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Drug Quality Assurance to Combat Counterfeit Streptomycin using Facile, Low Cost Colorimetry

MaKayla R. Foster
mrf4737@rit.edu

Drug Quality Assurance to Combat Counterfeit Streptomycin using Facile, Low Cost Colorimetry

By: MaKayla R. Foster

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MaKayla Foster

has been approved by the Thesis Committee as satisfactory for the thesis requirement for the Master of Science degree in Chemistry at the convocation of

May 2019

Thesis Committee:

Dr. Scott Williams, Primary Thesis Advisor

Dr. Michael Coleman, Graduate Program Coordinator

Dr. Hans Schmitthenner, Committee Member, SCMS

Dr. Lea Michel, Committee Member, SCMS

Dr. Suzanne O'Handley, Committee Member, SCMS

Dr. Michael Gleghorn, Committee Member, SCMS

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List of Abbreviations

A	Absorbance, used in Beer's Law Calculations
a*	Degree of redness or greenness
b	Pathlength, used in Beer's Law Calculations
b*	Degree of yellowness or blueness
c	Concentration, used in Beer's Law Calculations
CAMM	Chemical Assay to verify quantity and quality of Malaria Medications
CIE	International Commission on Illumination,
CIELAB	Uniform visual spacing of correlated colors
DESI	Desorption Electrospray Ionization
DQA	Drug Quality Assurance
FTIR	Fourier-Transform Infared Spectroscopy
GHB	Gamma-Hydroxybutyrate, commonly used as a date rape drug
GPHF MiniLab	Global Pharma Health Fund Minilab
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HSAB	Hard Soft Acid Base
LC-MS	Liquid Chromatography-Mass Spectrometry

L*	Lightness of color
MS	Mass Spectrometry
m/z	Mass to charge ratio of ions
NaOH	Sodium Hydroxide
NIR	Near InfraRed Spectroscopy
PADs	Paper Analytical Devices
TB	Tuberculosis
TLC	Thin-layer Chromatography
USAID	United States Agency for International Development
USD	U.S. Dollar
UV	Ultraviolet
WHO	World Health Organization
ε	Molar Absorptivity

Abstract

An easy to use, low cost colorimetric method to identify the quality and quantity of the tuberculosis (TB) medication, streptomycin was developed. TB is typically treated using a multi-drug regimen comprised of antibiotics such as streptomycin. According to WHO and USAID up to 50% of pharmaceuticals are counterfeit worldwide. The problem intensifies the closer one gets to the point of care. A facile counterfeit detection method, that is specific to only the authentic medication, is essential. Since the 1940s, streptomycin was colorimetrically identified using a 16-hour base hydrolysis process followed by an iron complexation reaction. A tris(maltonato)iron (III) complex was suggested, but not confirmed in the 1940s method. The maltol formation using a base hydrolysis approach was optimized for streptomycin. Monitored using HPLC, the original 16-hour hydrolysis time was reduced to 3 minutes using a heating process and a two-fold reduction in NaOH concentration. A two-fold increase in maltol hydrolysis per mole streptomycin was also achieved. The 2 M HCl acid, used to catalyze the iron complex formation, was eliminated by using iron (II) acetate in place of iron chloride in a capsule delivery system. The new assay system was improved to include fewer steps and to remove caustic solutions thereby making the reaction safer. In conducting optimizations to the 1945 method, we discovered a discrepancy in the 1945 reaction interpretations. HPLC results comparing the 1945 assay products with the known tris(maltonato)iron (III)

standard did not match. The solution colors were visually distinct. FTIR and UV-visible Spectroscopic analysis suggested that the 1945 method was not forming the tris(maltonato)iron (III) complex as reported. The safer and optimized assay method, however, does make the tris(maltonato)iron (III) complex. An effective, yet safer and better characterized colorimetric assay for streptomycin was developed.

Chapter 1

Introduction

Introduction

The World Health Organization's (WHO) goal is to build a better and healthier future through combatting disease, ensuring safe water, air, food and more.[1] The WHO is focused on many global health issues, however, there are two that the WHO is particularly interested in. These are tuberculosis and malaria.[2]

In 2016, approximately 10.4 million people became ill with TB. TB is especially dangerous because people can contract a multidrug resistant strain. If not properly treated the results can be fatal. Streptomycin is commonly used to treat adults with a resistance to the TB medication, Isoniazid, and to treat children with TB.[3]

Counterfeit medications have become a problem that is currently receiving international attention.[4] Medications are defined as counterfeit when they contain one or more of the following: no active ingredient (placebos), actively harmful ingredients, fake packaging or the incorrect quantities of active ingredients.[5] The most harmful counterfeiting method is when the concentration of the active ingredient is lower than effective dose. This can lead to drug resistance, treatment failure, or death.[6–8] This work demonstrates that a simple colorimetric assay can be used to detect counterfeit streptomycin as well as the detection of expired streptomycin. This colorimetric assay is a modification of a 1945 reaction with the addition of metal complex characterization.

Problem Statement

Counterfeit medications are a worldwide problem. This problem is intensified when the counterfeit medications are made with minute amounts of the active ingredient. When people have TB or other diseases that have a multidrug resistant strain, counterfeit medications can be fatal. A counterfeit detection method for the TB medication, streptomycin, was studied to determine if there is a quantitative, facile and low-cost authentication detection for streptomycin.

Reason for Interest

This project combined multiple interests of the researcher: forensics and analytical chemistry. Merriam-Webster defines forensic as *dealing with the application of scientific knowledge to legal problems*.[9] This research deals with a huge legal problem that leads to many deaths every year.

The researcher has an undergraduate degree in chemistry and did her capstone research project with a concentration in analytical research using HPLC. Through this work, the researcher was able to enhance her skills and knowledge using HPLC and other analytical chemistry methods and techniques.

Explanation of Problem

An authentication test for streptomycin was studied. This is a low-cost colorimetric assay that quantitatively and selectively identifies the streptomycin at the point of care. In addition to testing the authenticity, this test can also detect when streptomycin has expired. Strategies to detect other counterfeit medications were developed through this process. Characterization of a critical organometallic complex

was then performed to understand the color differences of two different papers that report that they are making the same complex.

Chapter 2

Theoretical Basis of the Study

Introduction

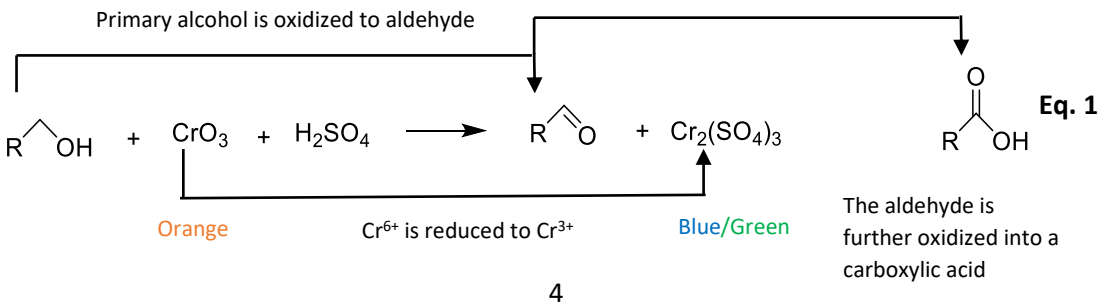
This section states the reasons behind the measurements, test target choices and the detection method techniques. The theories to follow set up the methods used for this research, can be used to recreate this study or to conduct similar research.

Functional Group Tests

Structures can be identified and characterized using their functional groups. There are different tests that can be performed to identify each functional group.

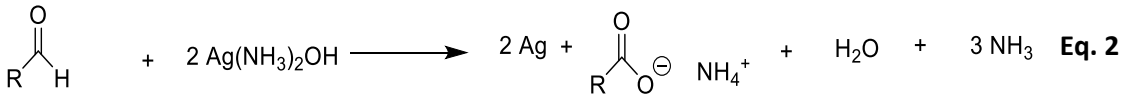
Jones Oxidation for Primary and Secondary Alcohol

The Jones reagent is 2.7 M chromic acid in 3.0 M sulfuric acid. When the orange-yellow reagent is added to a primary or secondary alcohol, a green-colored solution of chromium sulfate is formed, see Equation 1. Aldehydes and enols give a positive test result but tertiary alcohols do not. Phenols also result in a positive test, but the colored product is not the characteristic blue green.



Tollen's Aldehyde Test.

Equation 2 shows how the Tollen's aldehyde test works.[10]



Eq. 2

Tollen's reagent is made using 2 mL of 0.2 M silver nitrate and 1 drop of 3 M sodium hydroxide. A 2.8% ammonia solution is added dropwise and shaken until almost all silver oxide precipitate dissolves. This reagent can then be used to test for aldehydes but will also result in a positive test for easily oxidized compounds such as aromatic amines and some phenols. One drop or a few crystals of an aldehyde can be added to 1 mL of Tollen's reagent. A positive result will be shown by a silver mirror or black precipitate. If there are any oils in the test tube the silver mirror will not be observed.[10]

Hard-Soft Acid Base (HSAB) Theory

Coordinate compounds may be thought of as Lewis salts or adducts which are made from a combination of Lewis acids (electron acceptors) and Lewis bases (electron donors). In a coordinate complex Lewis acids are the metal atom or the ion and the Lewis bases are the ligands. Lewis acids and bases may be further separated into two more categories: hard and soft.[11] The hard acids and bases possess a common property of being non-polarizable; that is, typically compact, highly charged and high electronegativity. On the other hand, soft acids and bases are large, diffuse and therefore polarizable. Soft acids bond with soft bases and hard acids bond with hard bases. This theory is often used to empirically predict relative stabilities of transition

metal complexes. There are tables that contain lists of the hard and soft acids and bases, as seen in Table 1.[12]

Table 1: List of common elements and compounds and their grouping in HSAB theory

Hard Lewis Acids	**Borderline Lewis Acids**	**Soft Lewis Acids**
H^+, Na^+, K^+ Be^{2+}, Mg^{2+}, Sr^{2+} Sc^{3+}, Fe^{3+}, Ln^{3+}	Fe^{2+}, Ni^{2+}, Zn^{2+}	Cu^+, Ag^+, Au^+
Hard Lewis Bases	**Borderline Lewis Bases**	**Soft Lewis Bases**
H_2O, NH_3, R_2O Cl^-, NO_3^-	Br^-	RSH, R_2S I^-, CN^-

Chelate effect

Reactions are energetically driven either through the lowering of internal energy or increasing system disorder. The overall reaction tendency to proceed to products is governed by Gibb's Free Energy (ΔG), Equation 3.

$$\Delta G^o = \Delta H^o - T\Delta S^o \qquad \boldsymbol{Eq.3}$$

The chelate effect explains the higher stability of coordination compound that contain multidentate ligands than those containing monodentate ligands. This increased stability can be explained by the entropy of the reaction. Before the ligands react with the metal, the metal is bonded to six waters (hexa(aqua) iron (III)). Water bonds are broken, and the metal then binds to the ligands. When the ligands are monodentate, one ligand

replaces one water and the number of reactants is the same as the number of products. When the ligands are bidentate one ligand replaces two water molecules which increases the number of products. When the number of products is higher than the number of reactants there is a higher entropy on the products side of the reaction which pushes the reaction forward. The entropy increases with more multidentate ligands. This can be further explained through the free energy equation (Eq. 3) The higher the entropy, the more negative Gibbs free energy is (ΔG). The calculated ΔG can then be put into Eq. 4.

$$\Delta G^o = -RTln(K) \qquad \boldsymbol{Eq.4}$$

The more negative Gibbs free energy is, the larger the positive value for the equilibrium constant, further proving that the multidentate ligands are more stable than the monodentate ligands.[12]

Visual Color Perception: CIE Lab

The International Commission on Illumination (CIE) made a way to organize color space (CIELAB). This is a uniform visual spacing that is correlated with color perception. This three-dimensional model describes lightness, hue and chroma. L* represents the lightness, a* is the redness or greenness and b* is the yellowness or blueness. The L* that is used for this model is the Munsell lightness. Munsell's lightness model considers lightness of the background before looking at that of the object. CIELAB has the L* on the z axis where the more positive the value of L*, the lighter it is. The a* value is on the x axis and when it is positive it is more red and when it is negative it is more green. The last value of b* is on the y axis, when it is positive it is more yellow and when it is negative it is more blue.[13]

Color Perception

Color is based on perception and subjective interpretation. One object may look like it is different colors to different people. This is due to different light sources, backgrounds, directions, observers, and sizes. Every individual's eyes are different, even if someone has "normal" vision and "normal" color vision. The vision of human beings can be biased towards certain colors, and can also change with age.[14]

Color Vision and Color Vision Deficiency

Humans can only perceive specific wavelengths as colors in the region known as the visible region. This includes wavelengths from 300-800 nm. The region with the longest wavelength is seen as red while the shortest is seen as violet. Light and color stimulate the retina of the human eye which making the color vision possible.[14]

People can see in color due to photoreceptors that are in the retina of the eye. The retina contains a thin layer of tissue at the back of the eye that contains millions of these photoreceptors. The photoreceptors are shaped like rods and cones. The rods and cones contain photopigment molecules that undergo a chemical change when absorbing light. When excited, a chemical change acts like a switch. Once these signals are triggered, the signals are passed from the retina to the visual parts of the brain. The rods and cones serve different purposes once excited. Rods respond to dim lights and are used for night vision. Cones are more responsive to bright light and are sensitive to long (red), medium (green), and short (blue) wavelengths of light. Another difference between the two is rods only contain one photopigment while the cones contain one of the three different photopigments. The different photopigments allow the human eye to be sensitive to different parts of the visual spectrum, allowing more rich color vision. Since

these pigments are all the same, most people have a very similar color experience. The variations of the colors experienced by human beings stem from the way the eye and the brain work together to translate light and color.[15] The central part of the retina, called the macula, is the part of the retina responsible for cones sensitivity to red, green or blue light given their different wavelength sensitivities. A person becomes color vision deficient if one of the light-sensitive pigments is missing.[16]

Color vision deficiency is when the person cannot distinguish between certain shades of color. Color vision deficiency is commonly referred to as color blindness even though there are very few people that are color blind. People that are color blind have a condition called achromatopsia. When a person has achromatopsia they can only see in black, white, and shades of gray. Color vision deficiency can range in severity from mild to severe and can be inherited or cause by illness or injury. If the deficiency is caused by an illness or injury it can affect one or both eyes. Genetically inherited deficiencies usually affect both eyes. When color vision deficiency is inherited, it is linked to the X recessive gene; therefore, men are more likely to be color vision deficient. Approximately 0.5% of women are color vision deficient while 8% of males are color vision deficient.[16]

Color vision deficiency is diagnosed through a comprehensive eye exam. In this exam, the patient is shown pseudoisochromatic plates, also known as Ishihara plates. These plates are a series of specifically designed pictures designed within colored dots. People that have normal color vision see a number, but those who are color vision deficient do not.[16]

Reflective Spectrodensitometer

Reflective spectrodensitometers are commonly used in printing. These instruments measure process colors. Process colors are cyan, magenta, yellow and black. They are also used to check the density of the color.[17]

Beer's Law

Beer's Law states that absorbance is directly proportional to the concentration of the light absorbing species. This is explained using equation 5.

$$A = \varepsilon bc \qquad \textbf{Eq. 5}$$

Where A is the absorbance, c is the concentration, b is the pathlength and ε is the molar absorptivity or extinction coefficient. The molar absorptivity is a way to characterize the substance by how much light it absorbs for a specific wavelength. This coefficient is the proportionality constant between the absorbance and the product of the pathlength and concentration, the greater epsilon the greater the absorbance given a fixed chromophore concentration and pathlength. Using Beer's Law, an absorption spectrum can be made. An absorption spectrum is a graph that shows the absorbance or molar absorptivity at various wavelengths.[18]

The atom or group known as a chromophore is an important part of this process and is responsible for the light absorption that is being analyzed. A molecule that absorbs visible light appears to have a specific color. The human eye detects the colors that are transmitted or reflected. These colors are complimentary colors to what is being absorbed.[18] A list of the colors absorbed and observed, along with the wavelengths that the colors are absorbed at can be seen in Figure 1.

Observed Color of Compound	Color of Light Absorbed	Approximate Wavelength of light Absorbed
Green	Red	700 nm
Blue-green	Orange-red	600 nm
Violet	Yellow	550 nm
Red-violet	Yellow-green	530 nm
Red	Blue-Green	500 nm
Orange	Blue	450 nm
Yellow	Violet	400 nm

Figure 1: Observed and absorbed colors with the wavelengths at which they are absorbed

High Performance Liquid Chromatography (HPLC)

HPLC is a separation method that uses high pressure to force a solvent through a closed column containing fine particles, giving a high-resolution separation. This system consists of a solvent delivery system, a sample injection valve, a high-pressure column, a detector and a computer display system. The efficiency of the column is dependent on the packed column. The smaller the stationary phase, the larger the plate number, this higher plate number results in sharper peaks. As the particle size of the stationary phase decreases the efficiency increases. The particle size of the stationary phase typically ranges from 3-5 µm.[18]

The column is commonly steel or plastic and is 5-30 cm long with a 1-5 mm diameter. All separation happens in the column, however, the column is easily degraded. To protect the column, a guard column is used. The guard column contains the same stationary phase as the main column, but it retains the fine particles and strongly adsorbed solutes before they get to the main column. In some cases, the main column is heated. This decreases the solvents viscosity, therefore reducing the pressure that is needed to have a faster flow. This can improve the resolution, while decreasing the retention times. One problem with heating the column is the stationary phase can degrade and decrease the lifetime of the column.[18]

The stationary phase is most commonly highly pure, spherical, microporous particles of silica. These silica particles are permeable to solvents. There are a few limitations to using silica. One must avoid dissolving the silica in a pH above 8, otherwise it dissolves. The other limitation is that the silica surface also contains some silanol groups (Si-OH); these groups are protonated at a pH of approximately 2-3. These can dissociate to $Si\text{-}O^-$ at a pH above 3. These protonated groups can also lead to tailing. The biggest problem is that the siloxane bond goes through hydrolysis at a pH below 2. The pH of anything analyzed on HPLC needs to be between 2 and 8.[18]

In adsorption chromatography, the solvent molecules compete with the solute molecules for a place on the stationary phase. The solvent and solutes ability to elute depends on their nature. Elution occurs when the solvent displaces the solute from the stationary phase. In normal-phase chromatography, the adsorption is on bare silica. The nature of the solvents depends on polarity. In this case, there is a polar stationary phase with a less polar solvent. The more polar the solvent, the higher its eluent strength.

Reverse-phase chromatography is when the stationary phase is nonpolar while the solvent is more polar. This has a higher eluent strength with less polar solvents. Reverse-phase chromatography is more common in HPLC due to its more favorable results, such as, the stationary phase having fewer sites that strongly adsorb the solute, leading to less tailing and it is less sensitive to polar impurities.[18]

There are two different elution methods that can be used. The first is isocratic elution, this is when a single solvent or a constant mixture of solvents is used. The other is gradient elution, this is when there are increasing amounts of solvent B added to solvent A to create a continuous gradient. Solvent A is commonly an aqueous solvent and solvent B is an organic solvent. A gradient elution leads to more separation and is faster. The solvents used are very pure HPLC grade solvents that are required to prevent the degradation of the column.[18]

In HPLC, the peaks should always be narrow and symmetric to show full separation. To maintain this symmetric band, shape the column should be washed periodically to prevent strongly adsorbed compounds from building up on the silica. If the sample solvent has a larger eluent strength than the mobile phase with a double peak may be seen. The volume before the injection is called dead volume. This needs to be accounted for by subtracting this time from the time that the peaks are seen. [18]

How steady and reproducible the flow of solvent is in the HPLC is determined by the quality of the instrument. This steady flow minimizes the noise. The injection valve has interchangeable sample loops that holds a fixed volume. When the injection valve is in the load position, a syringe is used to wash and load the loop with the fresh sample at

atmospheric pressure. When the valve is rotated 60° counterclockwise, the sample contents that are in the sample loop are injected to the column at a high pressure.

Once the sample is injected and is separated in the column, it is eluted off the column and passes through a detector. There are many different detectors that can be used. Ultraviolet detectors using a flow cell are very common. These absorb the ultraviolet or visible light. The photodiode array is used to record the spectrum for each solute that is eluted.[18]

Mass Spectrometry

The mass spectrometer characterizes molecules through the measurement of the molecular weight of each molecule. Individual molecules are converted to ions, which are then moved and manipulated by electric and magnetic fields. The main components of the mass spectrometer are the ion source, mass analyzer and the detector. The ions to be measured are under pressure, and this can be done in a few different ways. One of the common procedures is that ionization is affected by a beam of high energy electrons, the ions are then separated by accelerating and focusing the ions in the beam. This beam is bent by the external magnetic field and the ions can then be detected.[18]

High energy electrons collide with molecules, breaking apart and ionizing the molecules. The energy from the collision may cause the molecular ion to fragment into neutral pieces and smaller fragment ions. These fragments can be radical cation, carbocations, or neutral. Fragments can fragment further into smaller parts.[18]

A mass spectrum looks like a vertical bar graph, where the bars represent the ions mass-to-charge ratio (m/z). The length of the bar represents the relative abundance of the ions, with the highest abundance being 100. The relative abundance of 100 is referred to as the base peak. The ion with the highest mass in the spectrum is usually considered to be the molecular ion and the lower mass ions are usually fragments, this is assuming that the sample is a single and pure compound.[18]

Mass spectrometers separate and detect ions based on their exact masses. These ions can be detected when they have slightly different masses, such as with isotopes. Common types of mass spectrometry are quadrupole, time-of-flight, orbitraps, and ion traps.[18]

Chapter 3

Literature Review

Tuberculosis

TB is one of the world's most deadly diseases with 10.0 million cases of TB in 2017.[3] This totaled to 1.3 million deaths among HIV-negative people and 300,000 deaths from TB from HIV-positive people.[3] TB is a worldwide problem, even affecting the United States.[19]

In the US alone, there were a total of 9,105 TB cases in 2017.[3] This is often surprising to people because TB is thought to be an "old disease."[20] TB is one of the oldest infectious diseases to affect mankind. A 4,000 year old skeleton's cause of death was determine to be from TB.[21] TB was thought to be under control in the 1980s and there were predictions that it could be eliminated completely, however, it is still prevalent today. Unfortunately, TB came back stronger than ever, this time with multi-drug resistant strains that continue to spread.[22] TB came back so strongly because HIV depresses the immune system making it easier for people to contract TB. Today, TB is one of the top 10 causes of death worldwide.[3]

TB is caused by a bacterium known as *Mycobacterium Tuberculosis*.[21,23–25] This bacterium can affect a person's bones, central nervous system and other organ systems but it most commonly is seen in the lungs. *Mycobacterium Tuberculosis* is contained in aerosol droplets on the lung alveolar surfaces. This makes it easy to transmit through the air.[25,26] When a person sneezes, coughs, or spits the bacterium is propelled through

the air. One third of the world's population has latent TB because it is so easily transmitted. Latent TB is when the person is infected but is not yet ill. The active form of TB is when the person will show symptoms and start being able to spread the disease to others. Initial symptoms are very mild including chills, a cough, fever, fatigue, night sweats, no appetite, weight loss, and can progress to a bloody cough. Since the symptoms are so mild and resemble the symptoms of a common cold for months, there is a delay in the infected person seeking the medical attention that is necessary to avoid the transmission. Without the proper treatment two thirds of the people that have the active *Mycobacterium Tuberculosis* bacterium die. Properly diagnosed and treated TB is both curable and preventable.[25] The treatment for TB is most commonly a multidrug regimen, also called chemotherapy.[27]

TB has a long history. The first TB medication was invented during World War II when a biochemist by the name of Frederick Bernheim was working at Duke University in North Carolina. At the time he believed that the war was increasing the number of people that were suffering from and dying from TB. For this reason, he came up with one of the first medications to treat TB, which was an aspirin derivative. Then, in 1943 streptomycin was discovered. This was less toxic to animals than the first medication but had very similar properties. The first set of clinical trials started in 1944 and were successfully completed. The limitations to streptomycin use were that it had to be injected and the patients with long term treatment quickly grew a resistance to the medication.[28]

Patients with the drug resistant strain have a high risk for treatment failure, relapse, further resistance, or even death. TB treatment consists of first-line regimens

plus require the use of four to six antimicrobial medications over a six-month period. The most common frontline medications are: isoniazid, rifampicin, ethambutol, and streptomycin. Resistances to these medications can be caused by the anti-TB medication being used inappropriately. This is done through incorrect prescriptions provided by the health care providers, poor quality medications, patients stopping their treatment prematurely, and counterfeit medications.[24]

Counterfeit Medication

The counterfeiting of medications has become a two hundred billion dollar annual business.[29] Many counterfeit medications are made from chalk, corn starch, potato starch, paracetamol tablets and co-trimoxazole tablets.[5,30] Counterfeit medications are currently receiving international attention which has also increased public awareness.[31] WHO has estimated that 10% of the world's pharmaceutical trade and 25% of the trade in developing countries is counterfeit.[32,33]

Counterfeit drugs are medications that are deliberately and fraudulently mislabeled. This could mean that they contain no active ingredients (placebos), actively harmful ingredients, have fake packaging, or incorrect quantities of the active ingredient.[5] The counterfeit medications often have the same packaging, pill color, pill shape, pill size, marking, and the package may have an electronic barcode that the pharmacist can scan. The most harmful counterfeiting method is when the medication has a low concentration of the active ingredient because it can lead to drug resistance.[6]

Current Counterfeit Detection Methods

There are several current detection methods for counterfeit medications. These include: HPLC, TLC, mini-lab, mass spectrometry, near infrared spectroscopy, and x-ray powder diffraction.[6]

When TLC is used to identify counterfeits, it is only semiquantitative. TLC detection methods can be evaded by adding a small amount of the active ingredient to the counterfeit medication. HPLC, mass spectrometry and x-ray powder diffraction are all instrument-based detection methods. These instruments are expensive, require stable electrical power supplies, and a skilled technician to operate them.[34]

The Global Pharma Health Fund (GPHF) Minilab[35] was created with the objective to make a kit with inexpensive field tests containing simple methods for rapid drug quality verification and counterfeit medication detection. This kit contains a manual that is printed in many languages and the kit can be shipped anywhere in the world. To make these tests low-cost, the kits contain the supplies for 1,000 assays but none of the tests exceed two euros (2.35 USD). Everything that is needed to perform these tests are compacted into a single, heavy duty suitcase. Each kit includes: glassware for sample extraction, preparation, pipetting, spotting, high performance chromagraphic plates, developing chambers, detection chambers, an electronic pocket balance, UV lamps with different wavelengths, a hotplate, a ruler, pens and pencils. Even though each individual test in the kit is inexpensive, one kit with all materials ranges from 10,000-20,000 USD.[35] Additional options for products can be added to the supplies listed above.

In some parts of the world there is a limited capacity and limited number of trained chemists. There are also a limited number of reference materials that the

samples can be referenced with and compared to. The methods that are used for field detection of counterfeits in developing countries needs to be portable, relatively easy to use, sturdy, and inexpensive, while still providing the user with reliable and useful data. This is a hard set of demands to meet given the more reliable methods typically result in more expensive test kits that cannot always be used in the field.[34]

Colorimetry is mostly used to quantitatively detect counterfeit medications. Even though colorimetry is a technique that destroys the samples and gives limited information, these tests are considered invaluable to field inspectors for ease of use and reduced expense. To make colorimetry a more quantitative technique a photometer can be used.[34]

A quantitative counterfeit detection method was made for Malaria medications by South Dakota School of Mines and Rochester Institute of Technology. This is a chemical assay to verify the quantity and quality of malaria medications (CAMM). The active ingredient sesquiterpene lactone was targeted for this colorimetric assay. This test result turns purple when the active ingredient is present. This can then be compared to a blue violet Crayola crayon. If it matches the crayon, the correct quantity of the active ingredient is present. This particular test is performed in a test tube.[36]

Low-cost paper analytical devices (PADs) were made at Notre Dame to detect low-quality medications. These PADs are cards that can quickly determine if the drug contains the correct medication. They are cheap and easy to use, do not require power, chemicals, solvents, or any expensive instrumentation. They also can be deployed rapidly at a large scale when there is a suspected problem with the pharmaceutical quality. PADs have been made for 60 different pharmaceuticals.[37]

Green et al. developed methods that characterize counterfeit medications using Liquid Chromatography-Mass Spectrometry (LC-MS), raman spectroscopy, near IR spectroscopy (NIR), and desorption electrospray ionization (DESI) MS at Georgia Institute of Technology.[38–46] All of these methods, plus a handheld device were used to study the antimalarial medication, artesunate.[38,40,44] DESI-MS is a rapid and quantitative method that probes the solid surface of the artesunate and Tamiflu capsules.[39,41] DESI-MS proved the top two layers of the capsules were authentic. Raman spectroscopy was used because it can discriminate between genuine and counterfeit tablets quickly.[40,43] A handheld instrument was also developed to detect counterfeit artesunate tablets using a visual fluorescence comparison. This counterfeit detection device contains an LED light source that covers the UV to IR region and a handheld microscope. The device can be used in real time for onsite comparisons of the pharmaceutical dosage and the packaging of the medications.[44]

Conclusion

There have been tests that have been made for counterfeit malaria and diabetes medications, but it seems that there has not been much research on TB medications. The scope of reliably, verifiable medications, utilized in great quantities the world over, could be broader.

Chapter 4

Problem Statement

WHOs could use assistance with research dedicated to reducing a major communicable disease like TB, while at the same time curbing the quantity of antibiotic resistant strains of diseases. Development of a simple and low-cost colorimetric assay that quantitatively and selectively identifies the frontline TB medication, streptomycin, could serve as an authentication test. This test was targeted for point of care implementation while simultaneously meeting the requirements of simplicity, low cost, and minimal training for those with limited resources.

Streptomycin was analyzed considering its use to treat children and people who have a TB strain that is resistant to isoniazid, a drug commonly used to treat TB.[3] An established colorimetric assay was then studied and modified to improve the implementation requirements. A roadmap to aid future researchers in developing simple, low-cost, and quantitative colorimetric tests for identifying other medications will be provided.

Chapter 5

Materials and Methods

Materials

Streptomycin sulfate, iron (II) acetate 95%, iron (III) chloride hexahydrate 99+% and standard 3-hydroxy-2-methyl-4-pyrone 99% (maltol) were purchased from Acros Organics and used without further purification. Sodium hydroxide pellets and glacial acetic acid were purchased from Macron Fine Chemicals. Size 0 gelatin capsules were used in this study. All HPLC grade solvents (Acetic Acid and Methanol) were obtained from J.T. Baker.

1945 method

Colorimetric assay in accordance to Schenck with overnight reaction time for maltol formation was performed without modification.[47]

Colorimetric Assay for Streptomycin

Samples were prepared using 58 mg/milliliter stock solution of streptomycin in water. Streptomycin concentrations ranged from 0 to 145 mg/mL in each assay trial. These solutions were combined with 0.5 milliliters of 1.1 M NaOH. Water quantities of 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 milliliters were added respectively. These assay trials were heated at 55-75°C for three minutes. At the end of this time, samples appeared faintly yellow in color due to the formation of a maltol. Next, a gelatin capsule containing 0.05 grams of iron acetate was added to the solution while it was still hot and mixed to allow the capsule to dissolve. This was repeated with each of the solutions. A red color developed in each sample. One drop of each sample was added to a piece of filter

paper. When the drops dried, they were orange, the intensity varying with the different concentrations of streptomycin.

Colorimetric Assay for Streptomycin with Various Salts

Samples were prepared using 0.34 M stock solution of streptomycin in water. Streptomycin concentrations ranged from 0 to 145 mg/mL in each assay trial. These solutions were combined with 0.5 milliliters of 1.1 M NaOH. Water quantities of 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 milliliters were added, respectively. These assay trials were heated at 55-75°C for three minutes. At the end of this time, samples appeared faintly yellow in color due to the formation of a maltol. Next, 0.002 moles of copper, nickel, cobalt, and iron salts was added to the solution while it was still hot.

Tris(maltonato)iron(III) Complex Synthesis

One mole of ferric chloride was dissolved in 50 mL of distilled water and 3 mole of maltol in 50 mL of distilled water was stirred and slightly heated until dissolved. After both solutions were dissolved they were mixed and diluted.

Freeze Drying

All samples that were freeze dried into powders were frozen with liquid nitrogen and freeze dried using a Labconco FreeZone freeze drier.

UV-Vis and FTIR Spectroscopy

All UV-visible spectrums were analyzed from a Shimadzu UV-2600 UV-Vis Spectrometer. All FTIR results were recorded from a Pike Technologies GladiATR ATR-FTIR. All spectrodensiometer samples were recorded using an X Rite model 500

Spectrodensiometer. The absorbance measurements of pellets were obtained using a Coloreye 7000 Spectrophotometer.

HPLC

The HPLC instrument used was a Hewlett Packard Agilent Series 1100 with Diode Array Detector and an Agilent column 1.0 mm by 100 mm and 3.5-micron particle size. The injection size was 10.0 microliters. The flow rate was 0.5 mL/min with a mobile phase gradient starting at 100% Solvent A (1% Acetic Acid) and 0% Solvent B (Methanol) to 0% solvent A and 100% solvent B at eight minutes. A broad gradient is used to assay the product relatively quickly (several minutes). Increasing the organic solvent to 100% also ensures that there are no non-polar impurities.

Chapter 6

Results: Counterfeit Streptomycin Detection Method Development

The first streptomycin detection method was developed in 1945 by Schenk et al.[47] This method uses a base hydrolysis step to break down streptomycin into maltol. The maltol can then complex with iron using ferric salt.[47] The 1945 method was later updated to state that 5 mg of streptomycin should be tested with four milliliters of water. This should be mixed with one milliliter of one molar sodium hydroxide and heated for four minutes. The hydrolyzed products are then acidified by adding hydrochloric acid. A violet color complex is formed by the addition of iron chloride.[48] The updated method was the starting point, in this study, to develop a point of care assay that would require as little training as possible. Substantial advances and optimizations of the original Schenk streptomycin authentication method are reported.

Chemistry

The colorimetric reaction consists of the hydrolysis of streptomycin, followed by the iron chelation. The streptomycin (seen in Figure 2) first undergoes a base hydrolysis (seen in Figure 3).

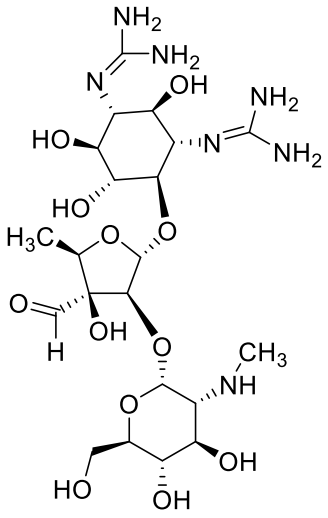


Figure 2: Streptomycin's structure.

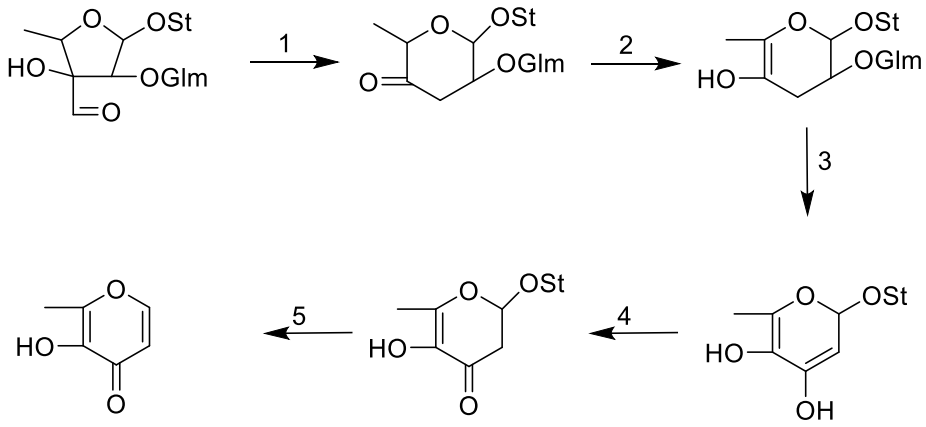


Figure 3: Hydrolysis of streptomycin to form maltol.

In Figure 3, the large streptomycin subgroups are abbreviated as St and Glm. In the first step there is a ring expansion, where carbon 2 releases its bond to carbon 4 and binds to carbon 3 instead. In step two, there is a keto-enol tautomerization. This is when the ketone group on carbon 4 transfers its electrons to form a double bond between carbon 4 and carbon 5. There is an elimination point in step three where the large Glm

streptomycin subgroup is released and a double bond is formed between carbon 2 and carbon 3. Next, there is an enol-keto tautomerization step where the double bond between carbon 2 and 3 transfers its electrons to carbon 3 and the alcohol on carbon 3, then becomes a ketone. Another elimination occurs in the final step where the ST streptomycin subgroup is released and a double bond is formed between carbon 1 and carbon 3. The maltol product can be distinguished by its yellow color. After this hydrolysis, maltol then reacts with iron acetate to form a tris(maltonato)iron (III) complex.

Heat time

The 1945 method stated that the reaction was left alone for 16 hours to form maltol through base hydrolysis. The updated method stated that the reaction had to be heated for 4 minutes in a hot water bath.[47,48] First, a maltol standard was analyzed using reserve phase HPLC (see Figure 4).

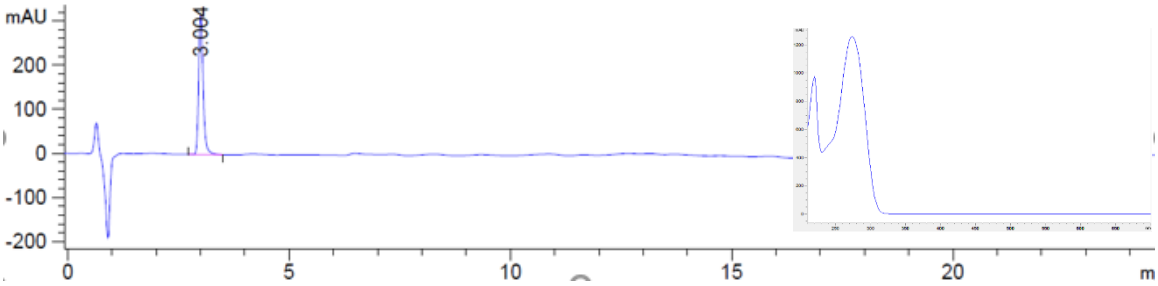


Figure 4: Chromatogram and spectrum of standard maltol.

The HPLC chromatogram of maltol after a 16-hour hydrolysis at room temperature is shown in Figure 5.

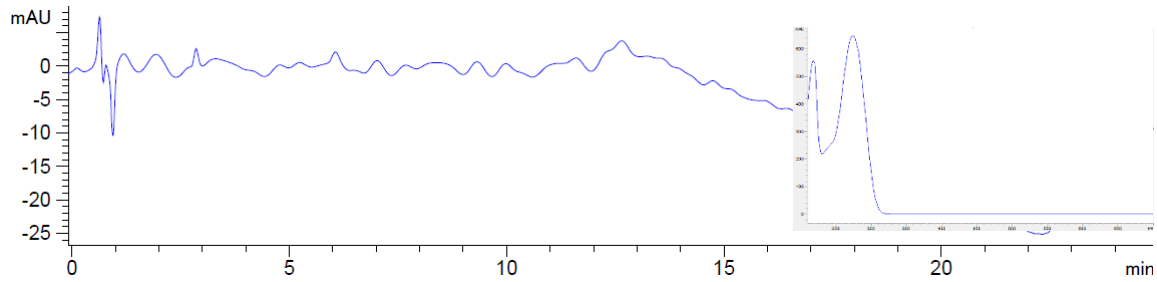


Figure 5: Chromatogram and spectrum of maltol formed through a 16-hour base hydrolysis.

The Schenck method did not resolve a peak corresponding to that obtained with a maltol standard (see Figure 5).

For this test to be a counterfeit test performed at the point of care, a much faster assay must be developed. The reaction was heated to determine if maltol could be formed faster through this process. The heating times that were studied started at ten minutes at 55-80 °C. These heat times were first studied with HPLC, and then Beer's law analysis to determine linearity. Figure 6 shows the HPLC chromatogram for the ten-minute heat time.

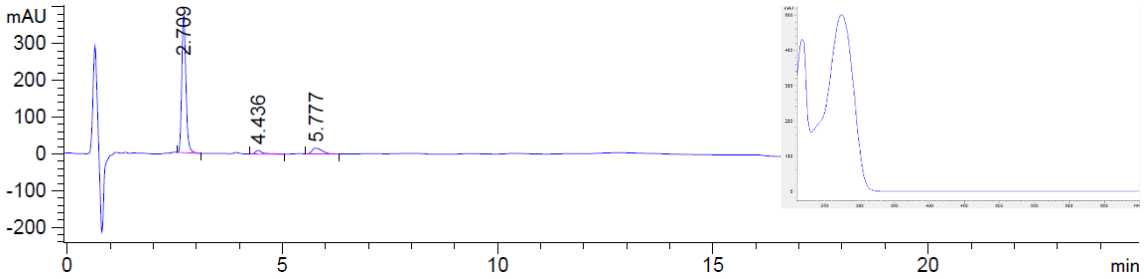


Figure 6: Chromatogram and spectrum of maltol after being heated for 10 minutes.

Heating the streptomycin solution for 10 minutes resolved a maltol peak at 2.799 minutes with a peak area of 2513.02100 mAU*s. Additional peaks, however, at 4.436 and 5.777 minutes indicate the presence of nonpolar impurities. The heat time was then decreased until these smaller peaks disappeared, and the area for the maltol peak reached a maximum thereby indicating maximum maltol formation. The optimized heat time and temperature was determined to be 3-minutes at 55 °C (Figure 7).

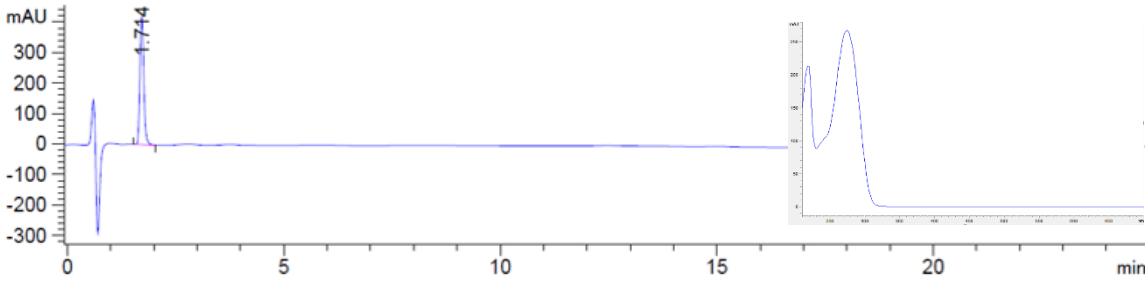


Figure 7: Chromatogram and spectrum of maltol after being heated for 3 minutes.

The HPLC peak area for the 3-minute heat time was 2605.95630 mAU*s. This suggests that there is maltol being formed with 3 minutes of heat at 55 °C with no detectable impurities. After the HPLC analysis was complete the 3-minute heat time was studied using Beer's Law analysis (see Figure 8).

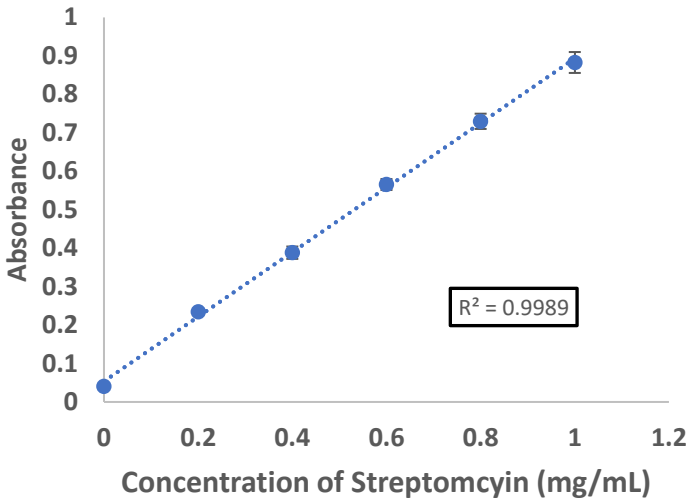


Figure 8: Beer's Law plot of iron-maltol complex heated for 3 minutes (x=3 trials).

Base Concentration Optimization

Sodium hydroxide concentration was varied to determine the optimal amount required for the experiment. The concentration of sodium hydroxide was decreased from the starting concentration of 2 M and was analyzed using reverse phase HPLC (see Figure 9).

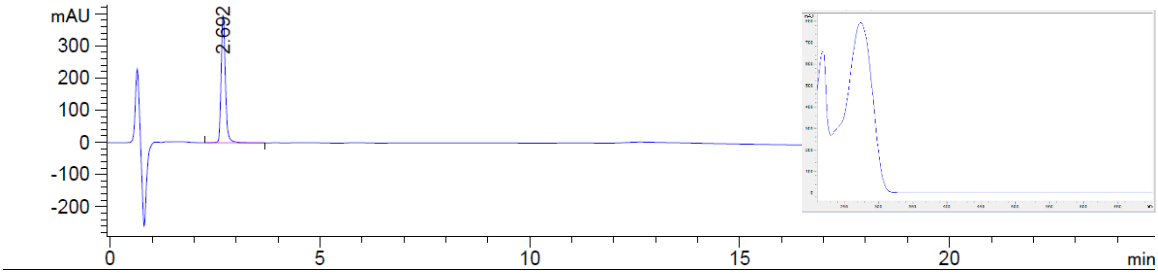


Figure 9: Chromatogram and spectrum of maltol when streptomycin is degraded with 2.0 M NaOH.

The peak at 2.6 minutes had an area of 2814.52246 mAU*s when 1 millimole of NaOH was used. After conducting a range of base concentrations from 0.4 to 1 millimole NaOH the optimal concentration was determined to be 0.55 millimolar NaOH with a curve area of 5381.96338 mAU*s, as shown in Figure 10.

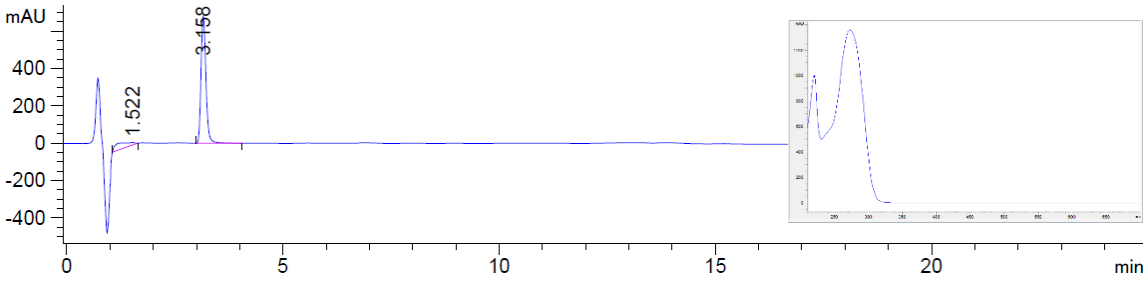


Figure 10: Chromatogram and spectrum of maltol when streptomycin is degraded with 0.55 millimolar NaOH.

At 0.55 millimole of NaOH, nearly two times the maltol amount was formed. Maltol yield formation was found to decrease when the base concentration was less than 0.55 millimole. The optimized base concentration resulted in pH reduction from 12.71 to 11.58.

Hydrochloric Acid Concentration Optimization

The 1945 method did not mention using hydrochloric acid and the updated Pharmacopeia method stated that dilute hydrochloric acid was used in excess.[47,48] In the Pharmacopeia method, 4 mL of 2.0 M (eight millimoles) hydrochloric acid was used because the reaction requires acidic conditions for iron complexation.[49] A pH of 0.62 was measured under these conditions. For someone with little training to be able to perform this test, the acidification process needed to be optimized. Iron hydroxide can

easily be visually identified as it forms an orange precipitate when the pH is adjusted to approximately five or greater. By decreasing the HCl concentration to 0.7 M HCl, the iron maltol complex was still found to be linear (see Figure 11). A weaker acidic condition was then analyzed to improve the safety of the field assay.

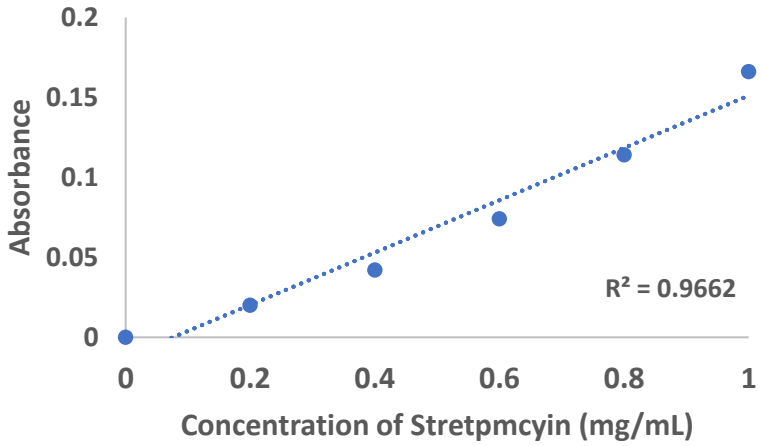


Figure 11: Beer's Law plot of the current method using 0.7 M HCl.

Acetic Acid Concentration Optimization

HCl concentration was decreased substantially. To continue to make this a safer method, a weak acid (acetic acid) was analyzed using Beer's Law (Figure 12).

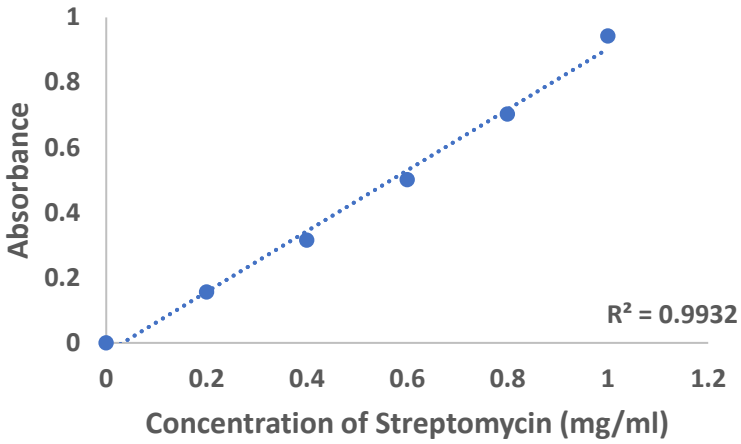


Figure 12: Beer's Law plot of the current method with acetic acid instead of hydrochloric acid.

Using acetic acid as the acid source provides critical improvements to the assay. As described, pH plays a key role in determining the final complex structure. At a pH of 1-2, as in the case when HCl was used, the preferred tris(malonato)iron(III) complex was not formed. Rather, an iron complexed with a mixture of chlorine and water ligands is proposed. Such an undefined mixture would have a profound impact on the linearity of a Beer's Law plot. As shown in Figure 11, a non-linear Beer's Law plot was obtained at the low pH assay condition. With acetic acid as the acid source, however, an assay pH of 3.5 was measured. At this more moderate pH, the tris(malonato)iron(III) complex was exclusively formed. A singular molecular species would be confirmed by a linear Beer's Law analysis. As shown in Figure 12, the novel assay of this study, using acetic acid as the acid source, produced a highly linear (R^2=0.9932) Beer's Law plot.

In addition to improved assay linearity, a five-fold increase in absorbance was obtained, per mole streptomycin hydrolyzed, with the improved assay procedure of this study. Two reasons are offered for this higher assay sensitivity. One, the preferred complex, tris(malonato)iron(III) complex, is formed at high yield without interfering impurities or mixtures. Secondly, there was more maltol being formed per mole streptomycin in the base hydrolysis step, as seen in the HPLC results.

Acetic acid, therefore, works well with the current reaction to adjust the pH enough to form a color complex with maltol and is much safer to use for someone that is untrained. White vinegar contains 0.8 M acetic acid and this reaction contained 1.0 M acetic acid, making this a safer reaction than selecting a caustic HCl acid.

HCl vs Acetic Acid Color Complex

When the different acid sources were visually compared, two contrasting colors were observed. The complex formed when hydrochloric acid was used was purple (Figure 13) but when acetic acid was used the complex was red (Figure 14).

Figure 13: pH 0.62 iron-maltol solutions with increasing streptomcyin concentrations made with hydrochloric acid.

Streptomycin concentrations range from 0 (left) to 1 (right) mg/mL.

Figure 14: pH 3.51 iron-maltol solutions with increasing streptomycin concentrations made with acetic acid.

Streptomycin concentrations range from 0 (left) to 1 (right) mg/mL.

Red is the color that is described for the iron-maltol complex.[46] Previous work stated that the same iron-maltol complex, formed with the maltol made from the base hydrolysis of streptomycin is purple when complexed with iron.[47,49] These inconsistencies led to a deeper study of the complexes. The results of this study will be discussed in Chapter 7.

Metal Salt Analysis

Previous work stated that the iron should be used to form a complex with maltol yielding a violet color.[47–50] It was determined that maltol can complex with other metals to form different color complexes. These were all analyzed with and without acid, and for visual analysis, using three different lighting conditions (indoor, outdoor direct and indirect lighting). Copper, nickel, cobalt, and iron salts were all studied. Only copper and iron complexes formed a visual color complex (see Figures 15 and 16). Complementary spectroscopic analysis was conducted to assess assay linearity (see Figure 17 and 18).

Figure 15: Iron-maltol complexes from 0 (left) to 1 (right) molar streptomycin containing 0.003 M iron.

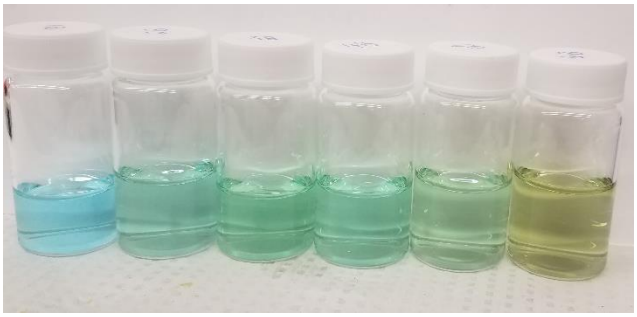


Figure 16: Copper-maltol complexes from 0 (left) to 1 (right) molar streptomcyin containing 0.003 M copper.

The Beer's Law analysis of the iron-maltol complex can be seen in Figure 17 and the copper-maltol complex can be seen in Figure 18.

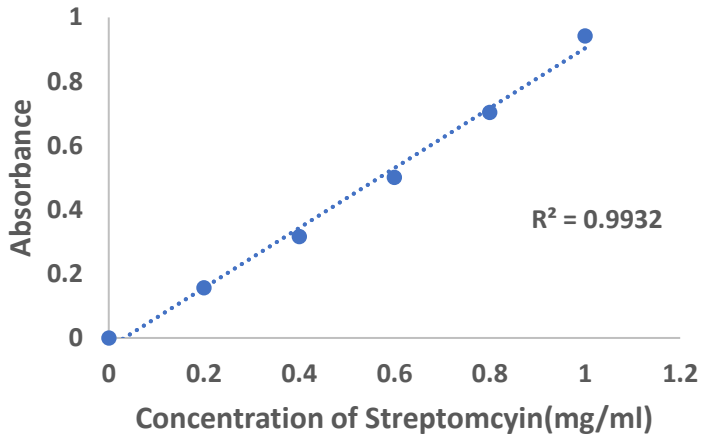


Figure 17: Beer's Law analysis of iron-maltol complex.

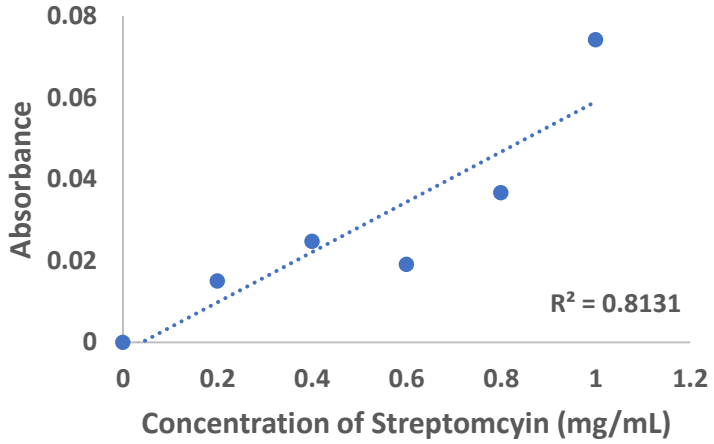


Figure 18: Beer's Law analysis of copper-maltol complex.

Figures 18 and 19 show that the iron-maltol complex was much more linear with an R^2 value of 0.9932 compared to the copper-maltol's complexes R^2 of 0.8131. When metal and maltol are compared at equal concentrations, the iron complex forms a response ten times greater than that found with copper. From these results, iron was determined to be the best metal to use for this test.

Iron Acetate

To make a test method that requires little-to-no-training iron acetate was studied. The use of iron acetate decreased the amount of solution preparation required. Instead of making a 0.37 M ferric chloride solution and having to measure and pipet both the ferric chloride and acetic acid solutions, iron acetate was investigated.

Paper Test Using Iron Acetate Assay Procedure

The iron acetate reaction results were very dark making it hard to distinguish between the different solutions. The solutions also contained an undesirable precipitate from the excess iron acetate. A paper test was developed that would provide two advantages: one, droplet deposition on paper would result in further separation of the reaction product from the excess iron acetate through chromatographic diffusion (see Figure 19); and two, the thinner optical pathlength and white background would enhance visual discrimination of color differences. The formed color patches could be analyzed using a reflective spectrodensitometer to determine the optical density, and reaction linearity.

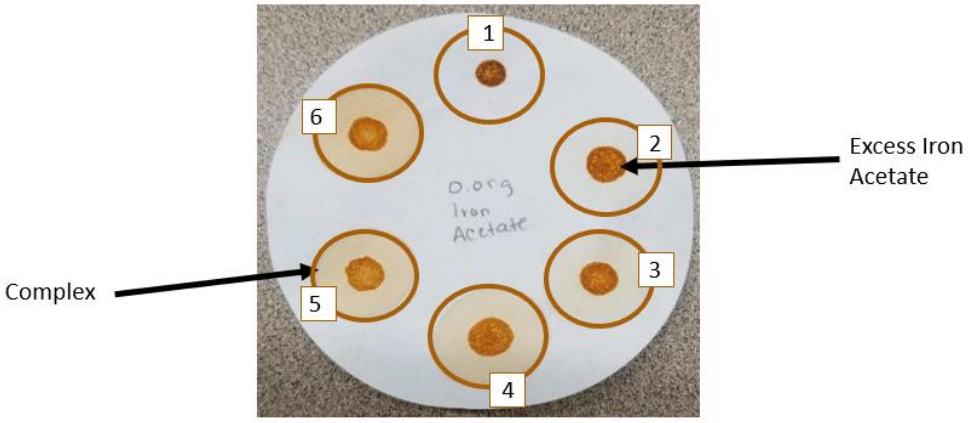


Figure 19: Paper assay ranging from 0 to 148 mg/mL streptomycin

These samples were studied in triplicate to test for linearity, the results can be seen in Figure 20.

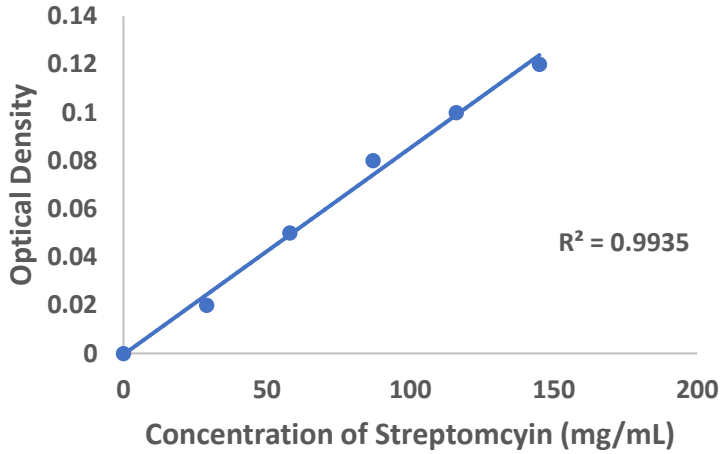


Figure 20: Beer's law analysis of iron-maltol complex on the paper test when solid iron acetate was added to the solution.

The paper test made the method much simpler. Due to the iron acetate precipitate the paper test was used and analyzed using a spectrodensitometer for a Beer's Law analysis. The amounts of iron acetate were studied in the range of 0.01 to 0.2 millimolar and the amount that led to the most linear results was determined to be the optimal amount of iron acetate. This amount was determined to be 0.1 millimolar iron acetate. Using the solid iron acetate instead of hydrochloric acid changed the pH of the complex from 1.16 to 7.62.

Capsules

To make the iron acetate addition easier and to facilitate a fool-proof method for point of care use, the iron acetate was added to the solution via a gelatin capsule. Gelatin capsules were chosen for safety and ease eliminating the need for measurement of iron acetate and contact with chemicals. The solution was heated making the gelatin dissolve in the reaction. The use of gelatin capsules was repeated in triplicate and the optical density was studied. The Beer's Law analysis, when the capsules were used, can be seen in Figure 21.

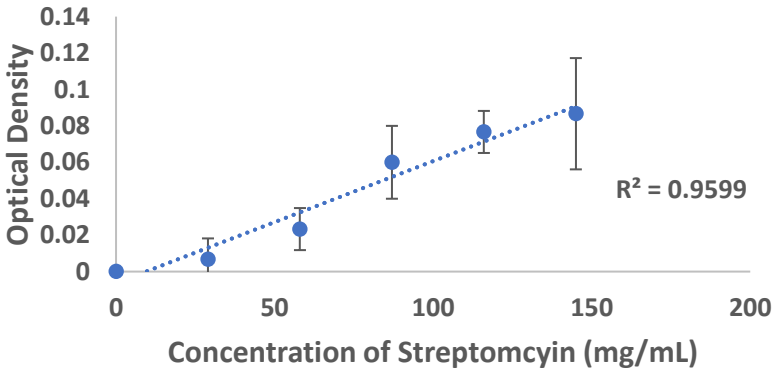


Figure 21: Beer's Law analysis of the iron-maltol complex when gelatin capsules are used to add the iron acetate.

Testing Common Counterfeit

Counterfeit detection was the purpose for developing this assay. Such an assay may positively detect the analyte, or positively detect a counterfeit. Detection of the positive analyte, in purity and dose is preferred. The improved streptomycin test was compared to many household medications and chalk to make sure the same color was not produced from any of those reactions from readily available substitutions commonly used in counterfeit medications. The medications included: magnesium sulfate, generic allergy tablets, Triphala gold, Aleve, Ibuprofen, and Claritin. A paper test (Figure 22) was used to compare the results of all of the different medications. This test included six different concentrations of streptomycin.

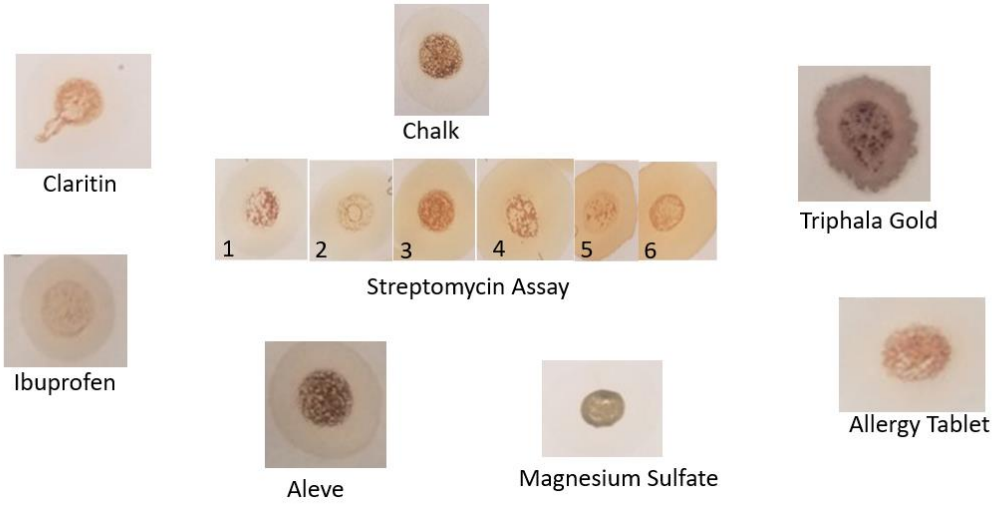


Figure 22: Counterfeit detection test used on common counterfeits.

The inner ring is the excess iron acetate and the outer ring is the color complex. The excess acetate in all the samples was orange or brown like that of the complex samples. All the medication samples and chalk are colorless with the exception of Triphala Gold; not at all matching the samples with streptomycin. Triphala Gold in this reaction leads to a purple product; this is most likely attributed to an iron-carbohydrate complex. Though Triphala Gold formed a color complex with iron, it has a distinctively different visual color. It is important that non-streptomycin samples do not give the same color results as streptomycin to detect when a “filler” is used in samples.

Detection of Counterfeit Medications

If the authentication test can be manipulated to give positive results the counterfeiters will do so (assuming the manipulation is neither too expensive nor requires significant chemistry knowledge). To avoid the test being manipulated by adding maltol to the counterfeits, this authentication test can be performed directly on streptomycin. If iron-acetate is added to streptomycin in water and the paper test is performed the results will be hexa(aqua)iron (III). This will give an orange precipitate for the inner circle and the outer circle where the iron-maltol complex is seen, will be colorless given there is no maltol present. If the test is performed with and without the hydrolysis step and the results show that the test is negative before the hydrolysis and positive after the hydrolysis, it can be determined that the streptomycin is authentic.

Chapter 7

Results: Tris(maltonato)Iron (III) Characterization

During the counterfeit detection test development, solution color from the 1945 method did not match those from the optimized test from this study, as well as, from tris(maltonato)iron (IIII) complex characterization reports.[46,47] The 1945 Schenck method described a complex derived from streptomycin hydrolysis, and a reported 2007 method from Pizzanelli et al.[46] used maltol extracted from plants. These complexes varied in visual color -purple in the 1945 method compared with a red color from the Pizzanelli study. In addition to a significant difference in pH conditions, maltol formation was not obtained when following the 1945 method, as seen in the HPLC results in Chapter 6. Counterfeit test validation requires that the products of any chemical test be robustly and verifiably determined. Detailed, chemical and spectroscopic, analyses were conducted to resolve the discrepancies between the two methods.

pH Study

Samples were made with a mole ratio of 1:3 ($FeCl_3$: Maltol) ranging in pH from 1-13 using sodium hydroxide or hydrochloric acid (Figure 23).

Figure 23: Range of standard iron (III) maltol complexes at different pH's adjusted with HCl and NaOH

A purple color was obtained at pH 2. At pH 3 and 4, a red color was formed as described in the Pizzanelli method. In previous reported studies, the predominate iron-maltol complex composition was found to be pH dependent.[51] A 1:1, iron: maltol complex, maltonatoiron (III), was determined to be stable at pH 1 and was violet in color. A red 1:2 complex, bis(maltonato)iron (III), is formed at pH 3, and a yellow 1:3 complex, tris(maltonato)iron(III) complex forms at pH 6.[51] Under the conditions of this study, a red complex was formed in the range of pH 5-9. A yellow complex was not formed until about pH 10. The change in color from red to yellow will be explained in more detail in this chapter. The rapid transition, however, at pH 9-10, corresponds with the maltol pKa (pKa = 8.6). Spectral changes[50] have been observed for maltol in this pH range. Changes in electronic chargstate would be expected to shift the ligand field splitting on iron (III), and influence solution color. In this case, a stronger field would be exerted by maltol above pH 9 as evidenced by a shift to a yellow solution color. Also, evident in Figure 1, was the absence of any iron hydroxide precipitate beyond pH 5.

The chelate effect explains why there are so many colors and complexes (see Figure 24).

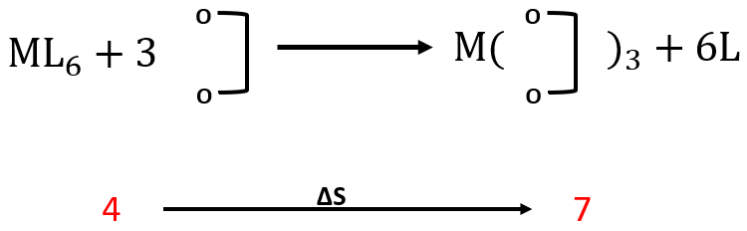


Figure 24: An example of how the chelate effect influences complex stability.

In Figure 24, a six coordinate metal complex reacts with three bidentate ligands. The product yields the metal chelated to three bidentate ligands, leaving six free monodentate ligands. Figure 24 contains four reactants and seven products. The chelate effect states that such a reaction is entropically driven towards the products.

Chloride and Non-Chloride Containing Acid Analysis

While making the detection method assay the acid was changed from hydrochloric acid to acetic acid. During this transformation, the color changed from purple to red. It is known that polymorphic iron chloride is purple and there was excess chloride when chloride containing acids were used. Chloride and non-chloride containing acids were analyzed to determine if this polymorphic iron chloride was being formed.

Samples were made of a 1:3 ($FeCl_3$:maltol). These were analyzed by bringing the color of the reaction to pH 2 with hydrochloric acid, then back pH 4 with sodium hydroxide and pH 2 with different acids (Figure 25 and 26).

Figure 25: A tris(maltonato)iron(III) solution was adjusted to pH 2 using HCl (left), adjusted to pH 4 with NaOH (middle), the same sample adjusted back to pH 2 with HCl (right).

Figure 26: A tris(maltonato)iron(III) solution was adjusted to pH 2 using HCl (left), adjusted to pH 4 with NaOH (middle), the same sample adjusted back to pH 2 with phosphoric acid (right).

These experiments confirm the pH dependent form of the iron complex. In this study, however, the novel addition of a non-halide containing acid revealed that there is more explanation required to justify the purple complex result.

The spectrochemical series shows that halides are weak field ligands. Weak field ligands have a small crystal field splitting energy; and therefore, have a red shift in absorbance. This red shift causes the complex with halides such as chloride to be purple. In the spectrochemical series the oxalate is very similar to maltol. This has a stronger field than the halides, causing a larger crystal field splitting energy and a blue shift. The blue shift in absorbance causes the solution to be red. Water is a stronger field ligand than the maltol causing it to have an even larger crystal field splitting and shift more towards the blue explaining the yellow iron hexahydrate. Using the molecular orbital diagram for octahedral iron complexes further contributes to understanding the result of a purple complex (Figure 27).

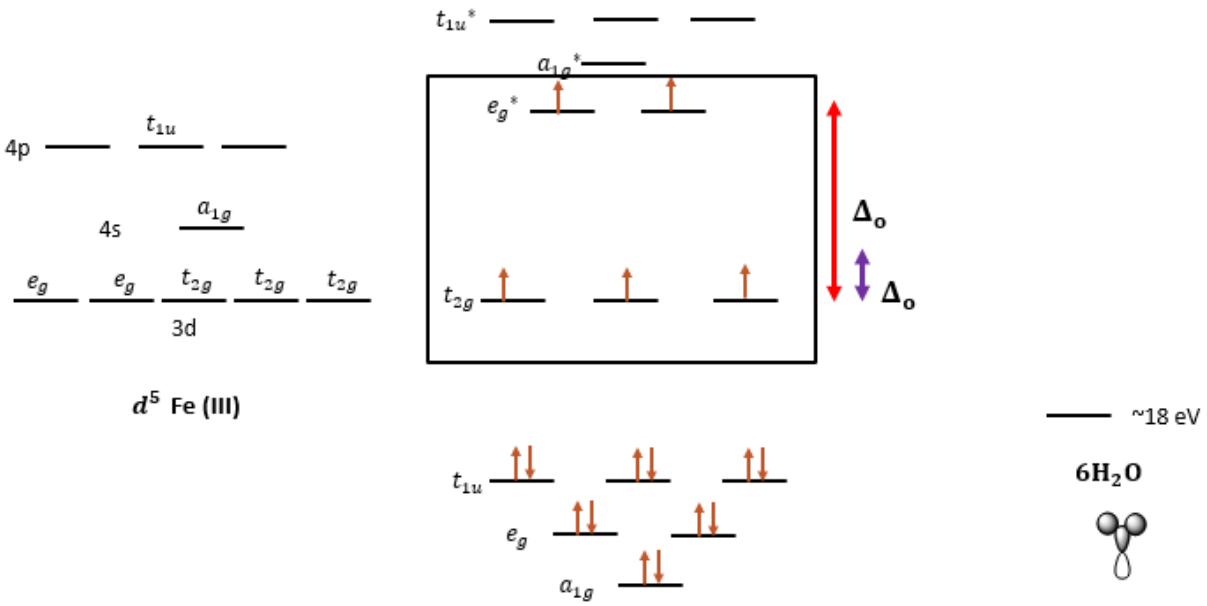


Figure 27: Molecular orbital diagram for octahedral iron complexes

The colors originate from the d orbitals that are centered on the iron. These orbitals originate in the d box. The separation in energy is related to the absorbance of color. In this example a strong field ligand combined with a large crystal field splitting energy results in a red solution. If the t_{2g} and e_g^* were closer together, for example with the halides, then the crystal field splitting energy would decrease causing the solution to appear purple.

Any complexes formed that were made using an acid containing chloride sources turned purple and the samples that did not contain a chloride source retained red color. This led to the conclusion that the purple complex contains chloride ligands. The spectrochemical series explains how the chlorides are bonding to the iron to form a polymorphic iron chloride while the red reaction shows the maltol bonded to the iron.

Hydration State of Iron (III) Chloride: A Solid-State Pellet Analysis

Anhydrous and hexahydrate ferric chloride pellets were fabricated and analyzed using UV-visible spectroscopy reflection and FTIR analysis to determine if the solution-derived purple iron complex could compare with pure chemical standards. The absorbance vs wavelength (nm) of the ferric chloride hexahydrate (blue) and the anhydrous ferric chloride pellet (orange) was obtained (Figure 28).

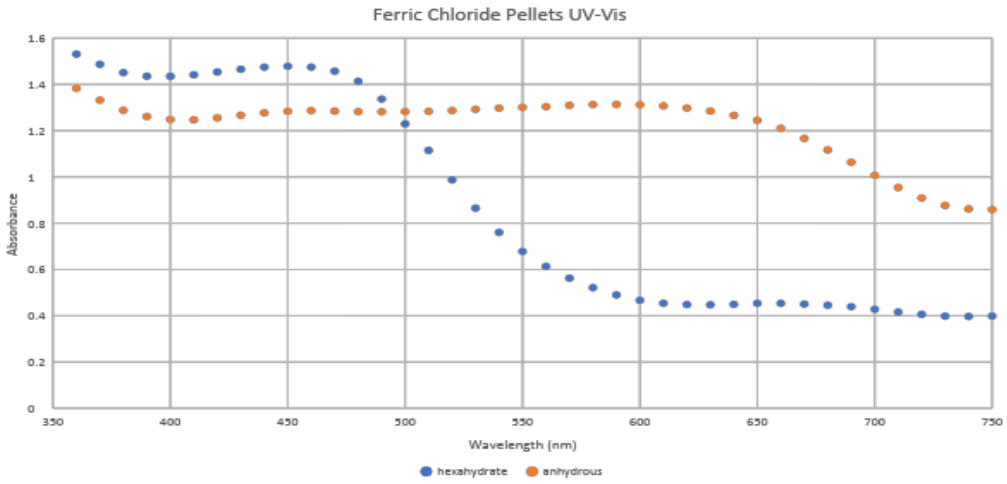


Figure 28: Reflective absorption spectrum of ferric chloride hexahydrate (blue) and anhydrous ferric chloride (orange)

The results show that the hexahydrate was yellow, and the anhydrous ferric chloride was almost black. The FTIR of these chlorides (Figure 29 and 30) allow for further analysis.

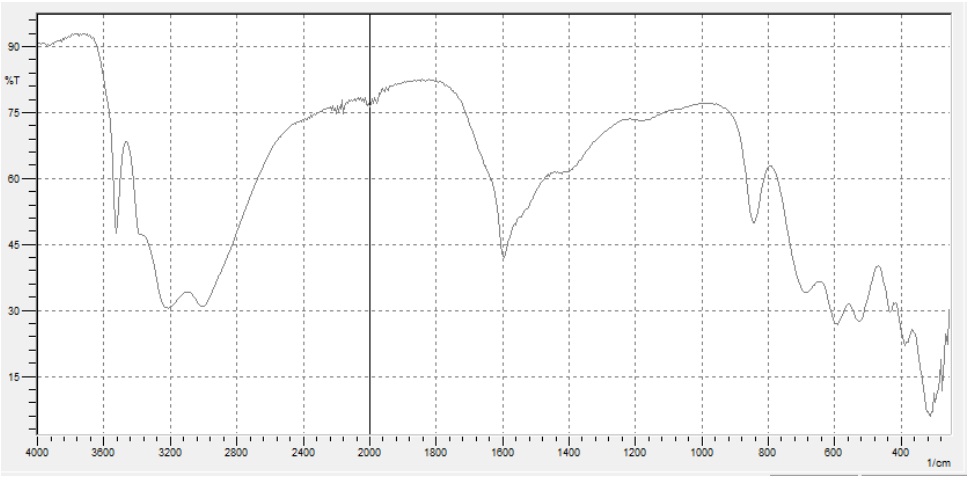


Figure 29: FTIR spectrum of ferric chloride hexahydrate

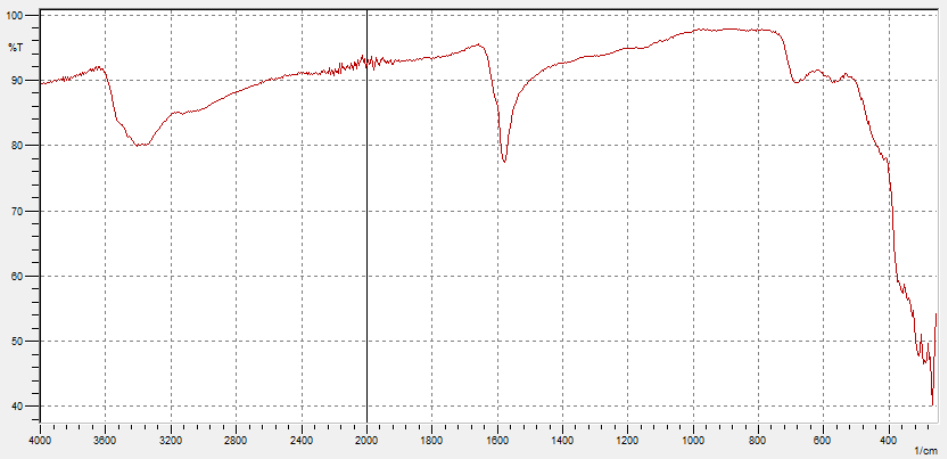


Figure 30: FTIR spectrum of anhydrous ferric chloride

While the anhydrous ferric chloride pellets were exposed to air, they slowly turned yellow. As discussed, using HSAB theory, iron is a hard acid and will complex with water, a hard base. Water is a harder base than chloride ions, therefore iron (III) will bond to water before it will bond to chloride. Chlorine displacement by water ligands led to a small water peak in the anhydrous ferric chloride FTIR results (see Figure 30).The FTIR data confirms that the ferric chloride hexahydrate contains water and the anhydrous ferric chloride does not, causing the yellow and purple colors, respectively (see Figure 28).

Mole ratios UV-Visible Spectra

The 1945 method and different mole ratios of maltol and ferric chloride were analyzed at pH 2 (Figure 31) and 5 (Figure 32) with UV-visible spectroscopy and compared to Gerard et al.[52]

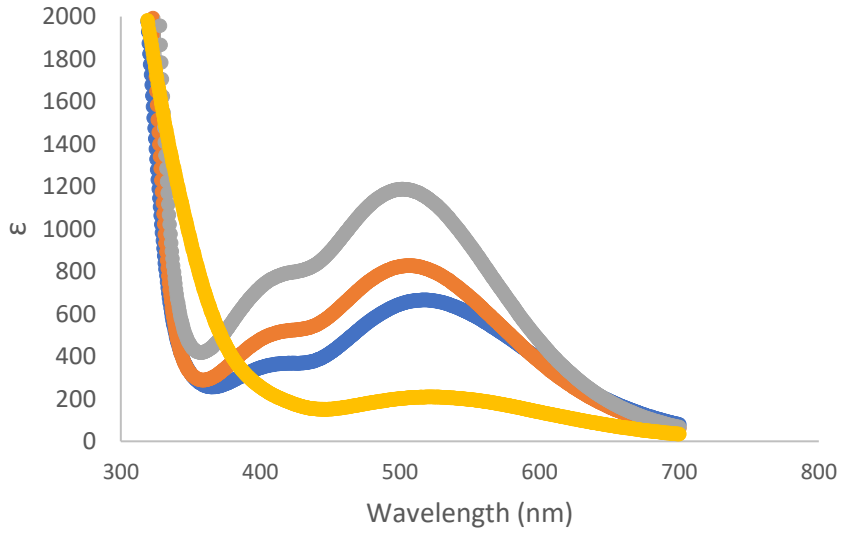


Figure 31: UV-visible Spectrum of the 1945 method at pH 2 (yellow) and mole ratios 1:1 (blue), 1:2 (orange), and 1:3 (gray) of ferric chloride: maltol at pH 2.

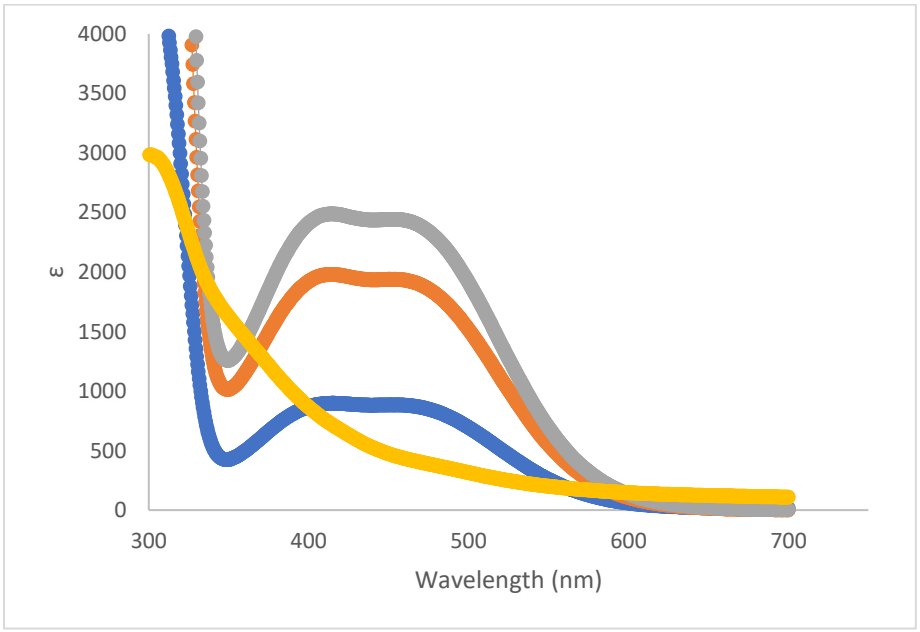


Figure 32: UV-visible Spectrum of the 1945 method at pH 5 (yellow) and mole ratios 1:1 (blue), 1:2 (orange), and 1:3 (gray) of ferric chloride: maltol at pH 5

According to Gerard et al., all the absorbance spectra for all the mole ratio mixtures at pH 2 (Figure 31) are making the 1:1 complex. At the same time all the mole ratios at pH 5 (Figure 32) with a 1:3 complexation were being formed. The 1945 samples do not match any of these complexes of the absorbance spectra, further proving that this method does not make the complex that was reported. The spectral profiles shown in Figures 31 and 32 can be explained using a spectrochemical series description.

In reference to the spectrochemical series, halides are weak field ligands. Oxalates and then water have the strongest field of the three ligand types. When the ligands are weak field ligands they have a small crystal field splitting energy and exhibit a red spectral shift.11 For stronger field ligands, larger crystal field splitting causes spectral shifting toward blue wavelengths. Oxalates are comparable to maltol in field splitting strength. Tris(malonato)iron(III), therefore, has a red color in aqueous solution, and hexa(aqua)iron(III) exhibits a yellow color in aqueous solution. The chlorides have the weakest field causing a red spectral shift, giving a purple color.

HSAB theory provides that iron (III) is a hard acid and therefore will bond with hard bases. It is originally bound to water; water is a hard base. It will only bond to the weaker base (chloride) when chloride is in excess to form a ferric chloride complex. Although maltol is a softer base than the water or chlorine ligand, maltol is a bidentate ligand. The iron-maltol reaction, therefore, is entropically driven, following the chelate effect. The chelate effect states that when a metal bonds to a multidentate ligand the reaction is entropically driven, favoring disorder.

FTIR of the Hydrolysis of Streptomcyin

Standard maltol (Figure 33), the 1945 method (Figure 34) and a standard 1:3 mole ratio (Figure 35).

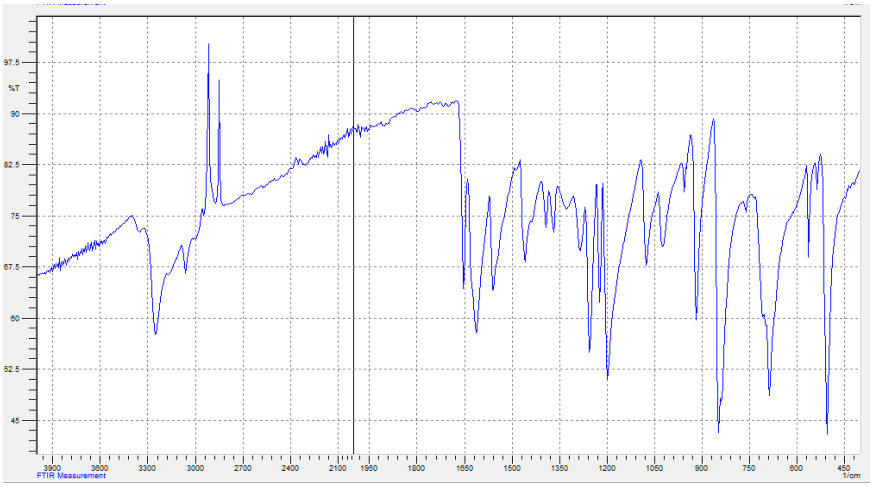


Figure 33: FTIR Spectrum of standard maltol

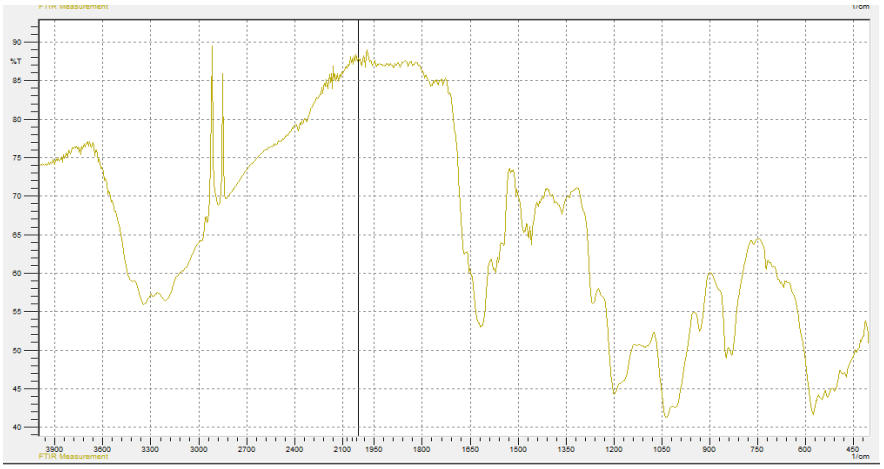


Figure 34: FTIR Spectrum of the 1945 method

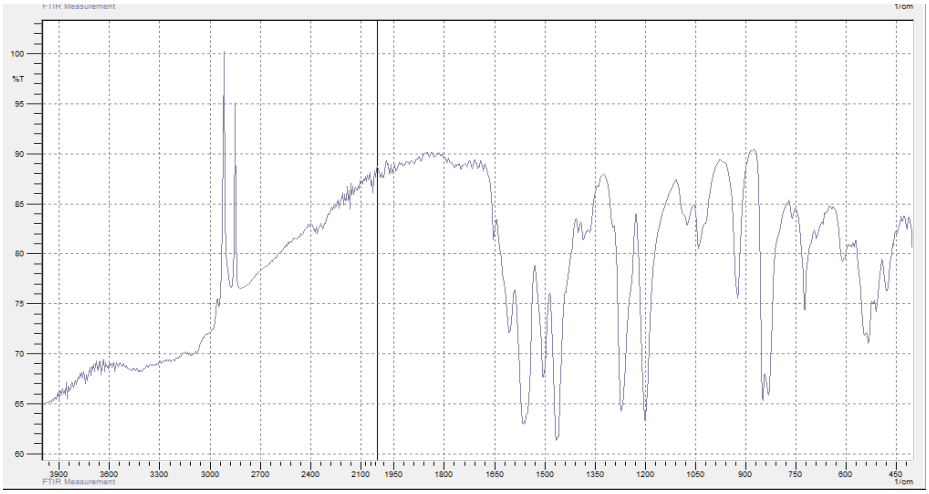


Figure 35: FTIR of the 1:3 mole ratio (ferric chloride hexahydrate: maltol)

When comparing these spectra, it can be determined that the 1945 method does not contain maltol. This confirms the HPLC results discussed in Chapter 6 that determined that the 1945 method was not making maltol during the hydrolysis.

Chapter 8

Summary and Conclusion

Summary

A colorimetric assay for the TB medication streptomycin was developed. This assay is rapid, safe, easy, low tech, requires little electricity and can be performed near the point of care. Counterfeit medications need to be studied as close to the point of care as possible by someone that is untrained anywhere in the world.

This colorimetric assay is something that is simple, allowing someone that is untrained to be able to do the analysis. Economically the test enhances the likelihood of it being performed in developing countries and poor areas in developed countries. This test is completed through the comparison of colors and therefore would be difficult for someone to perform if they are color vision deficient. One way to avoid this problem is having a female perform the test or at least the color comparison because they are much less likely to be color vision deficient.

The 1945 streptomycin detection test that was used to develop the new colorimetric assay was then invalidated through a comparative study using FTIR, UV-visible Spectroscopy, and HPLC. These technological processes were also used to characterize the improved method.

Recommendations for Further Investigations

This streptomycin assay can be improved upon by adding sodium hydroxide as a solid instead of making a sodium hydroxide solution. This could make the reaction even easier for someone that is untrained to perform. Using a specified dose amount, a print

could be made of the drop. The print can then be used for visual comparison without instrumentation to this test, yet, the detection method is still quantitative.

At this point in the assay's simplification the print making would need to be tested with a focus group. Having a focus group, comprised of people having taken no more than one semester of chemistry to perform this colorimetric assay and the visual test. The focus group would serve two purposes. First, it would show how simple this test is for someone that is not trained in chemistry and where it might need to be simplified even further in the future to make this something that most everyone can do. Secondly, it will help determine who would need to make the visual comparison given men more often than women have a color vision deficiency. Finally, statistical analysis needs to be completed to determine if the test is the same color every time.

There are many different directions that the drug quality assurance (DQA) project can go in. TB can have multidrug resistant strains.[3,24,25] To treat and/or avoid multidrug resistant TB chemotherapy is performed. Chemotherapy is when multiple medications are used as a treatment method. If TB streptomycin medication could be counterfeit, so could other medications used in conjunction with it. The other TB medications will need detection tests to fully safeguard users.[34,53] Therefore the other TB medications need to have counterfeit detection tests for them as well. Some of the frontline TB medications that need to be studied are isoniazid, rifampicin, pyrazinamide and ethambutol among others.

DQA methods are important for all medications but especially important for commonly counterfeit medications. Unfortunately, since counterfeiting medications are a two hundred billion dollar industry and still growing, there are many medications that are

commonly counterfeit.[29] The most common types of counterfeit medications include: cardiovascular medications, antibiotics, cancer medications, ED medications, medications for chronic ailments (HIV, diabetes and Alzheimer), birth control, painkillers and weight loss medicines and psychiatric medications.[54] From this list it becomes clear that no medication without a quantitative counterfeit detection method is completely safe. This is a huge worldwide problem with up to 50% of pharmaceuticals being counterfeit.[55]

Colorimetric detection can be used to detect trace amounts of dangerous substances as well. An example would be date rape drugs. Date rape drugs are commonly put into drinks of people at bars or parties creating great concern in social situations. They are currently being studied, by many groups, with some products already currently on the market. Drink Safe Test Coasters is one example.[56] There are additional test methods made but are awaiting FDA approval. These tests include: nail polish, straws, cups that change colors and paper chips.[57]

Considering all the products mentioned above for date rape and/or robbery detection there remains the influence of sociological and economic factors. The methods that are currently being used are not methods that everyone would be able to easily use. The coasters, cups, straw and paper chips are all methods requiring the consumer to bring the test method with them to the bar. This will be an inconvenience to the consumer but they would not be able to bring just one. These products can only be used one time and therefore the consumer would have to bring this in excess. Carrying multiple tests are easier for women than men because most women bring a purse or bag while most men do not and therefore would have nowhere to store these items while at the bar. The bars will not pay more money for coasters, straws and paper chips that are

not reusable. Up to 2000 people were drugged with date rape drugs and then robbed in the UK alone. When date rape drugs are used in robberies, men are typically the target.[58] The most convenient detection method that has been developed is nail polish, however, most of the men being drugged and robbed are not going to wear the nail polish to detect these drugs and therefore there is a need for another way to detect these drugs. Another problem with the current detection methods is that the drugs that these detection methods analyze are ketamine and GHB. Flunitrazepam is often overlooked. A separate test to detect flunitrazepam would have to be bought to test for all three common date rape drugs. If there was a detection method for all three of these common date rape drugs that can be used by anyone and is compact and easy to use it would greatly benefit society.

Colorimetric detection could also be applied to detecting gunshot or explosive residues in small quantities for efficiency in the field of law enforcement. The current detection method for gunshot and explosive residues uses analytical techniques such as a scanning electron microscope.[59] There have been colorimetric methods that have been attempted but are not currently being used because these colorimetric methods did not detect trace amounts of the residues.[59] Making a colorimetric assay that could detect trace amounts of gunshot or explosive residues earlier in the line of duty would help officers detain suspects earlier, potentially keeping more people safe.

These colorimetric assays can be useful to detect many different substances for a worldwide impact. The consequences of counterfeit medications implicate a greater need for science to study and develop manageable products to safeguard recipients, reduce drug resistant strains of superbugs, and keeping in check health care costs.

Lastly, criminal behavior can be subverted or minimized with thoughtful chemical detection methods.

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