# Utilizing Electron Spin Resonance (ESR) spectroscopy to analyze amodiaquine

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## Abstract

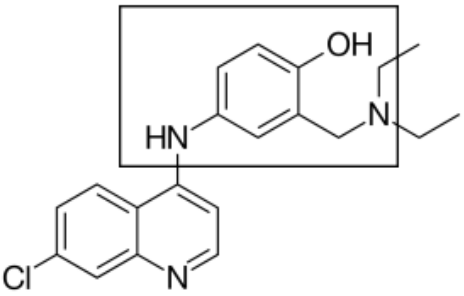
Amodiaquine is a drug that can treat malarial infections. However, this molecule is known to cause liver damage potentially attributed to phenoxyl radicals. These phenoxyl radicals are shared with an analogous compound of amodiaquine, acetaminophen, commonly known as Tylenol®. This project aims to produce a resolved ESR spectrum utilizing a variety of samples of amodiaquine to compare coupling constants to other molecules to further understand the impact of amodiaquine’s phenoxyl radicals on the liver.

## Introduction

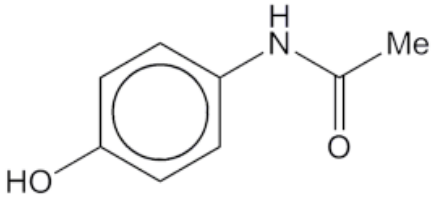
Malaria is a disease that relies on mosquito vectors to transport infectious protozoa from the *Plasmodium* genus [1]. This disease is quite prevalent, having infected 247 million people and causing 619,000 deaths in 2021 alone [2]. Most of these cases occurred in Africa due to the prevalence of the *P. falciparum* and *P. vivax* species. The symptoms of this disease are unassuming at first, presenting much like the flu with fever, chills, and headaches but can quickly become deadly. Despite this, treatments exist to prevent and cure a malarial infection.

One such treatment is amodiaquine, a synthetic aminoquinoline used to treat a malarial infection after its onset [3]. The reason being is that amodiaquine is known to be both hepatotoxic when administered in standard doses, yet effective at treating chloroquine resistant malaria strains. Similarly, an analogous compound (See Fig. 1 & 2) of amodiaquine, acetaminophen, also has been shown to be hepatotoxic in large doses [4]. It is hypothesized that the reason for the hepatotoxicity is due to the free radical properties of phenoxyl radicals produced in both amodiaquine and acetaminophen during liver metabolism. To study these free radicals, a technique known as Electron Spin Resonance spectroscopy or ESR was employed.

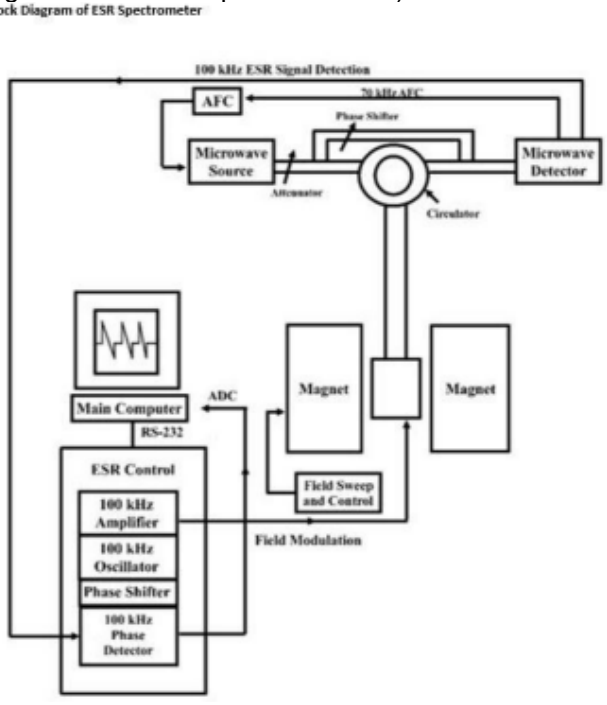
ESR utilizes a magnet and microwave frequencies to “excite” a sample containing free radicals resulting in a spectrum showing the derivative of the absorption of the microwave frequencies (Fig. 3). In turn, the resulting spectra can then be compared, potentially showcasing similarities between the two molecules’ phenoxyl radical. Due to the unstable nature of free radicals, different methods must be employed to produce observable concentrations of free radicals. This leads to a method known as fast flow ESR, in which a sample of a strong oxidizer and the sample to be oxidized are pumped into a flat cell, free radicals are formed, and a spectrum is produced.




(Figure 1: Amodiaquine Structure)




(Figure 2: Acetaminophen Structure)




(Figure 3: Block Diagram of ESR Spectrometer)

## Experimental

*Characterizing Amodiaquine.*

A capillary melting point tube was filled with 2mm of commercial amodiaquine. This sample was then placed into an EZ-Melt instrument. A general melting point range was acquired through a quick run of 10°C/Min. Using this range, a slower rate of 1°C/Min was used to determine the true melting point of the amodiaquine sample and compared to literature values. This process was repeated multiple times with differing samples of amodiaquine over the course of the experiment.

*IR Spectroscopy.*

Along with the melting point data, IR spectroscopy was also performed to determine the structure of the amodiaquine. To do this a Shimadzu IRTracer-100 Fourier Transform Infrared spectrometer was used. The plate and press of the instrument was cleaned using ethanol and a background spectrum was performed. A small sample of amodiaquine was then placed into the center of the plate and the press was screwed on. A spectrum was then recorded for the sample used and the process was repeated for different samples of amodiaquine.

*Practicing ESR and WinSim.*

The JEOL RE-x1 ESR spectrometer and control computer were turned on and a known sample was placed into the microwave chamber. The Q-Dip was then balanced by changing parameters on the computer. ESR spectra were then measured on the sample and the amplitude, time constant, time, modulation, and power were changed as needed to ensure a clean spectrum. The spectrum was then saved and transferred to the WinSim software and a simulated spectrum was created to coincide with the actual spectrum to determine the resolution of the sample spectrum and eliminate background noise.

*Recrystallizing Amodiaquine.*

Due to issues with the ESR spectra of the commercial amodiaquine, another sample was prepared through recrystallization. After determining a proper solvent, 10 grams of commercial amodiaquine was weighed out and transferred to a beaker. An excess 75 mL of DMSO was then heated on a hot plate and incrementally added to the beaker containing the amodiaquine until fully dissolved. The mixture was then left to rest for 15 minutes until cool enough to touch. The mixture was then cooled in an ice bath for 30 minutes to precipitate solids.

Another form of recrystallization was performed to remove potential waters of hydration from the commercial amodiaquine. Another sample of 10 g of amodiaquine was dissolved in deionized water with heat. 3 grams of NaOH were added to the water and the amodiaquine crashed out of solution. After solids had formed in both solutions, the solutions were suction filtered using a buchner funnel and suction flask. The remaining solids were then transferred to a vacuum desiccator and left to dry under vacuum for two days.

*Fast Flow ESR of Amodiaquine.*

Using stoichiometry, a 4 L sample of 1.8 mM Ce (IV) Sulfate was prepared using 2.392 g of Ce (IV) Sulfate, 0.225 M concentrated sulfuric acid, and 3.95 L of deionized water. A 4 L sample of 2.0 mM Amodiaquine was also prepared using 2.847 g amodiaquine, 2 L of deionized water, and 2 L of 95% ethanol. Both solutions were heated to 35°C to dissolve the solutes. These solutions were transferred to fast flow reservoirs and bubbled with nitrogen to remove oxygen potentially present in the solution. Each reservoir was connected to a peristaltic pump that fed into an 80 mm Wilmad WG-804-Hampden flat cell. ESR spectroscopy was then performed using differing solution flow rates and instrument settings to achieve the most resolved spectrum. This was then repeated for each sample of amodiaquine to produce a variety of spectra.

*NMR of Amodiaquine Samples.*

Each sample of amodiaquine was tested using both carbon and proton NMR. 40 mg of amodiaquine was dissolved in about 0.6 ml d-DMSO, enough to have 4 cm of solution in the NMR tube. The sample was then prepared and loaded into the JEOL NMR instrument and NMR was performed. The resulting spectra were then recorded and analyzed.

*Rotovap of Amodiaquine Sample.*

To ensure the highest yield of amodiaquine from the sample dissolved in DMSO, rotary evaporation was performed. The leftover solution from the recrystallization step was funneled into a 250 ml round bottom flask and secured to the Rotovap instrument. The water bath temperature was set to 80°C and the pressure was set to 200 mbar at 30 revolutions/minute. The round bottom flask was lowered into the water bath and left for 3 hours to produce a solid.

*Mixed Melting Point (MMP) of Amodiaquine Samples.*

To compare the samples of amodiaquine used, a mixed melting point was performed. This involved creating a sample of each amodiaquine being mixed 50:50 ratio and added to a capillary tube. Two

other capillary tubes were prepared using the pure forms of each unmixed sample of amodiaquine. The capillary tubes were then placed in the EZ-Melt instrument and set to 195°C-215°C at 1°C/min and the results were recorded.

*One-Pot Synthesis of Amodiaquine.*

After determining that the three previously collected samples of amodiaquine were unusable, a fourth sample was created. This was done by utilizing a one pot synthesis as detailed by Fortunak, et al. [5]. To start, 0.10 mol or 10.913 g of 4-aminophenol and 0.10 mol or 19.811 g of 4,7-dichloroquinoline were added to a round bottom flask with room temperature stirring. To the same RBF, acetic acid with a volume equating to 3 times the mass of the 4,7-dichloroquinoline, 60 ml, was added. This mixture was then heated with stirring at 110°C for 1 hour. The mixture was then cooled in an ice bath to 20°C. A 1.5 molar equivalent of formaldehyde from a 37% solution was added to the reaction vessel. This was followed by dropwise addition of a 1.5 molar equivalent of diethylamine. The reaction was then heated again with stirring to 50°C for 4 hours. After, the vessel was transferred back to an ice bath to cool to 20°C. 7.2 ml of a 37% concentration HCl was then added dropwise to the reaction vessel to insure the internal heat does not exceed 40°C. The reaction was then stirred for 2 hours to precipitate amodiaquine product. The precipitate was then vacuum filtered and dried in a vacuum desiccator and weighed for results.

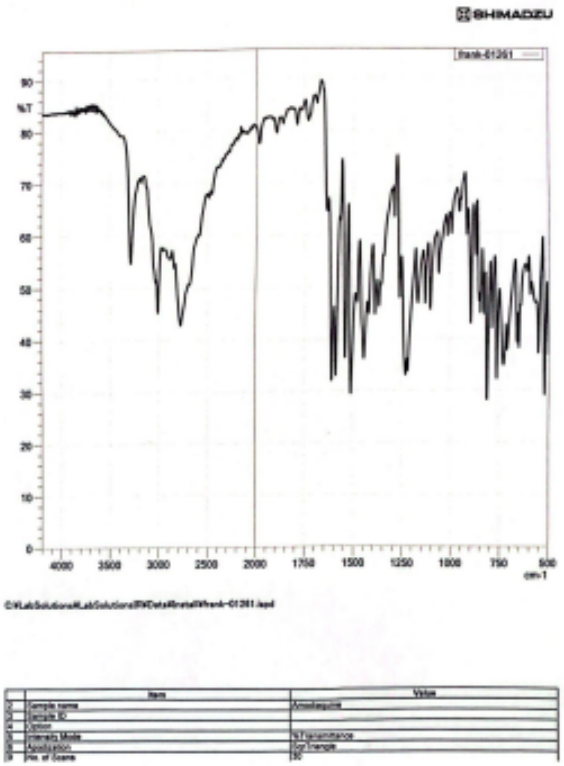
## Results and Discussion

*Characterizing Amodiaquine.*

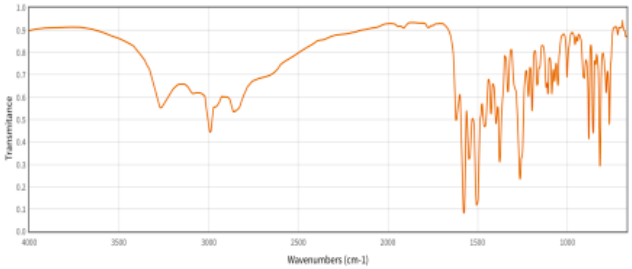
Throughout the course of this project, four distinct samples of amodiaquine were used. These samples are commercial amodiaquine (CA), recrystallized amodiaquine (RA) from commercial, previously synthesized amodiaquine (PS), and one pot synthesized amodiaquine (OP). The melting point of CA was determined to be 159.2°C which fell in the predicted range of 150-160°C for amodiaquine hydrochloride [6]. The melting point of RA was determined to be 203.5°C which was much higher than the assumed range but closer to the melting point of amodiaquine at 208°C [7] leading to IR being performed. RA also sublimated in the capillary tube making data collection difficult but multiple experiments proved that the melting point was indeed 203.5°C. This change in melting point was later attributed to the removal of HCl. PS and OP both had a melting point of 198.9°C. This was consistent as they were both produced using the same methods at different times.

*IR Spectroscopy.*

Of the spectra collected, only one, OP was able to be properly analyzed in conjunction with the literature. The comparison showcased similarities in the peaks proving that the OP was in fact amodiaquine.




(Figure 4: OP IR)




(Figure 5: Amodiaquine IR Spectrum according to literature [8])

*Practicing ESR and WinSim.*

Using known compounds with stable free radicals such as tetraphenyl ethylene, pyrene, and biphenylene led to decently resolved spectra. WinSim was then used to procure coupling constants and correlation values within 98%.

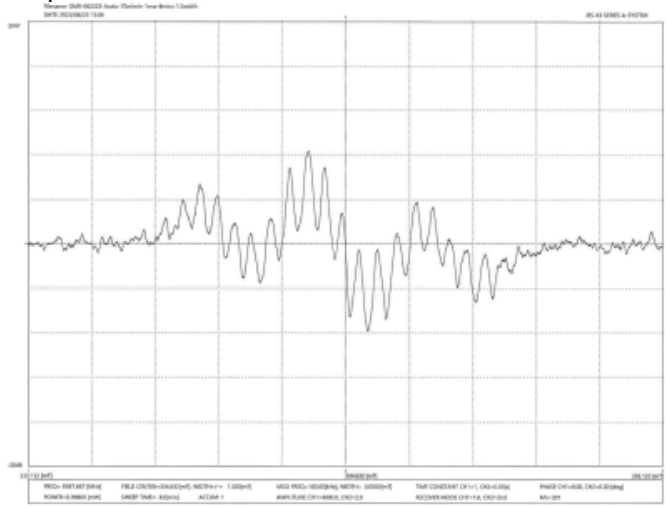
*Recrystallizing Amodiaquine.*

The first attempt was run using DMSO. This resulted in a lack of precipitation and the sample was transferred to rotovap. The second attempt was with ethanol which led to the sample completely crashing

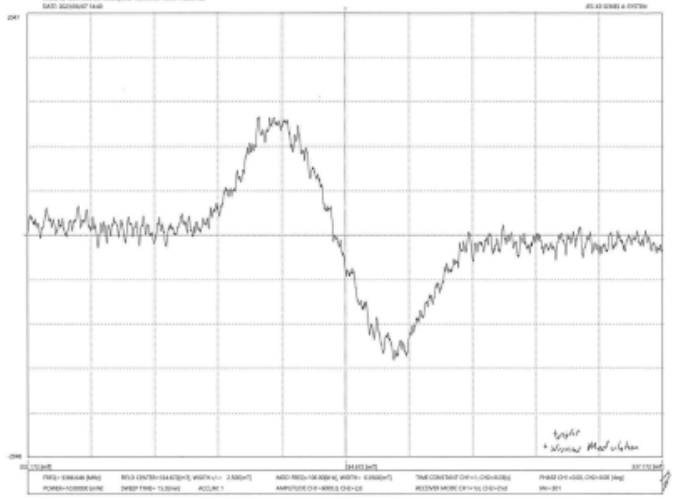
out of solution. The resulting yield was 70.7% which was consistent with the loss of HCl. This resulted in RA which had a slightly pinkish color and different melting point. Due to issues with the structure of CA, both CA and RA were ultimately not the proper compounds and did not show the expected splitting pattern with ESR.

*Fast Flow ESR of Amodiaquine.*

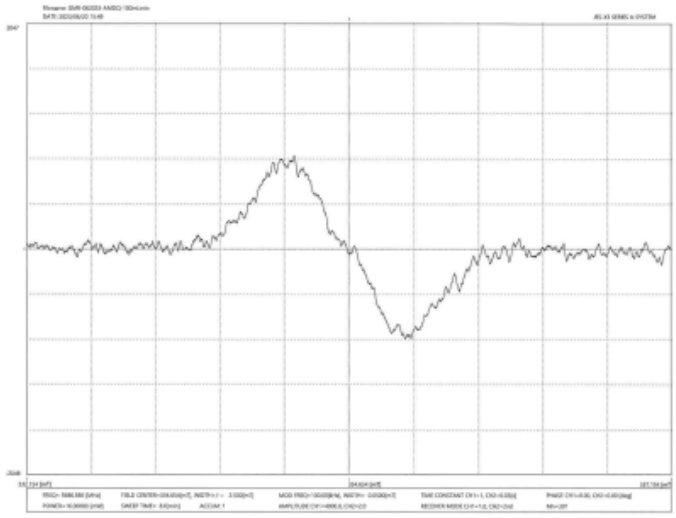
Fast flow ESR was run numerous times with each sample of amodiaquine. Each of the spectra were then compared with that of acetaminophen which should have a similar shape of a triplet due to the phenoxyl radicals present in each. Of the samples tested only two, PS and OP, (Fig. 9 & 10) presented splitting similar to that of acetaminophen. The spectra are presented below.



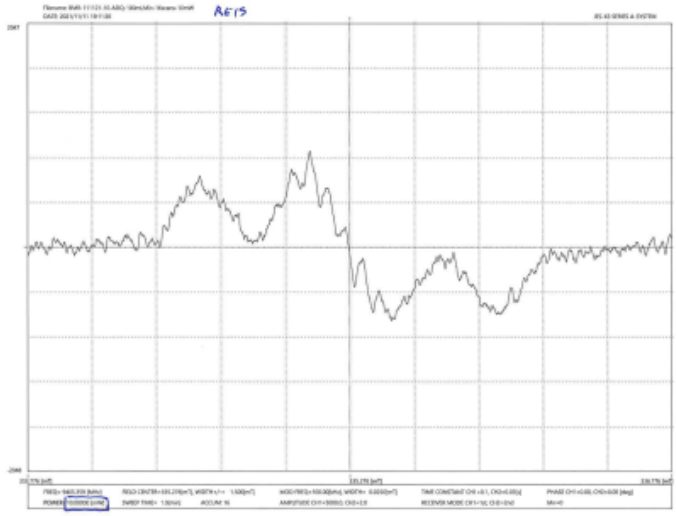
(Figure 6: ESR of Acetaminophen)



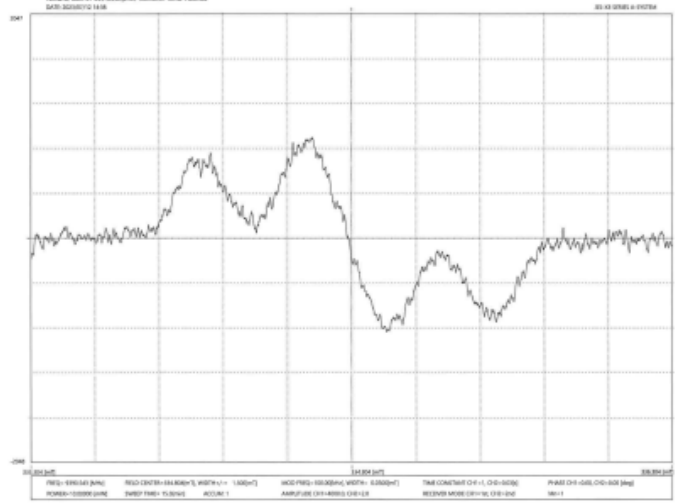
(Figure 7: ESR of CA)



(Figure 8: ESR of RA)



(Figure 9: ESR of PS)



(Figure 10: ESR of OP)

The lack of a triplet in CA and RA can most likely be attributed to being the wrong chemical entirely as it did produce a free radical but not in the correct location.

*Rotovap of Amodiaquine Sample.*

Due to the high boiling point of DMSO it is quite difficult to fully evaporate using rotary evaporation. Despite running for 3 hours, only a small amount of bright orange crystals formed which were unable to be used in fast flow ESR.

*Mixed Melting Point (MMP) of Amodiaquine Samples.*

Much like the ESR spectra, correlation between melting point was determined to be paired. Both CA and RA, compounds both resulting from the original CA sample, had no significant drop or gain in temperature when mixed and tested. Likewise, PS and OP, both resulting from the same procedure at differing times, had the exact same melting point separately and mixed. This ensured that the compounds were effectively the same as each other.

*One-Pot Synthesis of Amodiaquine.*

According to the literature [5], the one pot synthesis was projected to have a yield greater than 90%. PS, a compound synthesized by Eric Bowen following the same literature only resulted in a yield of 39.4%. OP however, resulted in a yield of 61.44% with 28.51 g which was much higher than previous experiments. While not reaching the expected yield above 90%, this was a great improvement from PS. The potential reason for a lack of yield may have been due to improper mixing during the heating stages of the experiment as solids formed preventing the stir bar from properly mixing, as well as violent boiling resulting in liquid being splashed to the top of the RBF and not reacting under heat.

## Conclusion

Although steps have been made to ensure progress, this is still an ongoing project. The OP spectrum could still be refined further and WinSim analysis needs to be utilized to record coupling constants. Both CA and RA led to dead ends in progression however, future tests will be conducted to determine the potential usability in future projects. In future projects, horseradish peroxidase as well as hemoglobin and hydrogen peroxide can be used as strong oxidizers in the fast flow reaction. This change may showcase how the amodiaquine might interact with the enzymes during liver metabolism and its similarity to acetaminophen. Currently the only usable sample is the OP as there is not enough PS to complete a fast flow ESR. However, this should not be an issue as it was determined that both OP and PS are the same
substance.

## Acknowledgements

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