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HPLC Method Validation: A Global Application for the Analysis of Amoxicillin

Senior Project

In partial fulfillment of the requirements for
The Esther G. Maynor Honors College
University of North Carolina at Pembroke

By

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Chemistry and Physics
1st of May, 2019

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Abstract

Several factors can determine the purity and efficacy of certain pharmaceutical compounds. These environmental factors play a huge role in how drug manufacturers store and handle compounds. Several countries that are without resources such as dependable electricity and clean water, also suffer from not receiving proper healthcare, which includes not receiving correct medication. **The main goal of this research is to validate the HPLC method which will be used to analyze the purity of amoxicillin capsules collected from selected African countries to show the importance of safe and effective pharmaceutical products.** Before the samples can be analyzed, it is necessary to meet several criteria for a validated HPLC method. **Thus, the aim of this research is to demonstrate the linearity, precision, and retention data of the HPLC method as well as to show that the method meets the requirements for tailing factor and column efficiency.**

HPLC Method Validation: A Global Application for the Analysis of Amoxicillin

Background

Amoxicillin is in the beta lactam family that also belongs to the Penicillin, which can be used to treat an overabundance of infections including: pneumonia, ear infections, bronchitis, and tonsillitis. According to Frontiers of Microbiology, Amoxicillin became available when it was introduced to the United Kingdom in 1972[1]. Amoxicillin is dispensed in a variety of forms from tablets to liquid drops, to be taken orally. These various dispensing techniques makes it difficult to manage the integrity of the drug. Amoxicillin is listed on the World Health Organization's "List of Essential Medicines"[2] as one of the most vital medications needed in a basic health system. Amoxicillin is one of the most frequently prescribed antibiotic used to treat infections in patients who are commonly children. However, Amoxicillin that is distributed in third world countries, like Uganda and Ethiopia, both countries within Africa, have different standards for handling and storage compared to the United States which may impact the efficacy of the drug[3]. Due to environmental factors, including temperature, humidity, storage containers/packaging, light, and humidity which degrade the purity of the medication and leads to the issue of antibiotic resistance. These medications are simply not as effective as pharmaceutical drugs in the United States[3]. In the world of pharmaceutical investigations, there are three problems which are poorly manufactured drugs, drugs that degrade due to improper storage conditions, and drugs that have been tampered with. Researchers are able to see each of these in the local street pharmacies of Uganda and Kenya.

Figure 1 (on the left): Counterfeit drugs in African communities which are not properly labeled or sealed in FDA approved containers. Figure 2 (on the right): Local distribution points that lack indoor coolant and refrigeration which could jeopardize the quality and potency of the medications.

This problem has caught the attention of researcher's everywhere which led the University of Notre Dame and the Moi Teaching and Referral Hospital to create a

research trial that would test the purity of these samples taken from street pharmacies in West Kenyan Communities by covert shoppers. The samples are collected and sent to the Moi Teaching and Referral Hospital, where they are cataloged and then sent to University of Notre Dame. The samples are then boxed and sent to the participating colleges that will be conducting the testing of the samples. However, this research is considered a blind study, which means that the participating institution does not know if they are testing a sample collected from Uganda and Kenya or a placebo created by the oversight institution. This allows for the integrity of the research to be carried out and for no discrimination to occur in both the researcher or the participating institution. There are certain parameters met to fulfill the requirements to conduct this research.

Methodology

To conduct this research, a reversed-phase high-performance liquid chromatography (HPLC) method with UV detection, the subsequent components of these drugs are able to be compared to the original compound to differentiate whether or not they contain the same properties. The HPLC instrument must be calibrated and the accuracy insured to be operating within the right guidelines. Precision, linearity, and accuracy are the three criteria required to test and fully satisfy the protocol established by University of Notre Dame of the Amoxicillin. The instrument is set to a wavelength of 230 nm, a 95:5 v/v% monosodium phosphate buffer and methanol is used at a 0.5 mL/min injection rate. These guidelines are constant for every injection. To test these parameters, pharmaceutical grade Amoxicillin (Source: Sigma, Lot Number: 066M4760V) is used and certain dilutions are created to test the linearity of the compound. These samples are then injected (20 µL injection volume) into the chromatography column in the HPLC instrument and the instrument generates a chromatogram that lists the peak area, peak height, theoretical plate, tailing factor, and k prime data. The data illustrates how the

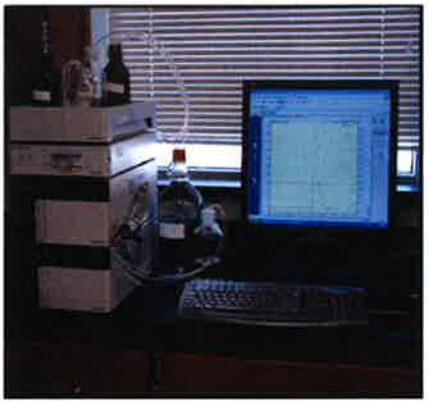
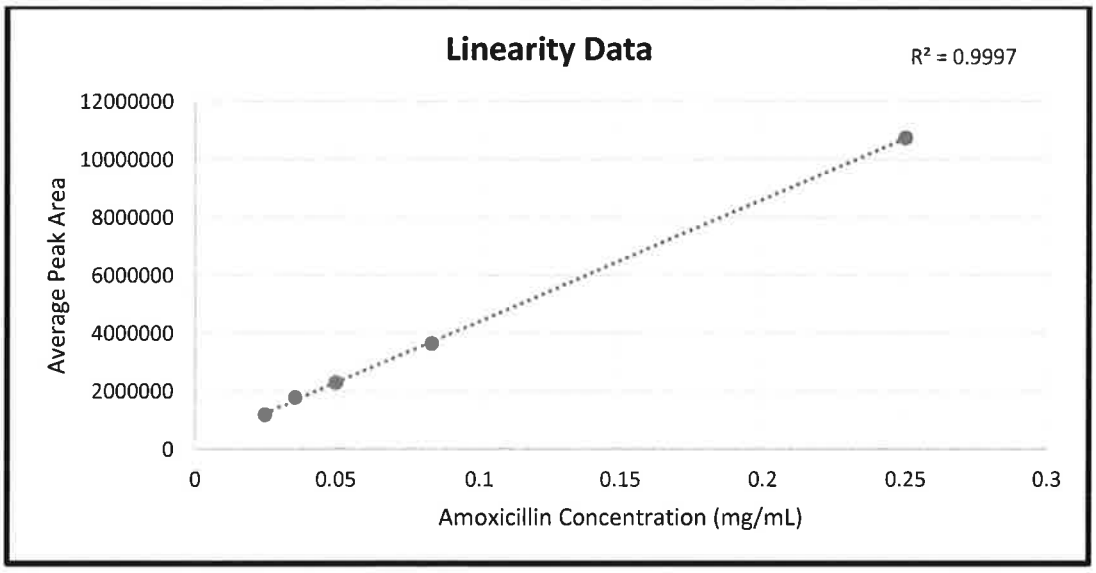


Figure 3: HPLC Instrument at the UNCP Science Laboratory

compound breaks down over a 15 minute time period which also shows how the pure Amoxicillin how the peak area this is the time for elution compared to counterfeit samples. This same procedure is completed to calculate the precision part of the data. Compared to linearity, the retention variability aspect measures the retention time, the time it takes for Amoxicillin to elute from the column. Instead of using different concentration of Amoxicillin, the same concentration was used repeatedly. Also, an optional test to validate is the Limit of Detection (LOD) and Lower Limits of Quantification (LLOQ) which is defined as the lowest concentration at which 95% of positive samples are detected. Simply, this shows the smallest concentration of Amoxicillin that is detectable in the HPLC instrument. These above mentioned test areas have specific guidelines concerning the acceptance or rejection of data, which points the researchers in the "right" direction of the calibration of their HPLC instruments. Without these guidelines, it would be impossible to fully accept any data collected from other instruments since each would be set up so differently.

Results and Discussion

Linearity



According to Dr. Lieberman, designer of the analytical method used in this research, she recommended to "...prepare and run at least five calibration standards over the concentration range of 5% to 200%." After reviewing the data as a whole, a graph

was created. Each single data point on the calibration curve represents triplicate injections of various amoxicillin concentrations. Based on the method requirements, the acceptable R^2 point is to be ≥0.98. As noted on the calibration curve, the method resulted in a R^2 value of 0.99 which is creditable.

Quality Control

Quality Control Chart			
Date	**Theoretical Plates**	**Tailing Factor**	**k'**
11-Feb	3808.634	1.133	2.024
18-Feb	3111.859	1.185	1.523
18-Feb	3148.853	1.198	1.522
18-Feb	3028.968	1.240	1.554
19-Mar	2724.039	1.009	1.041

To ensure the validation of the method, an external standard is used to ascertain various quality control parameters. These parameters are theoretical plates, tailing factor, and retention factor. A theoretical plate in many separation processes is a hypothetical zone or stage in which two phases, such as the liquid and vapor phases of a substance, establish an equilibrium with each other. The tailing factor is a measure of peak tailing. It is defined as the distance from the front slope of the peak to the back slope divided by twice the distance from the center line of the peak to the front slope, with all measurements made at 5% of the maximum peak height. Determination of Retention Factor (k) A high k value indicates that the sample is highly retained and has spent a significant amount of time interacting with the stationary phase.

- The number of **theoretical plates** must be >1700.
- The **tailing factor** must be < 2.5.
- The **retention factor (k')** must be between 1.1-2.2.

Based on the data presented in the Quality Control Chart, all three criteria are met with this HPLC method.

Precision

Precision Table		
Run	**Peak Area**	**Retention Time**
1	2848397	6.796
2	2913860	6.552
3	2927961	6.608
4	2967067	6.760
5	3005648	6.695
6	2895903	6.768
	Mean: 2926472.667	
	Standard Deviation: 54969.523	
%RSD: standard deviation/mean * 100	%RSD: 1.88%	

The Relative Standard Deviation (RSD) for the integrated intensities of 6 consecutive injections of the known standard should be below 2%. The RSD found from our study was 1.88%, so the parameters were met. The peak area is the area from the chromatogram. The retention time is the time it takes for Amoxicillin to elute from the column. The range of the retention time is less than 0.5 minutes, which is another parameter that was met.

LOD/LLOQ

LOD/LLOQ Table

Concentration	Run 1 (peak area)	Run 2 (peak area)	Run 3 (peak area)	Average Peak Area	Std. Dev of Peak Area	%RSD
6 µg/mL	1411810	1417620	1439726	1423052.00	14729.42	1.03
10 µg/mL	2294849	2366629	2378886	2346788.00	45396.07	1.93
10 µg/mL	2411442	2405287	2424846	2413858.33	10000.88	0.41
15 µg/mL	3348757	3396954	3320738	3355483.00	38550.60	1.14
20 µg/mL	4904042	4902144	4933249	4913145.00	17436.42	0.35

The LOD/LLOQ was determined by using varying concentrations of Amoxicillin each at a smaller concentration until it was undetectable by the HPLC instrument. The %RSD will also be measured and has to be under 2%. Since each value was under the mentioned value, we were able to accept each one and check off the LOD/LLOQ portion of the test.

Conclusion

The purpose of our research is to validate the HPLC method which will be used to analyze Amoxicillin samples purchased in African countries. At this point, our research demonstrates that the linearity of the data (0.999 R^2) , quality and efficacy of our standards, parameters for precision (1.88% RSD), and the range for retention time (0.244 min) has been satisfied. We also determined the LOD to be 6 µg/mL with the LOQ equivalent to 10 µg/mL. Our results from the precision study met the specific parameters allowing us to continue with our research. After final validation and approval, our hopes are to begin testing the samples which will then be sent to the Medical Regulatory Authority (MRA) and the World Health Organization (WHO) Medical Rapid Alert System (MRAS).

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