Faculty of Science

DETERMINATION OF THE OCTANOL:WATER PARTITION COEFFICIENT OF INDOLICIDIN AND INDOLICIDIN45 USING MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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B.Sc. Honours thesis – Chemical Biology

THOMPSON RIVERS UNIVERSITY

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by

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ABSTRACT

Antimicrobial peptides are a potential new generation of drugs that are extremely diverse and could help to fight the imminent threat of antibiotic resistant bacteria. In this study, we attempt to determine the octanol:water partition coefficient for the antimicrobial peptides indolicidin (amino acid sequence: ILPWKWPWWPWRR-NH2) and its derivative indolicidin45 to better understand their potential mechanisms of action when attacking pathogens. The partition coefficient is defined as the ratio of concentrations of a solute in two immiscible liquids at equilibrium. It helps determine the hydrophobicity/ hydrophilicity of an analyte. Indolicidin45 has its fourth and fifth highly hydrophobic tryptophan residues relative to the N-terminus swapped with less hydrophobic alanines. This reduced the cytotoxicity while increasing the bioactivity of the derived molecule. Capillary electrophoresis is the instrument that separates analytes based on their relative electrophoretic mobilities and was used for this study. Micellar electrokinetic chromatography is a technique done with capillary electrophoresis which can help to determine the capacity factors and partition coefficients of the peptides for this study. This technique utilizes micelles as a pseudo-stationary phase; the analyte can then partition between the micelles and the aqueous buffer running through the capillary. The capacity factor of the peptides was first needed in order to determine the partition coefficients. The capacity factors of indolicidin and indolicidin45 were determined to be 0.21 +/- 0.04 and 0.23 +/- 0.04, respectively. From these values the partition coefficients were estimated based off a Log P vs. Log k' calibration curve of known values. The log of the partition coefficients for indolicidin and indolicidin45 were determined to be 1.55 ± 0.04 and 1.62 ± 0.04, respectively. Since these Log P values seen are greater than 1, they suggest that both peptides are hydrophobic. As a result, a potential mode of action for these AMPs to kill pathogens may be to bind and disrupt/ lyse the microbial membrane leading to cell death. As potential drugs, this is also promising as it is important that they would be able to cross the bacterial cell membrane to attack pathogens which more hydrophilic molecules cannot do. Some future directions would be to determine the partition coefficient but with the vesicular electrokinetic chromatography method as well as looking into other derivatives of indolicidin for better potential to one day be used as drugs to fight against antibiotic resistant bacteria.

Thesis Supervisor: Associate Professor Heidi Huttunen-Hennelly

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DEDICATION

To my mom,

For all the love, compassion, guidance, and care you have given to me and the success of my future. I will be forever grateful for your efforts and support.

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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.[1] These mutations causing the resistance are then transferred to other bacteria via horizontal gene transfer.[1] Bacteria can also be intrinsically resistant to specific antibiotics meaning they can resist the action of the antibiotic through mechanistic, structural, or functional barriers.[1] Mechanistically, bacteria can resist antibiotics by either preventing the drugs from entering the target cell, or by destroying/ altering the antibiotic itself.[2]

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.[3] All multicellular organisms have been in contact with a diverse number of microbes.[4] Throughout evolution organisms have been able to face and overcome pathogen attack due to an innate immune response.[4] AMPs have been discovered across virtually all living organisms with a very broad spectrum of antimicrobial activity.[4] The world of AMPs is extremely diverse and has potential to solve the superbug crisis with enough time and research. Even though these pathogens have had prolonged exposure to AMPs throughout evolution, resistance has never been acquired.[4] A potential reason for the lack of development of resistance towards these chemicals may be because many of them involve an extracellular mechanism of action to killing the pathogens. If a drug can work on the outside/ membrane of the bacteria, the microbe would have a difficult time developing resistance because the interaction between drug and pathogen would be quite limited. The AMPs studied for this experiment may be an example of this and may follow this concept with respect to the lack of resistance we see.

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 +4 charge.[5,6] This AMP has shown activity against Gram-positive bacteria,

Gram-negative bacteria, fungi, protozoa, and viruses.[7] This AMP has already shown activity against the superbug methicillinresistant *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 in the fourth and fifth positions relative to the N-terminus were substituted for mildly hydrophobic alanine residues which resulted in a >33000-fold decrease in cytotoxicity.[6] This new derivative is called indolcidin45 (indol45). These two AMPs will be the focus of my project.

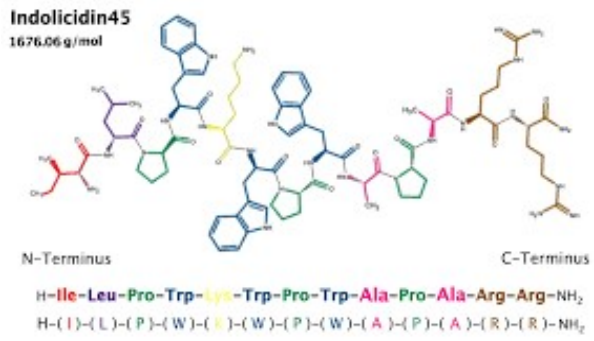

Figure 1. AA formula and structure of indol45. This AMP is a synthetically modified version of the naturally occurring 13 residue peptide linked indol where the fourth and fifth tryptophan residues were replaced with the pink alanine groups seen above.

In the same study as discussed above, an apoptosis/ necrosis assay was done to better understand indol/ indol45 mechanism of action.[6] Their tests found significant levels of apoptosis occurring in the cells they tested which was indicative of the drugs passing the membrane and activating intracellular apoptosis signaling cascades. This cascade could be a result of DNA damage or mitochondrial disruptions but the researchers stated that further study would be required to pinpoint the exact mechanism leading to the activation of the signaling cascade.[6] Finding the partition coefficient could help support these findings.

Hydrophobicity of pharmaceuticals is important for bioactivity analysis/ estimations as there are correlations with absorbtion and transportation. Finding the octanol:water partition coefficient for indol and indol45 would entail using the micellar electrokinetic chromatography (MEKC) technique. Finding the partition coefficient will bring us closer to understanding these

AMPs so future research can be done to hopefully one day see feasible therapeutic use of indol or its derivatives to fight against antibiotic resistant superbugs.

The goal of this study is to better understand the mechanism of action the AMP indol takes to kill/ destroy the harmful pathogen it attacks. The method behind this will be figuring out the partition coefficient which will tell us whether indol prefers a hydrophobic or hydrophilic environment. Based on the result we can conclude its' preferred mechanism of action again pathogens. This study will be repeated for indol45.

THEORY

Partition Coefficient

The partition coefficient (P) is defined as the ratio of concentrations of a solute in two immiscible liquids at equilibrium. It helps determine the hydrophobicity/ hydrophilicity of an analyte.

$$P = \frac{[Analyte]Organic}{[Analyte]Aqueous}$$

Equation 1. Formula for partition coefficient where the organic phase is octanol and the aqueous phase is water.

In the pharmaceutical industry, P is used as a measure of a drug's lipophilicity. This means P is used to determine a drug's ability to cross a cell membrane and whether the chemical substance is hydrophilic or hydrophobic. One of the most common ratios seen and the one used for this study is the octanol: water P, meaning the organic layer is made of octanol and the aqueous layer water.

Capillary Electrophoresis

Capillary electrophoresis (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. 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 factors affecting the electrophoretic mobility). The CE graphs an electropherogram from which migration time can be determined.

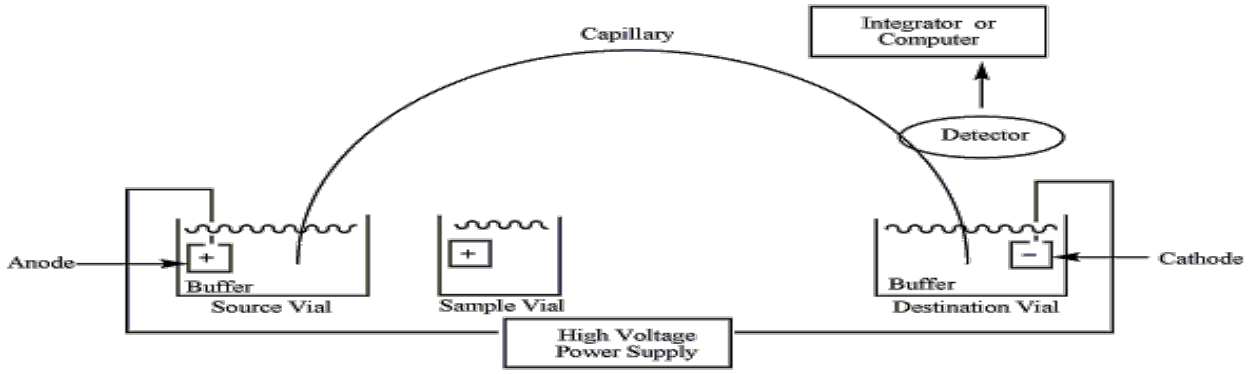

Figure 2. Schematic of the CE instrument set up.

As can be seen in Figure 2, 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.

Electroosmotic flow (EOF) is the movement of the buffer solution in the capillary towards the cathode due to the applied electric field. The walls of the capillary are electrically charged, and the surface of the silica capillary is coated with many silanol groups (Si-OH) (Donkor). When the pH of solution in the buffer is higher than 2, the silanol groups ionize producing solanoate ions (SiO^-); for this reason, we rinse the capillary with 1.0 M NaOH prior to any runs and with 0.1 M NaOH between runs. The silanoate ions are then able to interact and tightly bind to cations from the buffer which forms a fixed layer; however, a the silanoate ions are not completely neutralized from the buffer cations so other cations bind more loosely forming a mobile layer. Since the cations in the mobile layer are solvated, the applied electric field allows for the cations to drag the solution through the capillary producing an electroosmotic flow towards the cathode. Increasing the concentration of the buffer solution decreases the EOF.[8]

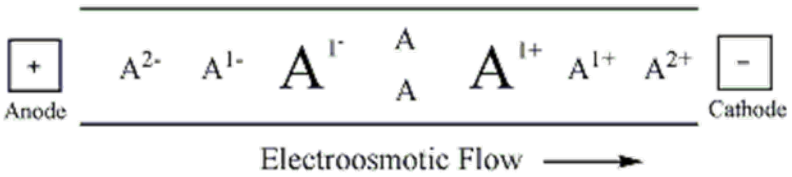

Figure 3. Schematic showing electrophoretic mobility of analytes.

The smallest, most positive analytes move through the capillary faster than the larger, more negatively charged ones. All analytes eventually elute as an electroosmotic flow is present; however, the speed at which the compounds elute can be drastically different based on factors discussed.

The Beckman P/ACE MDQ CE instrument was used for this study to conduct all testing and analysis. A UV detector was used at a wavelength of 214 nm. The CE was run with a fused silica capillary of 50 μm inner diameter and an applied voltage of 20 kV and a total length of 60 cm. Rinses were done with 0.1 and 1.0 M NaOH at 20 psi for 5 minutes each. Samples were introduced via an injection at 1.0 psi for 5 seconds. Some advantages of using CE, over other analytical methods, are that it requires very little sample quantity, it is very sensitive, and it has fast analysis times.

Micellar Electrokinetic Chromatography

MEKC is an analytical technique which uses the CE for a chromatography technique where the samples partition between the micelle pseudo-stationary phase and the buffer solution flowing through the capillary. This is a method that can be used to determine the octanol:water P. Previous methods of determining P include the "shake-flask method"; however, the MEKC method is significantly more efficient as it is faster, uses less samples, and has a high throughput for multiple samples. For this study, sodium dodecyl sulphate (SDS) was the surfactant used to form the micelles in the run buffer. Since sulphate is negatively charged (anionic), the micelles forming from SDS want to move in the opposite direction of the electroosmotic flow. This means the micelles move through the capillary slower than the neutral marker. The micelles do reach the detector eventually as the net movement is still in the direction of the buffer flow; however, as analytes partition between the hydrophobic micelle environment and the hydrophilic buffer, we observe differences in migration times when interactions between analyte and micelles are occurring. From these migrations times we can use derived equations to find the capacity factor (k').

$$k' = \frac{\mathrm{tr} - \mathrm{teof}}{\mathrm{teof}\,(1 - \frac{\mathrm{tr}}{\mathrm{tmc}})}$$

Equation 2. Formula showing the relationship between k' and migration times where t_r is the migration time of the analyte in the phosphate buffer, t_{eof} is the migration time of the DMSO marker, and t_{mc} is the migration time of the analyte in the presence of micelles.

The k' is known as a measure of retention of a peak that is independent of the mobile phase flow rate.

The log of this value can then be used to extrapolate the log P from a known log P vs log k' plot.

METHODS AND EXPERIMENTAL

Chemical and Sample Preparation

All prep work requiring water used 18 MΩ water so impurities were kept to a minimal. Indol and indol45 samples were prepared at pH 7.4 (physiological pH) with a concentration of 100 ppm for use as the analytes. Concentrations of 300 and 500 ppm were also run but 100 ppm was used for most of the study as increasing the concentration of the analyte increased the peak size/ area but had no effects on migration time. A 50 mM phosphate buffer was made with sodium phosphate dibasic This buffer was used as the run buffer without the presence of micelles. A 50 mM phosphate with 20 mM SDS buffer was made and used as the run buffer with micelles present. Optimizing buffer concentrations took a significant period of time. Phosphate concentrations were tested at 20 mM, 50 mM, and 100 mM; overall, 50 mM phosphate obtained the best results with the cleanest peaks, most consistent current, and appropriate peak elucidation times. The buffer in the presence of micelles was optimized at 20 mM SDS. This concentration ensures that the solution is kept over the critical micelle concentration of SDS meaning micelles would form and remain present spontaneously in the buffer [8.2 mM at 25℃].[8] NaOH solutions were made at 0.1 and 1.0 M for washes/ rinses of the capillary. All peptides used as samples were obtained from GenicBio Limited and all other chemicals and reagents used from Sigma-Aldrich.

Table 1. Optimized experimental and CE conditions.

UV Detector Absorbance	214 nm
Length of Capillary	50 um
Inner Diameter of Capillary	60 cm
Applied Voltage	20 kV
pH of Buffers	7.4
Micelle Buffer	20 mM SDS + 50 mM Phosphate
Stock Run Buffer	50 mM Phosphate

RESULTS

All migration time values were averaged from the triplicate data obtained to maximize accuracy. The triplicate sets of data all looked very similar with minimal deviations in migrations times so one of each electropherogram is shown (Figures 4-7) to represent the clusters. The triplicate runs were also done three times on separate days to ensure reproducibility of peaks. Due to the early campus closure, no t_{eof} value was obtained experimentally as this was the next step of the project. A t_{eof} value of 5 was used to calculate the k' based off the t_{eof} determined by Milne.[9] The k' values calculated from equation 2 can be seen in table 1.

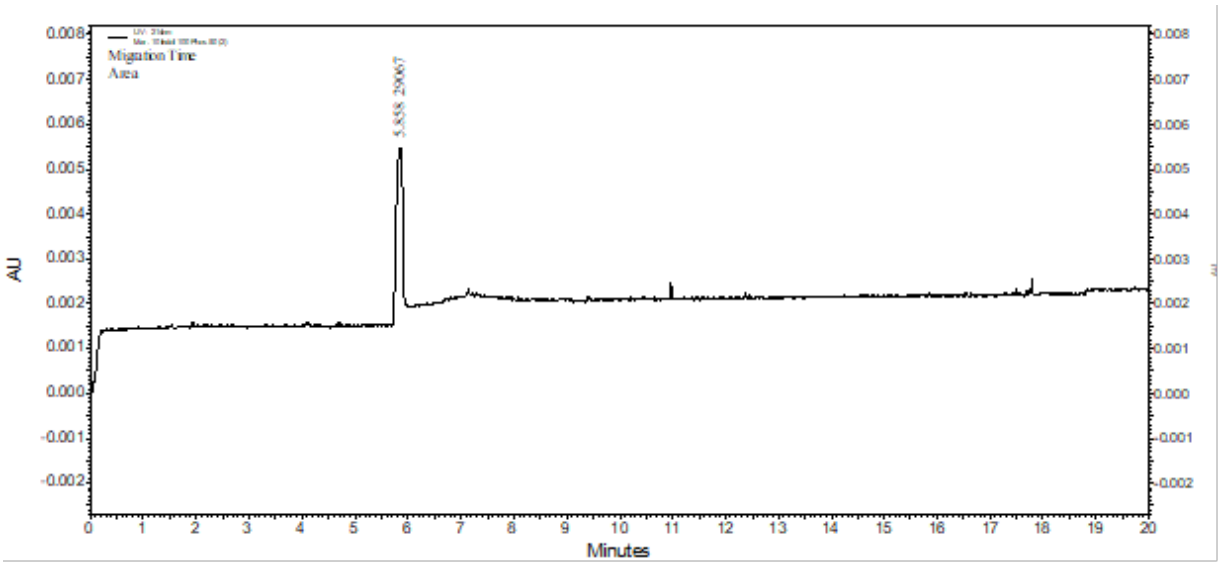

Figure 4. Electropherogram of 100 ppm indol run in 50 mM phosphate without the presence of micelles.

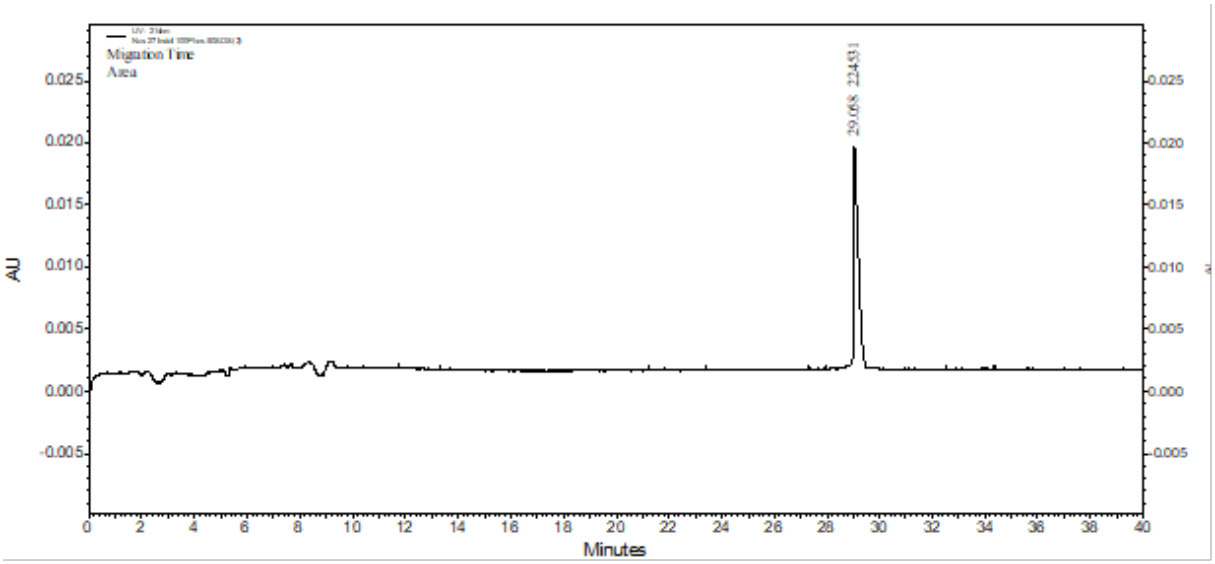

Figure 5. Electropherogram of 100 ppm indol run in 50 mM phosphate and 20 mM SDS with the presence of micelles.

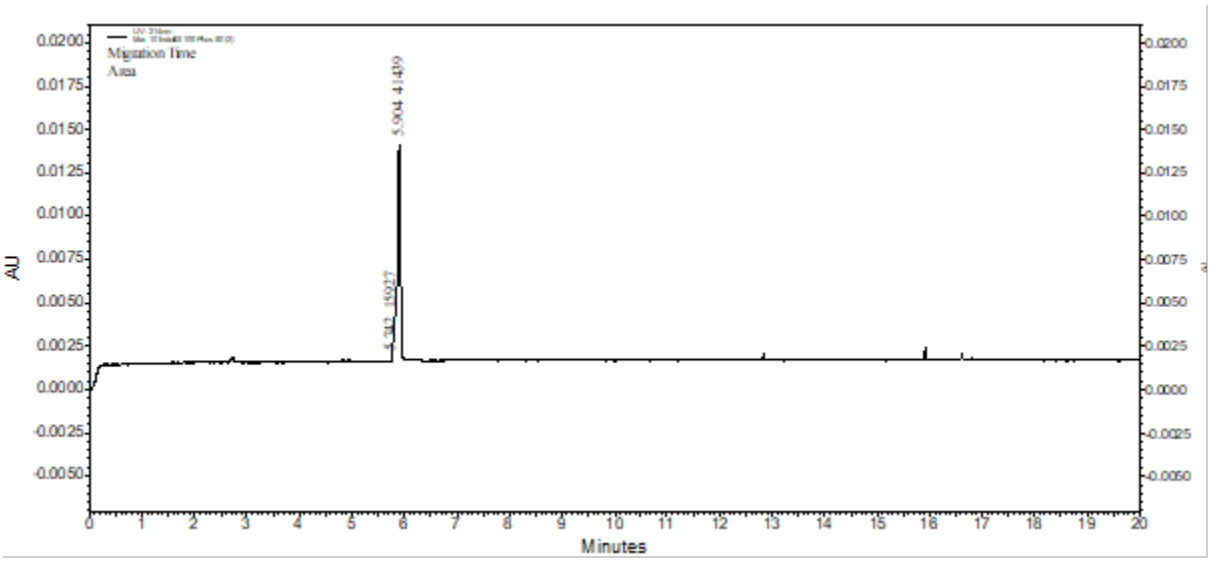

Figure 6. Electropherogram of 100 ppm indol45 run in 50 mM phosphate without the presence of micelles.

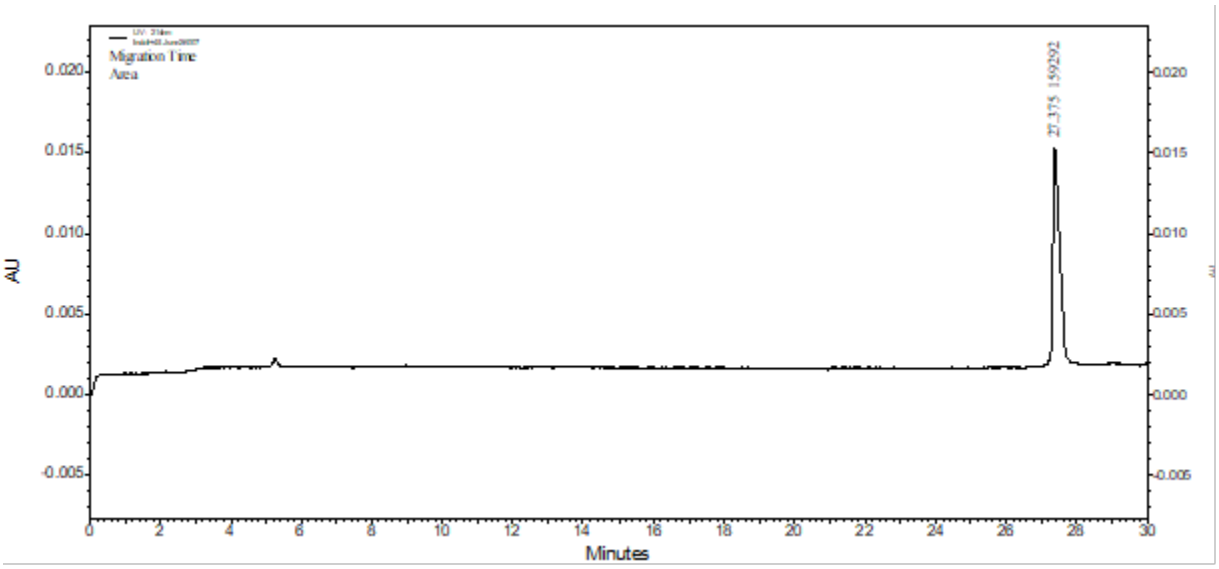

Figure 7. Electropherogram of 100 ppm indol45 run in 50 mM phosphate and 20 mM SDS with the presence of micelles.

Table 2. Results obtained for k' values calculated using equation 2 and migration times averaged from Figures 4-7 and their respective triplicate runs for indol and indol45.

Indol	Indol45
k' = 0.21 +/- 0.04	k' = 0.23 +/- 0.04

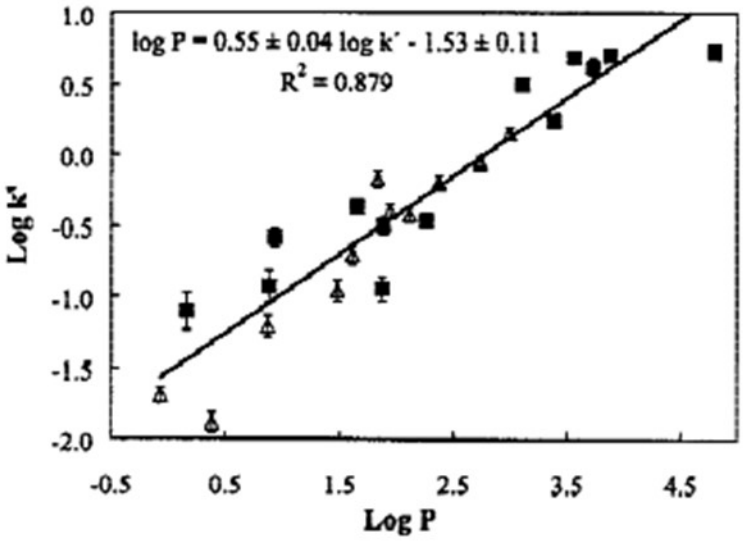

Figure 8. Calibration curve showing the relationship between calculated Log k' and known Log P values from which the Log P can be estimated from the equation of the line for indol/indol45 based on the calculated k' values.[10]

$$Log\ k' = 0.55 \times Log\ P - 1.53$$

Equation 3. Equation of the line from the calibration curve seen in Figure 8 used to estimate Log P values.

Table 3. Partition coefficient results for indol and indol45.

Indol	Indol45
Log P = 1.55 ± 0.04	Log P = 1.62 ± 0.04
P = 35.5 ± 0.1	P = 41.7 ± 0.1

DISCUSSION

Unfortunately, as most research goes, there were some setbacks with this study which lead to me not being able to complete the research. Throughout the semester there were many problems with the CE and it became an apparent difficulty obtaining usable data. After many attempts at troubleshooting, the data being obtained often still was not usable with a lot of noise and scrambled peaks. Weeks on end of runs resulted in poor data being collected and this was the second biggest setback for our timeline. CE1 (the CE used for this study) also went down for a period where no one was running on that CE. These problems added with the fact we had many students doing research on the two CE's led to the schedule being tight and a given student was restricted to essentially one run a week on average. These compounding issues along with the biggest problem/ setback of the early school closure due to the COVID-19 pandemic has me regrettably informing that this study was not fully completed.

The lack of control over the situation is unfortunate and the given unprecedented circumstances cut this study short; however, with the data that was acquired over the semester, we were able to determine the k' values for indol and indol45 respectively. Using equation 2 and the electropherogram data seen in Figures 4-7 we were able to calculate the approximate k' values of the AMPs. Just before the school closed, we had just began doing runs with the DMSO marker that was needed for equation (t_{eof} value for migration time of the neutral marker). Unfortunately, the data obtained was not conclusive so a t_{eof} value of 5 mins was used as this was the lower end of the t_{eof} value obtained by Milne.[9]

The k' values were determined to be 0.21 ± 0.04 and 0.23 ± 0.04 for indol and indol45, respectively. These values of k' were then used to estimate the Log P values for both AMPs using equation 3. The estimated values of Log P for these AMPs were determined to be 1.55 ± 0.04 and 1.62 ± 0.04 for indol and indol45 respectively. Since P is a ratio of analytes in two immiscible solvents (equation 1), a negative Log P value means the analyte prefers the aqueous environment, a value of 0 means the analyte equally partitions between the organic and aqueous phase, and a positive value means the analyte prefers the organic/ hydrophobic/ lipid phase. A positive Log P value was expected for both AMPs as they are both hydrophobic in nature. As potential drugs this is promising as it is important that they would be able to cross the bacterial cell membrane. If the AMPs had a negative Log P and were more hydrophilic, it could be a problem as the compounds

would potentially have difficulty interacting with the pathogens being targeted leading to undesirable drug traits. Seeing as the AMPs prefer a hydrophobic environment, a potential mode of action for these AMPs to kill pathogens may be to bind and disrupt/ lyse the microbial membrane leading to cell death. This mechanism gives some support as to why bacteria have had such a tough time developing resistance towards these AMPs; if an AMP like indol/indol45 binds to the membrane and lyses the cell, the bacteria may not have a response for the attacking compound and will have difficulty developing a resistance.

In the future, I would like to finish up this research by hopefully coming back and completing the runs with the DMSO marker. Obtaining an experimental t_{eof} value will give a more accurate k' value and as a result a better estimation of the Log P. Time permitting, it may also be beneficial to produce our own calibration curve from compounds more closely resembling indol/indol45 with known Log P. Making this new curve to estimate Log P from will be another way to increase the accuracy of our experimentally obtained Log P value for indol, indol45, and any other future AMPs our research group decides to investigate.

Vesicular electrokinetic chromatography (VEKC) is an alternative form of MEKC which utilizes vesicles formed in the buffer instead of the conventional micelles used in MEKC. These vesicles work better for larger compounds as MEKC has been seen to show problems with analysis involving larger compounds such as proteins.[10] The pseudo-stationary phase for this method is vesicles which form a bilayer compared to a micelle's single lipid layer. This bilayer is also a closer representation of physiological conditions as most human and bacterial cell membranes are formed of a lipid bilayer. The VEKC method was the initial plan for this study; however, after spending a large portion of time attempting to optimize the CTAB/SDS buffer (with minimal success), we opted for the MEKC method of which the buffers were able to be optimized. The VEKC method of determining P may be another aspect to relook/ retry in the future.

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