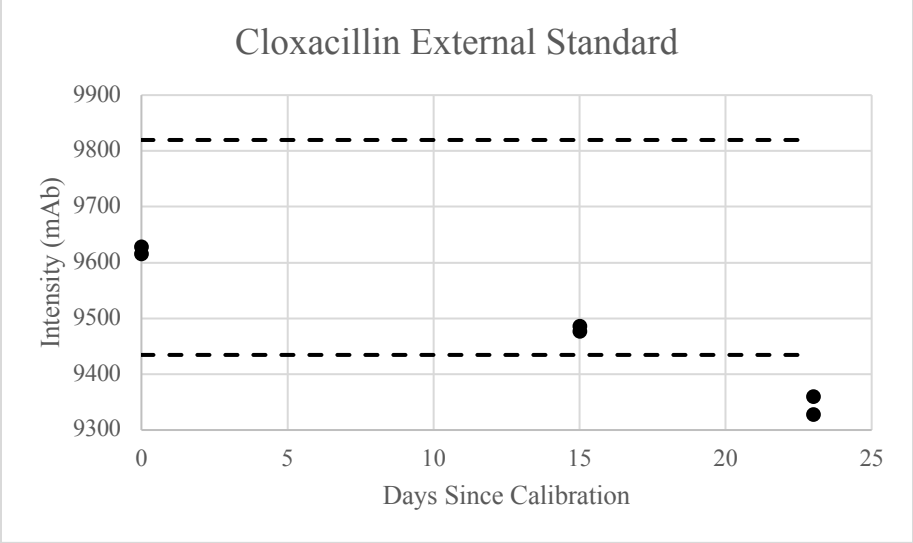


Figure 7: Intensity versus Time Plot for cloxacillin

The most significant concern regarding the analysis of pill solutions was the stability of the standard solutions over the period required for preparation and analysis of all pills. To most effectively remove the errors in the results associated with degradation of the standard over time, additional pill analyses will be completed within one week of

calibration and preparation of the standard solutions. New standard solutions will be made before the analysis of any new group of pills.

Although the majority of the data was deemed inconclusive and requires re-evaluation, there are several pills with a percent difference of greater than 35%. Most notably, NDID-19L-003 and NDID-19L-0010 had 89.49% and 89.13% deficiency in cloxacillin, respectively. Without further analysis, the significance of these results may not be directly concluded. Such a large deficit, however, is concerning and confirms that high-performance liquid chromatography (HPLC) analysis, with refinements, may be a valuable tool in quality control testing of pharmaceutical products.

Future directions of this project will focus largely on re-analyzing pill samples with consideration for the lack of stability of the standards. Once the re-evaluation of this initial subset of 21 Ampiclox capsules is completed, findings will be reported to DPAL. Subsequently, the method may be used to analyze additional pills obtained from DPAL.

The purpose of this investigation is multi-faceted. Not only was it intended to achieve the broad goal of yielding results that are meaningful in detecting substandard and falsified medications, but also to develop a novel method for the analysis of Ampiclox using RP-HPLC. It is important to highlight that although the results acquired in the first round of analyses are not believed to be a true and accurate representation of the quality of the pills, the data did provide important insights into the success of the method development, as well as the validity of the procedure of analysis. By assessing the success of each of these purposes, important changes to future procedures were determined. Furthermore, throughout the analysis process, the chromatograms were

consistent and reproducible. The tailing factors, resolution, and retentions times remained well within DPAL specifications indicating the method is an effective separation of the active pharmaceutical ingredients (APIs).

Reflection

This honors investigation was successful and facilitated growth and learning in a multitude of disciplines. The first and major goal of this project was the development of a reverse-phase high-performance liquid chromatography (RP-HPLC) method that could be used to simultaneously separate and quantify ampicillin and cloxacillin in Ampiclox. This method development process, although ultimately successful, was long, extensive, and frustrating. In the earliest stages, previously published literature was used as a guideline for development. It was rapidly realized that the methods published in the literature were not compatible with the instrumentation used in this project. As a result, I began to build a knowledge-base and skillset from which I could develop a method based on experimentation alone. Throughout this process, I faced many setbacks and challenges. Within the first 24 hours of beginning my research, I used a very concentrated buffer which precipitated with the organic acetonitrile mobile phase to clog the HPLC. This occurrence impeded progress in my work early on and for an extended period. I spent time on the phone with Agilent customer support to replace parts and remove precipitate from the tubing in the instrument. A few days later, I not only emerged victorious, but also well versed in the inner workings of the HPLC.

Conversations with instrument manufacturers (such as my conversation with Agilent Technologies described above) were important in developing the skill of troubleshooting. It is important to be able to convey the problem concisely and accurately when talking in real-time to experts in the field. Throughout the investigation, I was forced to identify and locate resources to help me solve encountered problems. Broadly,

this trouble-shooting process facilitated the development of a valuable skillset that is widely applicable in many situations.

Shortly after overcoming this first set-back, I encountered another challenge. After many manipulations of buffer composition and gradients of elution, I had achieved adequate separation of ampicillin from cloxacillin. The peak shape as indicated by the tailing factor, however, was far outside of United States Pharmacopeia (USP) guidelines. After extensive research and discussion, in addition to much trial and error, it was theorized that the poor peak shape was due to interaction of the charged groups on the active pharmaceutical ingredients (APIs) with ionized silanol groups inside of the column. To solve this problem, a competitor acid was added to the buffer. This acid protonates the ionized silanol groups in the column and prevents unnecessary interactions in the column from producing warped peaks. With this correction, the method passed all system suitability requirements as specified by the Distributed Pharmaceutical Analysis Laboratory (DPAL), and the method was accepted for analysis of pharmaceutical samples.

Analysis of Ampiclox pills facilitated the larger goal of the project to be achieved; the quality of an antibiotic obtained from a developing nation could be successfully and adequately assessed. Throughout the analysis of pill samples, there are strict guidelines and requirements that must be met to ensure the results are meaningful and significant. Thus, skills of communication, diligence, and organization are required to successfully complete the analysis. Upon collection of preliminary data, an evaluation of the validity of the analysis procedure was required. A critical evaluation of results revealed that there

was some error in the treatment of data to be addressed before proceeding in sample analysis. Specifically, it was important to realize that the external standards degrade over time. This finding has inspired me to pursue further analysis of this initial set of 21 Ampiclox pills. Completion of this task will ultimately fulfill my desire to make an impact on the lives of others by reducing the distribution of substandard and falsified pharmaceuticals.

All aspects of this project required critical thinking and problem solving; skills that are invaluable both personally and professionally. I use the skills I developed while completing this project every day. They are particularly applicable to me as I will be attending graduate school in chemistry in the fall and will be continuing with research in this field. Not only did I learn important scientific procedures and techniques, but I have developed skills of writing and communication that are important in sharing results with a larger group. Importantly, I found great joy and satisfaction in working on a project with such relevance and importance. I am so grateful to have been given the opportunity to learn and execute these skills and feel that they will facilitate much success in my future endeavors.

References

1. Demain, D.L., Sanchez, S., Eds. *Antibiotics: Current Innovations and Future Trends;* Caister Academic Press: Norfolk, UK, 2015; pp xi-15.

2. Amador, P; Fernandes, R; Prudencio, C. β-Lactams: chemical structure, mode of action and mechanisms of resistance. *Rev. in Med. Microbiol.* **2013,** *24,* 7-8.

3. Lin, J; Ximin, Z. Beta-Lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front. Microbiol.* **2013,** *4,* 1-2.

4. Falagas, M.E.; Kelesidis, T. Substandard/counterfeit antimicrobial drugs. *Clin. Microbiol. Rev* **2015,** *28,* 443-446.

5. Substandard and Falsified Medical Products. https://www.who.int/news-room/fact-sheets/detail/substandard-and-falsified-medical-products (Accessed January 27, 2019), World Health Organization, 2018.

6. Fernandez, F.; Green, M. D.; Newton, P. N. Impact of poor-quality medicines in the 'developing' world. *Trends Pharmacol. Sci.* **2010,** *31*, 99-101.

7. *WHO Global Surveillance and Monitoring System for Substandard and Falsified Medical Products;* World Health Organization: Geneva, 2017; 1-57.

8. Tool for Visual Inspection of Medicines. http://www.whpa.org/Toolkit_Be Aware_In -spection.pdf (accessed February 4, 2019), World Health Organization.

9. Arzhantsev, S.; Buhse, L.F.; Gryniewicz-Ruzika, C. M.; Kauffman, J.F.; Rodriguez, J.D. *Science and the Law: Analytical Data in Support of Regulation in Health, Food, and the Environment* [Online]; ACS Symposium Series; American Chemical Society: Washington D.C., 2014; 1167, 149-168. https://pubs.acs.org /doi/pdf/10.1021/bk-2014-1167.ch011 (accessed January 27, 2019).

10. Hawes, S.E.; Kovacs, S.; Maley, S.N.; Mosites, E.; Stergachis, A.; Wong, L. Technologies for Detecting Falsified and Substandard Drugs in Low and Middle-Income Countries. *PLOS One* **2014,** *9,* 1-11.

11. Boehle, K.E.; Carrell, C.S.; Caraway, J.; Henry, C.S. Paper-Based Enzyme Competition Assay for Detecting β-Lactam Antibiotics. ACS. Sens. 2018, 3, 1299-1307.

12. Barstis, T.; Benvenuti, M.; Ghosh, D.; Hunckler, M.; Joy, B.; Koenig, L.; Lieberman, M.; Raddell, K.; Reiser, H.; Weaver, A.A. Paper Analytical Devices for Fast Field Screening of Beta Lactam Antibiotics and Antituberculosis Pharmaceuticals. *Anal. Chem.* **2013**, *85*, 6453-6460.

13. *Countering the Problem of Falsified and Substandard Drugs* [Online]; Buckley, G.J., Gostin, L.O., Eds.; The National Academies Press: Washington D.C., 2013; 1-330. https://www.ncbi.nlm.nih.gov/books/NBK202530 /pdf/Booksh elf_NB K20 2530.pdf (accessed February 9, 2019).

14. Harris, D. C. Fundamentals of Spectrophotometry. *Quantitative Chemical Analysis.* 9; W.H. Freeman and Company: New York, NY, 2016; 609-690.

15. D'Atri, V.; Fekete, S.; Clarke, A.; Veuthey, J.; Guillarme, D. Recent Advances in Chromatography for Pharmaceutical Analysis. Anal. Chem. [Online early access]. DOI: 10.1021/acs.analchem.8b05026. Published Online: Nov 8, 2018. https://pubs-acs-org.ezproxy.uindy.edu/doi/ pdf/ 10.1021 /acs. analchem.8b05026 (accessed Dec 1, 2018).

16. Adeleke, O.E.; Coker, M.E.; Fatoyinbo, A.D.; Oluwagbohun, J.O. Brands of Ampiclox Against Clinical Strains of Staphylococcus Aureas. *Afr. J. Cln. Exper. Microbio.* **2010**, *11,* 129-136.

17. Ampiclox.https://gskpro.com/content/dam/global/hcpportal/en_NG/PDF/Home/Products/Ampiclox_GA_03.pdf (accessed Feb 13, 2019), GlaxoSmithKline.

18. Giang, D.T.; Hoang, V.D. Comparative Study of RP-HPLC and UV Spectrophotometric Techniques for the Simultaneous Determination of Amoxicillin and Cloxacillin in Capsules. *J Young Pharm.* **2010,** *2,* 190-195.

19. Grosse, R. Distributed Pharmaceutical Analysis Laboratory (DPAL): Citizen Scientists Tackle a Global Problem. *Chemists Without Borders,* ACS Symposium Series; American Chemical Society: Washington, D.C., 2017; 1, 117-127.

20. Atabe, A.N.; Ayukekbong, J.A.; Ntemgwa, M. The threat of antimicrobial resistance in developing countries: causes and control strategies. *Antimicrobial Resistance and Infection Control* **2017,** *6,* 2.

21. Patil, J.K.; Patil, K.A.; Pawar, S.P. Development and Validation of RE-HPLC Method for Simultaneous Estimation of Amoxicillin and Dicloxacillin in Bulk Drug and Capsules. *Pharma. Sci. Monitor.* **2014,** 2, 39-47.

22. Battula, S.; Shivaraj, A. RP-HPLC Method for the Simultaneous Estimation of Ampicillin and Cloxacillin in Capsule Dosage Form. *Int. J. App. Pharm. Sci. Research* **2017,** 2, 93-98.

23. Lieberman, M. HPLC Methodology Manual Distributed Pharmaceutical Analysis Laboratory (DPAL). unpublished work.

24. *United States Pharmacopeia and National Formulary* [online]; USP 42-NF 37-1107; United States Pharmacopeial Convention: Rockville, MD, 2019. https://online.uspnf.com /uspnf/docu ment/GUID-7 ED2261C-2EA5-457A-B076-7AB6D07017CF_1_en-US

25. Compound Summary for CID 6249: Ampicillin. https://pubchem.ncbi.nlm. nih. gov/comp ound/ampicillin#section=2D-Structure (accessed March 7, 2019), Pub Chem Open Chemistry Data Base.

26. Compound Summary for CID 6098: Cloxacillin. https://pubchem.ncbi.nlm.nih. gov/comp ound/cloxacillin#section=2D-Structure (accessed March 7, 2019), Pub Chem Open Chemistry Data Base.

27. Ashnagar, A., Naseri, N.G. Analysis of Three Penicillin Antibiotics (Ampicillin, Amoxicillin, and Cloxacillin) of Several Iranian Pharmaceutical Companies by HPLC. *E-J. Chem.* **2007,** *4,* 536-545.

28. *United States Pharmacopeia and National Formulary* [online]; USP 42-NF 37-322; United States Pharmacopeial Convention: Rockville, MD, 2019. https://online.uspnf.com /uspnf/docu ment/GUID-57A2342F-D116-4DC3-920C-5AFD817C099_3_en-US

29. The Secrets of Good Peak Shape in HPLC. https://www.agilent.com/cs/ library/eseminars/ Public/secrets %20of %20good%20peak%20shape %20in%20hplc.pdf

30. Neue, U.D.; Niederlanender, C.L.;Peterson, J.S. Liquid Chromatography Stationary Phases with Reduced Silanol Interactions. 5,374,755. Dec. 20, 1994.

31. Rowe, R.C.; Sheskey, P.; Quinn, M. Preface. *Handbook of Pharmaceutical Excipients,*6; Pharmaceutical Press: London, UK: 2009; x.

32. Certificate of Analysis: Ampicillin Sodium. https://www.sigmaaldrich.com/ catalog/CertOfAnalysisPage.do?symbol=PHR1424&LotNo=LRAA9206& brandTest=SIAL&returnUrl=%2Fproduct%2FSIAL%2FPHR1424 (accessed Mar 22, 2020). Sigma-Aldrich.

33. Certificate of Analysis: Cloxacillin Sodium. https://www.sigmaaldrich.com/catalog/CertOfAnalysisPage.do?symbol=PHR1922&LotNo=LRAB1227&brandTest=SIAL&returnUrl=%2Fproduct%2FSIAL%2FPHR1922 (accessed Mar 22, 2020). Supelco.

Appendices

Appendix 1: SDS Sheets

Ampicillin Sodium:

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=PHR1424&brand=SIAL&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsial%2Fphr1424%3Flang%3Den

Cloxacillin Sodium:

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=PHR1922&brand=SIAL&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsial%2Fphr1922%3Flang%3Den

HPLC Grade Acetonitrile:

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=437557&brand=SIAL&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsial%2F437557%3Flang%3Den

Tri-fluoroacetic Acid

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=T6508&brand=SIGALD&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fsearch%3Fterm%3Dtrifluoroacetic%2Bacid%26interface%3DAll%26N%3D0%26mode%3Dmatch%2520partialmax%26lang%3Den%26region%3DUS%26focus%3Dproduct

Potassium Dihydrogen Phosphate:

https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=1.04873&brand=MM&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fsearch%3Fterm%3Dpotassium%2Bdihydrogen%2Bphosphate%26interface%3DAll%26N%3D0%26mode%3Dmatch%2520partialmax%26lang%3Den%26region%3DUS%26focus%3Dproduct

Appendix 2: Certificates of Analysis for Ampicillin and Cloxacillin[32,33]

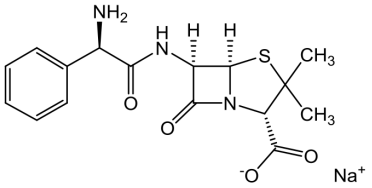
Certificate of Analysis

ISO GUIDE 34
ANAB Cert# AR-1470

ISO/IEC 17025
ANAB Cert# AT-1467

AMPICILLIN SODIUM

CERTIFIED REFERENCE MATERIAL



CERTIFIED PURITY: 91.6%, U_{crm} = ±0.9% k = 3.18
(Mass Balance/as is basis, as $C_{16}H_{18}N_3NaO_4S$)

NOMINAL PACKAGE SIZE: 1g

CATALOG #: PHR1424 **LOT #:** LRAA9206

CERTIFICATE VERSION: LRAA9206.2 **ISSUE DATE:** 30 September 2018

Note: Certificates may be updated due to Pharmacopeial Lot changes or the availability of new data.
Check our website at: www.sigma-aldrich.com *for the most current version.*

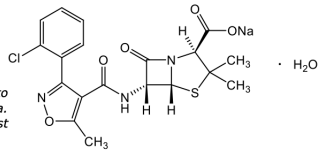
Supelco®

www.sigmaaldrich.com

Certificate of Analysis – Certified Reference Material

CLOXACILLIN SODIUM

Product no.: PHR1922-500MG
Lot no.: LRAB1227
Description of CRM: White Powder
Expiry date: 31 December 2022
Storage: Store in a Freezer/Protect from Light
Certificate version: LRAB1227.2 *(Note: Certificates may be updated due to Pharmacopeial Lot Changes or the availability of new data. Check our website at: www.sigma-aldrich.com for the most current version.)*
Chemical formula: $C_{19}H_{17}ClN_3NaO_5S•H_2O$
Molecular mass: 475.88
CAS No.: 7081-44-9



Analyte	Certified Purity ± associated uncertainty *U*, *U=k · u (k=)* (Mass Balance/ basis)
Cloxacillin Sodium	94.4 % Ucrm = ± 0.4%, k = 2 (as is basis)