Determination of the Octanol-Water Partition Coefficient of Indolicidin and Indolicidin45 Using Micellar Electrokinetic Chromatography

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Introduction

Average life expectancy before the invention of antibiotics was 47 years. The war against bacteria is a commonly known struggle. Common antibiotics are becoming less effective with the emerging amounts of antibiotic resistant superbugs. Antibiotic resistance is a result of overuse/ misuse of antibiotics where bacteria desensitize to the drugs. More and more superbugs are becoming prevalent where new antibiotic formation has slowed down. The fear of entering back into a post antibiotic era is a real threat.

Bacteria can become resistant to antibiotics through mutations (Blair). These mutations causing the resistance are then transferred to other bacteria via horizontal gene transfer (Blair). Bacteria can also be intrinsically resistant to specific antibiotics meaning they can resist the action of the antibiotic through mechanistic, structural, or functional barriers (Blair). Mechanistically, bacteria can resist antibiotics by either preventing the drugs from entering the target cell, or by destroying/ altering the antibiotic itself (Abraham).

A new generation of drugs is needed to fight against the superbugs of today. Antimicrobial peptides (AMPs) could be the key. AMPs are found in all organisms and are part of the organisms' innate immune systems (Reddy et al. 2004). All multicellular organisms have been in contact with a diverse number of microbes (Wilcox). Throughout evolution organisms have been able to face and overcome pathogen attack due to an innate immune response (Wilcox). Even though these pathogens have had prolonged exposure to AMPs throughout evolution, resistance has never been acquired (Wilcox). AMPs have been discovered across virtually all living organisms with a very broad spectrum of antimicrobial activity (Wilcox). The world of antimicrobial peptides is extremely diverse and has potential to solve the superbug crisis with enough time and research.

The AMP of particular interest for this study was indolicidin (indol - ILPWKWPWWPWRR-NH2) which is isolated from bovine neutrophils and is a 13-residue peptide amide with an overall +3 charge (Subbalakshmi and Krishnakumari, Podoriezach). This AMP has shown activity against Gram-positive bacteria, Gram-negative bacteria, fungi, protozoa, and viruses (Jones). This AMP has already shown activity against the superbug methicillin-resistant Staphylococcus aureus

(MRSA). The problem with indol however is its high level of hemolytic activity. Previous research has been done at TRU by my supervisor and her students with much success to reduce toxicity of this AMP. Two highly hydrophobic tryptophan residues were substituted for mildly hydrophobic alanine residues which resulted in a >33000-fold decrease in cytotoxicity (Podorieszach). This new derivative is called indolcidin45 (indol45)(Figure 1.) and will be the focus of this study.

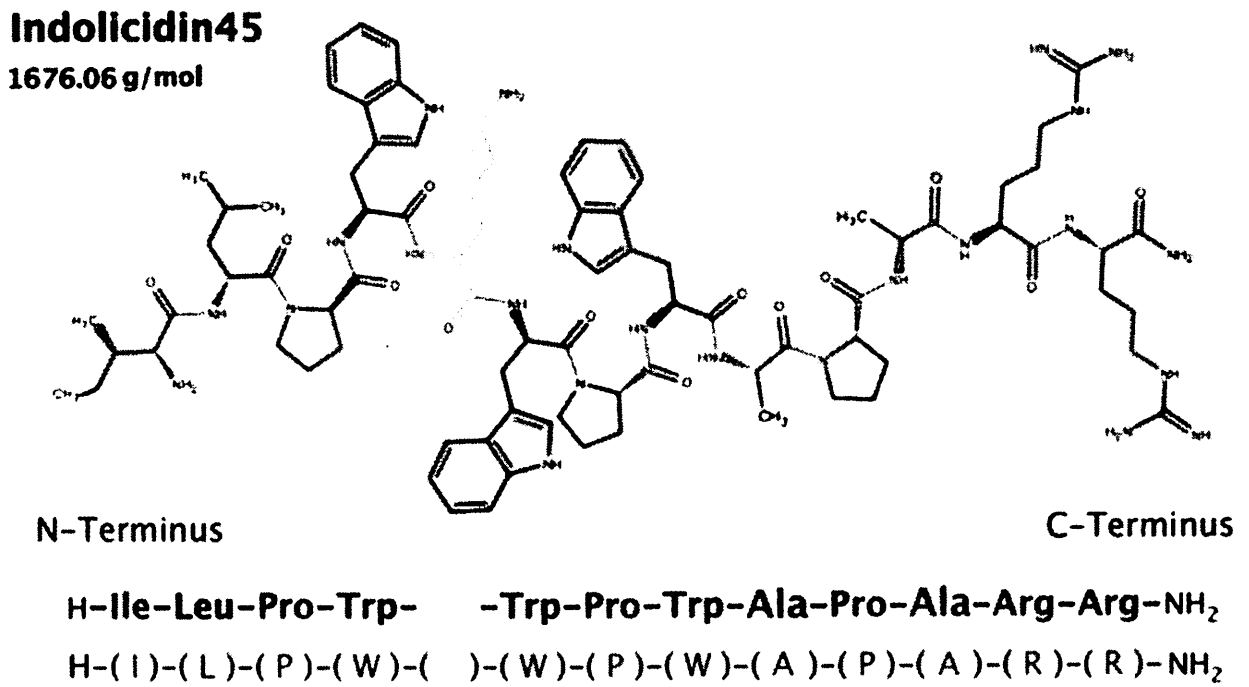


Figure 1. Organic structure of the synthetically modified AMP indol45. This analog is a derivative of the naturally occurring indol from which the 4th and 5th tryptophan residues were switched with alanine resulting in a decrease in hemolytic activity.

Finding the octanol:water partition coefficient for indol45 would entail using the Micellar Electrokinetic Chromatography (MEKC) technique. This is important to furthering our knowledge of the AMP and determining its method of action when killing a pathogen. The primary goal is to determine whether indol45 possesses an intracellular mechanism or if it binds and disrupts the microbial membrane of the pathogens it attacks. Finding the partition coefficient will bring us closer to understanding this AMP so future research can be done to

hopefully one day see feasible therapeutic use of this substance to fight against antibiotic resistant superbugs.

Theory

Capillary Electrophoresis (CE)

CE is an analytical instrument used to separate analytes based on their electrophoretic mobility. Analytes separate through a buffer under the presence of an electric field created by a high voltage power supply and a basic cell style anode/cathode relationship. The capillary used in this instrument is usually less than 1 mm in diameter. Analytes separate in the buffer solution due to their electrophoretic mobility. Some analytes will migrate quicker than others and this is due to size and charge (the two main components effecting the electrophoretic mobility).

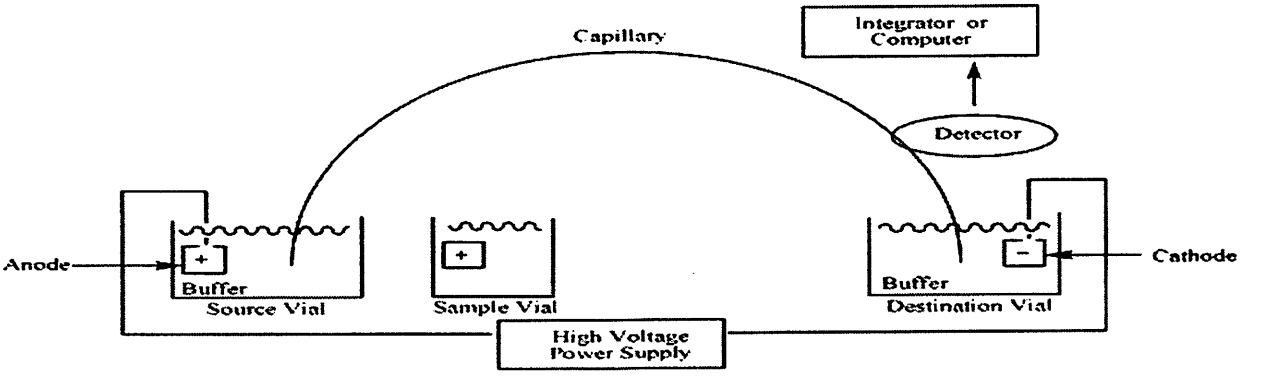


Figure 2. This is a schematic of how the CE instrument is set up. The analyte is injected from the sample vial followed by separation which occurs between the source and destination vials. The high voltage power supply allows for a consistent current through the capillary. The separation is often through a buffer system which allows for the analytes to migrate based on electrophoretic mobility.

The CE will graph an electropherogram from which migration time can be determined. The migration time can then be utilized to determine electrophoretic mobility (μ) which can be seen in the equation below.

$$\mu = \left(\frac{L_D}{t_m}\right) \times \left(\frac{L_T}{V}\right)$$

L_D: length to detection point

L_T: length of total capillary

t_m: migration time

V: Voltage

From the electrophoretic mobility, characteristic information of the analyte can be found from the equation shown below.

$$\mu = \frac{q}{6\pi\eta r}$$

q: charge

η: viscosity

r: radius

From these two equations, CE can be used to determine migration time and electrophoretic mobility of analytes. The physicochemical characteristics of an analyte can then be calculated to provide a wide range of information about the analyte in question.

Partition Coefficient

The partition coefficient (P) is an important physicochemical property to all pharmaceutical drugs. The octanol-water P is particularly important for this study as it specifically determined the tendency of a compound to move from aqueous phase to lipids. The P is shown as a ratio of concentrations of a specific analyte in two immisible solvents.

$$Partition\ coefficient\ (P) = \frac{[Solute]_{1octanol}}{[Solute]_{Water}}$$

The P value shows a degree of hydrophobicity versus hydrophilicity. Based on this value we can determine whether an analyte's tendency is to pass through a lipid membrane or to simply embed itself within the membrane. From this the method of action for an analyte, such as an AMP, can be determined for how it kills a pathogen. For a compound to enter into a cell (past the lipid membrane) it must be adequately hydrophobic; however, if the compound is too hydrophobic it would tend to remain in the membrane bilayer itself. A compound would

therefore require both hydrophobic and hydrophilic properties to have viable intracellular interactions.

Experimental

Methods

The CE instrument Beckman P/ACE MDQ was used for this study. The wavelength of light used was 214 nm. The capillary used had a 50 μm diameter and a 60 cm total length. Each run began with rinsing the capillary at 20.0 psi with 0.1 M NaOH (5.00 min), 18 MΩ water (1.00 min), and buffer (1.00 min). The rinsing is done to clean out capillary to remove all potential interfering impurities. After the rinses were complete, the sample was injected at 1.0 psi for 5.0 sec. Separation then began with the applied voltage set at 20 kV across the capillary and a 40.00 min run time to obtain all necessary peaks.

Chemical/ Buffer Preparations

All preparation was done using 18 MΩ water and all buffers were adjusted to pH 7.2 (physiological pH).

A stock MEKC micelle buffer solution was made with ..... sodium dodecyl sulphate (SDS) and ..... phosphate (in the form of sodium phosphate dibasic).

A stock phosphate solution was made with .... phosphate (again with sodium phosphate dibasic).

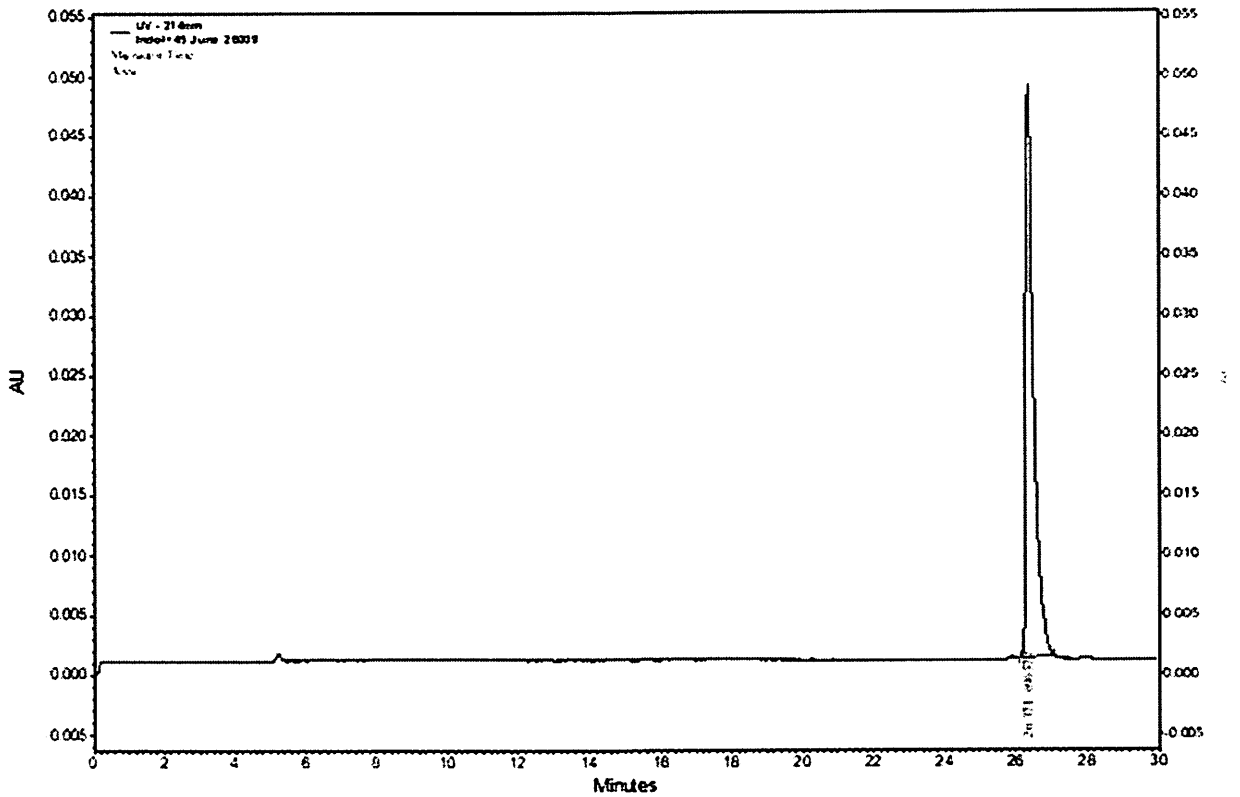
Stock indol and indol45 solutions were prepared at 500 ppm then diluted to 300 and 100 ppm concentrations. These concentrations were all run with both the phosphate and SDS buffers.

1 M and 0.1 M NaOH were also prepared for the rinses talked about previously.

All solutions were filtered through a 0.45 μm membrane filter prior to running through capillary.

Results

During semester, the CE was a very busy instrument. Two weeks into the summer one of the two working CEs obtained a problem which has yet to be resolved. As a result, runs were very limited due to the number of students running over the summer. Optimizing the method and buffers was done successfully by the end of summer and some good electropherograms were obtained. Unfortunately, due to the lack of run time available and time it took to optimize the methods, the goal of finding the partition coefficient was not met. Moving forward data acquisition should be much smoother. Below will be added an example electropherogram obtained.



This electropherogram is very clean and exactly the type of results to be looking for. This was obtained for the stock indol45 solution at 100ppm. Four other runs resulted in electropherograms looking almost identical to the one shown above. Those were for the stock indol at 100, 300, and 500 ppm as well as indol 45 at 300 ppm. The only sample that did not result in a clean peak was the indol45 at 500 ppm and the reason for this is unknown. The reason for not being able to determine the partition coefficient was that these were one-time

results. Replication of this data was never obtained but the project came very close to conclusion.

Discussion

As mentioned in the results section, time constraints with lack of instrumentation available lead to the inability to complete this project. Nearing the end of the summer however, good/ relevant data (like the electropherogram shown above) was obtained showing a lot of promise moving forward.

This project will be the topic of my honours. I will be continuing this research and hopefully completing it over the next two semesters as this leaves ample time for data acquisition and analysis. After completing the research, I hope to publish the work in a reputable journal and defend my thesis in early May 2020.

Now that method optimization is complete, obtaining clean/ usable data for analysis is the goal. Near the end of summer, the data obtained shows this can be done so the future of this research appears very bright.

After determining the partition coefficient for indol45 there are still two major stepping stones in research with this peptide before it emerges as a high potential drug for use one day. Further research needs to be done to lower toxicity of the peptide as its high level of hemolysis is still the major component standing in the way. When cytotoxicity is reduced, research goals can then be transitioned towards determining the specificity of the AMP.

If indol45 does possess intramolecular mechanisms of action towards killing microbes, further research should be conducted as to what those mechanisms are. Whether that be the AMP binding to specific proteins/ enzymes, DNA, or other intracellular components, determining specific mechanisms will later be very important. Once the exact mechanism is determined, the AMPs specificity can also be discovered as to exactly which type of microbes it is effective against. Making sure that indol45 is not harming the helpful bacteria in the body may also present a challenge for researchers of the future. It is safe to say indol45 and AMPs like it still require a lot of work and research before they become feasible for pharmaceutical use. The process in developing pharmaceuticals is always a very long process. I hope research continues

with these peptides and that this research helps significantly as one of the primary steps towards the potential development of AMPs as useful drugs to fight the war against antibiotic resistant superbugs.

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