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Chemotherapy Filter: An Evaluation of Methods for Measurements and Extractions of Doxorubicin from DNA

by

Jonathan Chan

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Biomedical Imaging

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by
Jonathan Chan

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Chemotherapy Filter: An Evaluation of Methods for Measurements and Extractions of Doxorubicin from DNA

Jonathan Chan

Department of Radiology and Biomedical Imaging, University of California, San Francisco

Abstract

Introduction: Liver cancer is on the rise in the US with an increase of 2.7% new cases per year and 2.6% increase of death rates (4). The current treatments include surgical, tumor ablation, embolization, radiation, and chemotherapy. Many patients are diagnosed much too late in their progression of liver cancer and cannot undergo surgical treatments. Chemotherapy has become a promising possible treatment for liver cancer although its largest limitation is systemic toxicity. It has been found that 50-70% of the chemotherapy agent is not absorbed by the liver cancer cells and is circulated through the rest of the body (6). This amount of chemotherapy agent in the blood circulation can cause severe cardiac toxicity. Luckily, chemotherapy filter devices are currently being researched and may solve the limitation of systemic toxicity. Current devices include ionic based, magnetic based, and DNA based (6). The experiments described in this paper will be focusing on DNA based chemotherapy filters and Doxorubicin as the chemotherapy agent. The goal of this research is to create a simple and quick assay to measure the efficiency of a functionalized DNA chemotherapy filter device.

Materials and Methods: In order to simulate an in-vivo usage of a DNA chemotherapy filter device, 50 mg of genomic DNA (Salmon sperm, Sigma-Aldrich, St. Louis, MO) was mixed together with 50 mL of 0.05M Doxorubicin in a beaker. The solution

was mixed using a magnetic stir bar, allowing the DNA to bind Doxorubicin. In order to optimize the assay, three different types of extraction agents were tested; 0.3M solution of sodium acetate, 10%wt/vol solution of trichloroacetic acid (TCA), and 35%wt/vol solution of silver nitrate. These extraction agents were mixed with the DNA and Doxorubicin solution to analyze how efficient they were at removing bound Doxorubicin from DNA. The resulting samples were analyzed using both a fluorospectrometer (SPECTRA max M2, Molecular Devices) and higher performance liquid chromatography (HPLC) (SPECTRA max M2, Molecular Devices). Finally, all resulting graphs were created using Microsoft Excel (Microsoft Office 2011).

Results and Discussion: It was found that the 35%wt/vol solution of silver nitrate was the most effective in releasing Doxorubicin from DNA. The 10% wt/vol solution of TCA was found to be the second most efficient followed by the 0.3M solution of sodium acetate. Silver nitrate was able to retrieve 98.12% of the Doxorubicin initially bound to DNA according to the HPLC data, and 80.66% according to the fluorospectrometer data. TCA was able to retrieve 79.21% of the initial Doxorubicin from DNA according to the HPLC data and 85.91% according to the fluorospectrometer data. Sodium acetate was able retrieve 74.35% of the initial Doxorubicin from DNA according to the HPLC data and 17.27% according to the fluorospectrometer data. The discrepancy between the HPLC data and the fluorospectrometer data is most likely due to the quenching limitation of the fluorospectrometer causing a lower Doxorubicin concentration reading than the actual concentration. HPLC appears to be the more reliable and accurate device for measuring Doxorubicin concentration in solution (13).

Conclusion: This paper describes a simple and quick method of extracting Doxorubicin from DNA and then measuring the amount of unbound Doxorubicin. This assay will assist in the optimization of a functionalized DNA-based chemotherapy filter device by allowing the users to test how efficient a device is at absorbing excess Doxorubicin in a simple and quick method. This research uses genomic DNA in place of iron oxide bound DNA. The current model of the functionalized DNA-based chemotherapy filter device uses iron oxide bound DNA that allows the DNA to be magnetically bound to the filter device. Future investigations will test this assay using iron oxide bound DNA instead of genomic DNA. This will allow for a more accurate representation of the efficiency of the extraction agents ability to release Doxorubicin from DNA. This is done in order to measure unbound Doxorubicin and thus the amount of Doxorubicin the DNA is able to bind.

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Introduction

Hepatocellular Carcinoma, or liver cancer is the third type of cancer mortality worldwide (1). In the US, according to the American Cancer Society (ACS), in 2017 there will be an estimated 40,710 new cases of cancer and of those diagnosed 28,920 of them will pass away due to the disease (2). Worldwide in 2012, there were 782,000 new cases of liver cancer with 746,000 of the diagnosed having passed away due to this disease (3). In the U.S., the prevalence of liver cancer is on the rise (4). From the years 2005 to 2014, new cases of liver cancer has increased by 2.7% on average per year with death rates increase by 2.6% on average per year (4).

In the U.S. the average age of patients diagnosed with liver cancer is 63 years old with men having twice likelihood of developing the disease compared to women. Some known risk factors that increase the likelihood of developing liver cancer are obesity, heavy alcohol use, smoking, anabolic steroids, arsenic, and diabetes (5). Current treatments for liver cancer include surgery, tumor ablation, embolization, radiation, and chemotherapy. The research described in this paper will be focusing on chemotherapy, specifically the improvement of this method of treatment. Common chemotherapy agents include Cisplatin, Carboplatin, and Doxorubicin. While these chemotherapy agents are effective in eliminating cancer cells, up

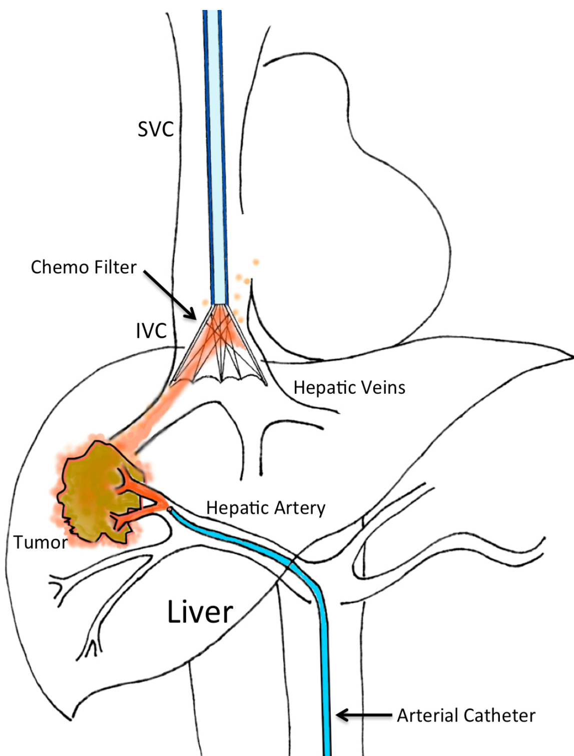
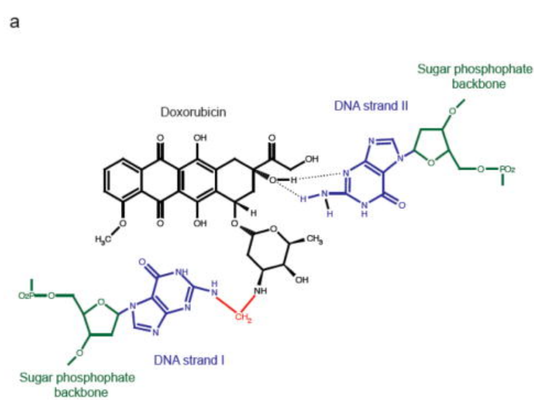


Figure 1: Deployment of Chemotherapy Filter along with Intra-Arterial Hepatic Chemotherapy Treatment

to 50-70% of the chemotherapy agent passes through the liver and circulates through the rest of the body (6). This can cause significant systemic toxicity and severely limit the efficacy of chemotherapy treatment. As shown in Figure 1, a solution to this limitation is to filter out the excess chemotherapy agent that passes through the liver by placing a chemotherapy filter in the hepatic vein during intra-arterial hepatic chemotherapy treatment. Current chemotherapy filters devices that are being researched include ionic-based, magnetic-based, and DNA-based filters. This research will be focusing on the mechanics of the DNA-based chemotherapy filter. DNA can be used to filter out excess Doxorubicin, this can be achieved by taking advantage of the intrinsic binding activity of Doxorubicin to DNA. As shown in Figure 2 Doxorubicin intercalates rapidly in-between the two strands of DNA (7). The planar anthracycline nucleus of Doxorubicin intercalates between the DNA double helix and allows the prevention of DNA replication (10). If the target goal of these chemotherapy filter devices is achieved, higher dosages of chemotherapy agents could be used during each treatment without the risk of systemic toxicity. It has been shown that increasing chemotherapy dose also linearly increases tumor suppression (8).



b

Figure 2: Structure of the doxorubicin-DNA complex. (a) Doxorubicin forms a covalent bond (shown in red) with guanine on one strand of DNA (b) A structure of intercalation of doxorubicin into DNA (7).

In order to further investigate the efficacy of the DNA-based chemotherapy filter, a method for measuring the amount of

Doxorubicin that DNA can bind must be produced. This research focuses on analyzing methods for releasing Doxorubicin from DNA and measuring the amount of unbound Doxorubicin, which represents the amount of Doxorubicin bound to the DNA. Three compounds were used as extraction agents in order to interrupt the binding kinetics between Doxorubicin and DNA. These agents include sodium acetate, trichloroacetic acid, and silver nitrate. Sodium acetate dehydrates the DNA and thereby releases the Doxorubicin from DNA while trichloroacetic acid acts as a precipitant to release Doxorubicin from DNA (12). Silver nitrate on the other hand, works creating a complex with DNA with reduced polarity and thus denatures DNA and release Doxorubicin. These methods were tested using both a fluorospectrometer and high performance liquid chromatography (HPLC), for their efficacy for releasing Doxorubicin from DNA. Both the fluorospectrometer and the HPLC were both set to the emission and absorbance wavelengths correlated to Doxorubicin, 560nm and 480nm respectively (9).

The aim of this study is to create a standardized assay for measuring the optimal amount of Doxorubicin that can bind to a functionalized DNA based chemotherapy filter. Three methods for releasing Doxorubicin from DNA were tested, these included either a 0.3M sodium acetate solution, a 10%wt/vol trichloroacetic acid solution, or a 35% wt/vol silver nitrate solution as extraction agents to release Doxorubicin from DNA. These concentrations of extraction agents were chosen based on previous literature (10, 12). It is predicted that 35% wt/vol silver nitrate should have the highest efficacy for releasing Doxorubicin from DNA because it is known to denature DNA by forming a complex of reduced polarity with DNA (10). The fluorospectrometer was used as a quick measuring tool to assess each of the three methods before the samples were measured using the

HPLC. In addition, standard curves were made for each method and on both the fluorospectrometer and HPLC. Standard curves were created for each separate experiment in order to obtain a slope equation used to convert the fluorescence or luminescence units, produced by the fluorospectrometer or the HPLC, into a concentration (ng/mL).

Materials and Methods

General Experiment

In order to accurately assess the efficacy of a DNA based chemotherapy filter, a 50mL solution of 0.05M Doxorubicin was added with 50mg of genomic DNA (Salmon sperm, Sigma-Aldrich, St. Louis, MO) in a 200mL beaker and stirred using a magnetic spin bar for 15 minutes allowing the Doxorubicin to bind to the genomic DNA. Initially, time points at 1, 3, 5, 10, and 15 minutes were taken during the mixture of DNA and Doxorubicin. 100µL of each time point were pipetted into individual wells on a well plate (Costar® 96-Well Black Plate, Solid Bottom) and analyzed with the fluorospectrometer (SPECTRA max M2, Molecular Devices).

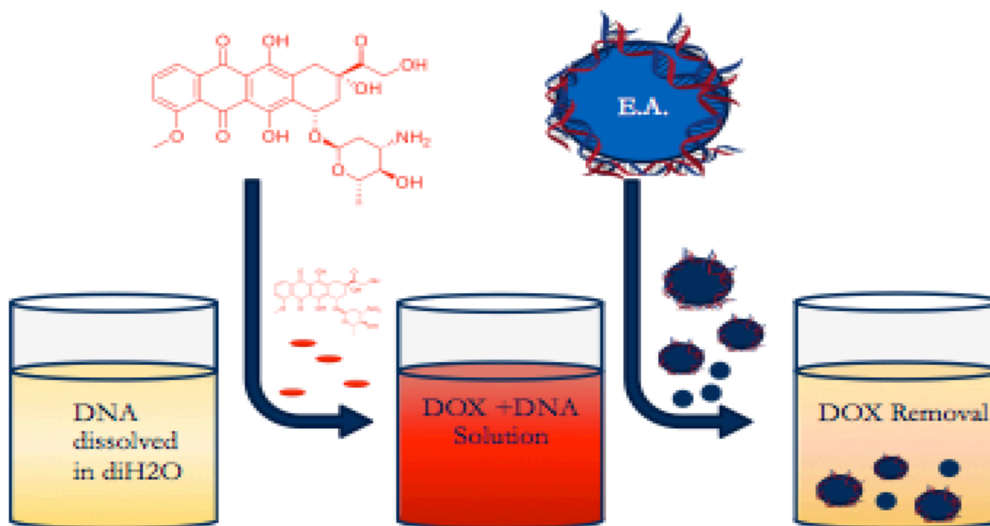


Figure 3: A work flow diagram of DOX+DNA binding and then removal of DOX with addition of an extraction agent (E.A.).

As shown in the work-flow diagram in Figure 3, either a 200μL solution of 0.3M sodium acetate, 10%wt/vol trichloroacetic acid (TCA), or 35%wt/vol silver nitrate was added to a micro-centrifuge containing 200μL of the mixed DNA and Doxorubicin solution in order to extract the bound Doxorubicin from DNA. The concentrations for these extraction agents were chosen from past literatures that describe similar DNA extraction experiments. (10, 12) The micro-centrifuge tube was then mixed using a vortex and then spun down in a micro-centrifuge for 10 minutes so that the DNA precipitant sunk to the bottom and the unbound Doxorubicin rose was on the top. The supernatant (top layer) was then extracted using a pipette, leaving the DNA precipitant at the bottom of the tube. Next, 100μL of the unbound doxorubicin solution was pipetted into a well plate (Costar® 96-Well Black Plate, Solid Bottom) and then analyzed using the fluorospectrometer (SPECTRA max M2, Molecular Devices). Another 100μL of the unbound Doxorubicin solution was pipetted into a HPLC vial and analyzed with the HPLC (Agilent 1100 Series, Hewlett Packard).

In addition to assessing the efficacy of each extraction agent, standard curves were created for each of the different extraction agent trials using both the fluorospectrometer and the HPLC. All graphs produced from both the fluorospectrometer and HPLC were analyzed using Microsoft Excel (Microsoft Office 2011).

Fluorospectrometer and HPLC Equipment

Fluorospectrometer (SPECTRA max M2, Molecular Devices)

-Temperature set at room temperature (20-25°C).

-Absorbance set at 480 nm and Emission set at 560 nm for Doxorubicin.

-96- Well Black plate with Solid Bottom used (Costar®).

-100μL samples used per well.

HPLC (Agilent 1100 Series, Hewlett Packard)

-Temperature set at room temperature (20-25°C).

-LCGC Certified Amber Glass 12 x 32mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 2 mL Volume, 100/pkg.

-Solvents: 50% Acetonitrile and 50% Phosphate Buffer (pH 4).

-Run speed: 1mL/min.

-Set at 30μL injections per samples.

-Absorbance Detector set at 480nm for Doxorubicin.

-Fluorescence Detector set at 480nm absorbance and 560nm emission for Doxorubicin.

Preparing DNA and Doxorubicin Solution

1. 50mg of genomic DNA (Salmon sperm, Sigma-Aldrich, St. Louis, MO) was dissolved in 20mL of diH20 for 15-20 minutes.

2. 1.25mL of Doxorubicin(1mg/mL) was added to 28.75mL of diH2O and also stirred using a magnetic spin bar for 15-20 minutes.

3. When both solutions have reached a homogenized consistency, they were mixed together and stirred using a magnetic spin bar for another 15 minutes to allow Doxorubicin to bind to DNA.

4. 100µL samples were taken at time points 1, 3, 5, 10, and 15 minutes and then analyzed using the fluorospectrometer.

Preparing 0.05 mg/ml Doxorubicin Control Solution

-Added 1.25mL of 1mg/mL Doxorubicin to 48.75 mL diH2O in a beaker and then stirred using a magnetic stir bar for 10 minutes to obtain a 50mL solution of 0.05M Doxorubicin.

Preparing Extraction Solutions

Sodium Acetate (0.3M)

-To obtain a solution of 0.3M concentration of sodium acetate, 4.1015 grams of Sodium Acetate Anhydrous (Molecular Weight of 82.03) to 50mL of ethanol and then stirred using a magnetic spin bar for 20 minutes.

Trichloroacetic Acid (10%wt/vol)

-To obtain a 10% weight per volume concentration of TCA, 5 grams of Trichloroacetic Acid (TCA) Anhydrous was dissolved in 50 mL of diH2O in a beaker and mixed for 20 minutes using a magnetic stir bar.

Silver Nitrate (35%wt/vol)

-To obtain a 35% weight per volume concentration of silver nitrate solution, 17.5 grams of Silver Nitrate Anhydrous was dissolved in 50 mL of diH2O in a beaker and mixed for 20 minutes using a magnetic stir bar.

Testing Extraction Agents

1. 500µL of the DNA+DOX solution was added to a micro-centrifuge tube.
2. 500µL of one of the three extraction agents was added to the same micro-centrifuge tube.
3. The solution was vortexed for 4-7 seconds.
4. This solution was spun down in a centrifuge for a minimum of 10 minutes so that the precipitants have aggregated to the bottom of the tube.
5. Next, the supernatant (upper layer) was pipetted out and placed in a micro-centrifuge tube, which should contain only the released unbound Doxorubicin.
6. 100µL of the supernatant was pipetted into a well plate.
7. Solution was analyzed using the fluorospectrometer.
8. The leftover 400µL was pipetted into a HPLC vial and analyzed by the HPLC.
9. Repeated steps 1-7 for the all other extraction agents (Sodium Acetate, TCA, Silver Nitrate).

Preparing Solutions for Standard Curve

1. Six micro-centrifuge tubes were obtained,
2. Next, 500µL of one of the extraction agents was added to each of the micro-centrifuge tubes.
3. After all micro-centrifuge tubes were filled, 500µL of the 0.05M Doxorubicin control solution was added to the first micro-centrifuge tube.
4. The micro-centrifuge tube with the added Doxorubicin was then vortexed for 6-10 seconds.

5. Next, 500μL of solution from the first micro-centrifuge tube was pipetted into one of the other micro-centrifuge tubes that only contain one of the extraction agents.
6. Steps 1-5 were repeated until all 6 micro-centrifuge tubes were diluted by the Doxorubicin solution.
7. 100μL of each of the 6 different concentrations of Doxorubicin were pipetted into their own respective wells on a well plate (Costar® 96-Well Black Plate, Solid Bottom).
8. Solution was Analyzed using a fluorospectrometer.
9. The leftover 400μL of solution from each of the micro-centrifuge tubes was then pipetted into a HPLC vial and analyzed with the HPLC.
10. Steps 1-8 were repeated for each of the three extraction agents (Sodium Acetate, TCA, and Silver Nitrate)

The data obtained from both the fluorospectrometer and HPLC with units of relative fluorescence units (RFU) and Luminescence Units (Lu*s) respectively, were analyzed using Microsoft Excel. The best-fit line was found for each of the standard curves and the slope equation obtained from the best fit line was then used to convert RFU and Lu*s units obtained from the fluorospectrometer and HPLC into a concentration (ng/mL).

Results

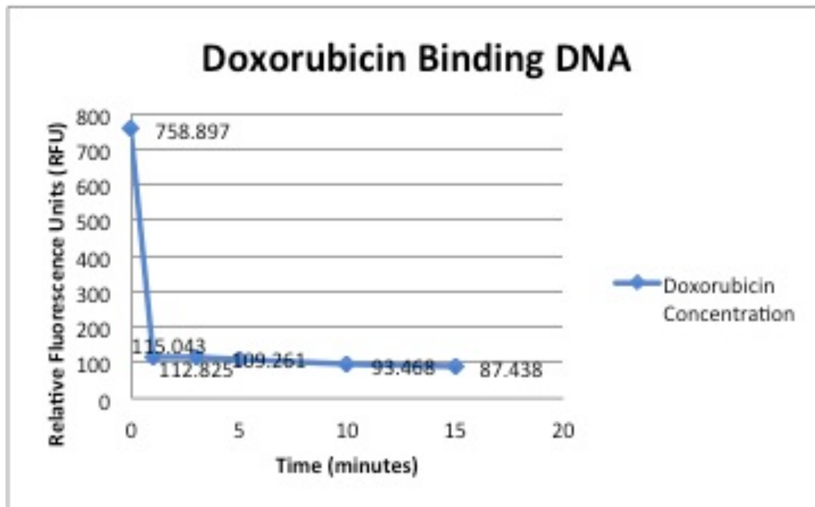


Figure 4: Time-points (1, 3, 5, 10, 15 minutes) of Doxorubicin Binding DNA. The concentration of unbound Doxorubicin, represented by RFU, decreases from 758 RFU to around 100 RFU almost instantaneously. After the 1-minute mark, the concentration of unbound Doxorubicin only decreases incrementally compared to the initial reduction before the 1-minute time-point.

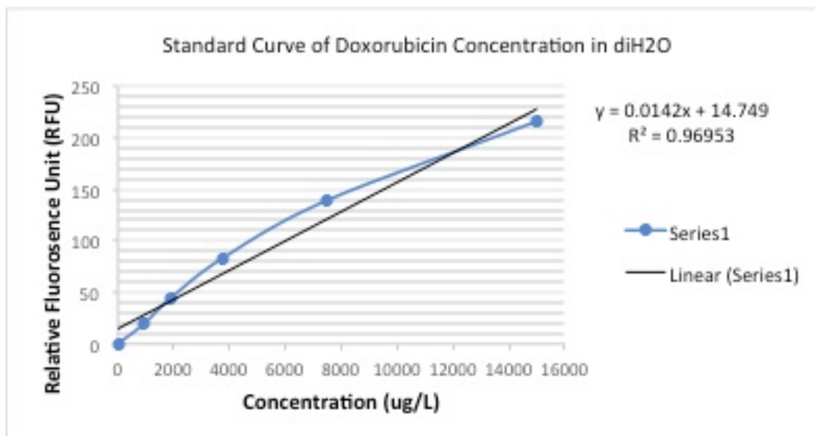


Figure 5: Standard Curve of Doxorubicin Concentration in diH2O (Fluorospectrometer).

When attempting to measure how quickly the Doxorubicin would bind to DNA using the time point samples at 1, 3, 5, 10, and 15 minutes, all of the Doxorubicin appeared to bind almost instantaneously with the DNA. This can be seen in Figure 4, after the time-point at 1 minute, the rest of the samples produced similar RFU values obtained from the fluorospectrometer. At the 1-minute time-point, the DNA absorbed 84.84% of the Doxorubicin. After the 1-minute time-point the DNA absorbed only 3.638% of the Doxorubicin, contributing to a total of 88.48% unbound Doxorubicin absorbed by DNA at the 15-minute time-point. The average of the values after the 1-minute time point was found to be 103.6 RFU with a standard deviation of +/- 12.37 RFU. When converting this average, using the slope equation ($y=0.0142x+ 14.75$ $R^2 = 0.9695$) obtained from the

standard curve shown in Figure 5, the concentration of unbound Doxorubicin was found to be 6258 ng/mL. When converting the initial amount of Doxorubicin in solution, 758.9 RFU, the equation yields 52405 ng/mL. By dividing the amount of unbound Doxorubicin leftover by the initial amount and subtracting that value from 1 and then multiplying it by 100, yields the percentage of Doxorubicin that bound to genomic DNA. It was found that 86.5% of the initial Doxorubicin was intercalated into the genomic DNA after 1 minute of mixing the two solutions together.

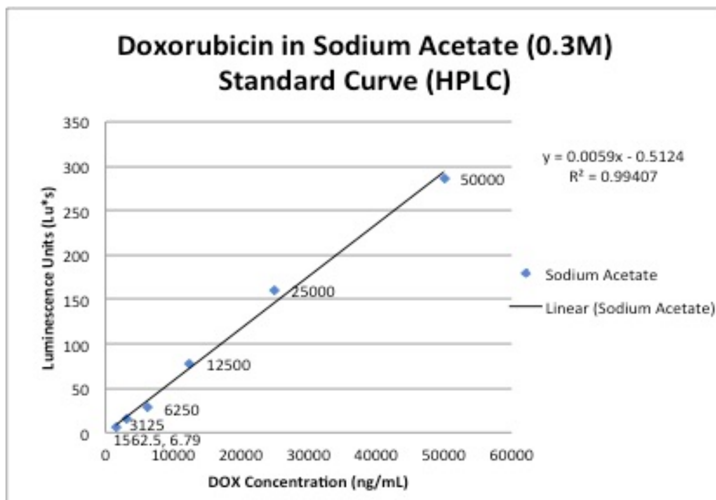


Figure 6: Doxorubicin in Sodium Acetate (0.3M) Standard Curve (HPLC).

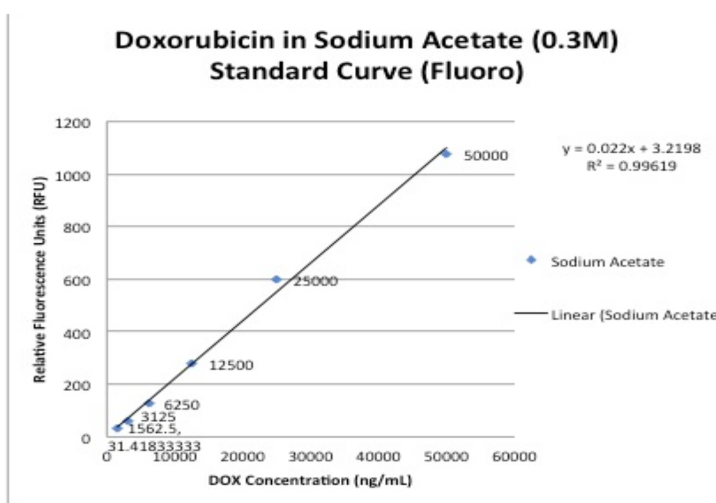


Figure 7: Doxorubicin in Sodium Acetate (0.3M) Standard Curve (Fluorospectrometer).

The slope equation from the best-fit line obtained from the standard curve from the fluorospectrometer as shown in Figure 7 with sodium acetate was y=0.022x+3.220 with an R^2 of 0.9962. As shown in Figure 6, the slope equation obtained from the HPLC was y=0.0059x-0.5124 with an R^2 of 0.9941.

As shown in Figure 9, when a solution of 0.3M sodium acetate was added to the Doxorubicin and DNA mixture the RFU increased from 83.94 RFU to 210.7 RFU with a standard deviation of +/- 8.128 RFU. Using the slope

equation from Figure 6, RFU was converted into concentration of unbound Doxorubicin in solution. From this conversion, it was found that the concentration of unbound Doxorubicin increased to 9429 ng/mL from 3669 ng/mL. Of the maximum concentration of Doxorubicin, 50,000ng/mL, according to the fluorospectrometer data, 0.3M sodium acetate was able to extract 17.27% of the Doxorubicin from DNA. In Figure 8, the sodium acetate data from the HPLC can be seen.

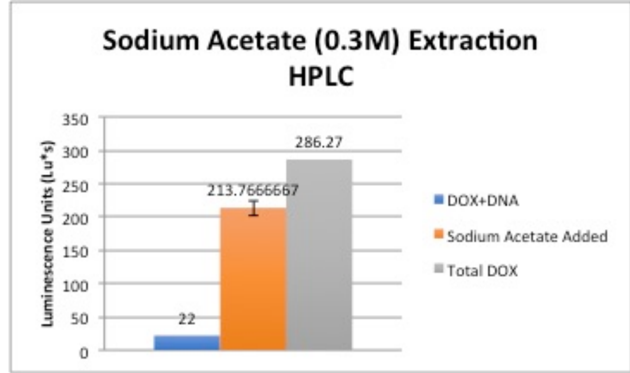


Figure 8: Doxorubicin Extraction With Sodium Acetate (0.3M) (HPLC) First column shows a solution of DOX+DNA. The second column shows the solution after 0.3M solution of Sodium Acetate was added. The third column shows a solution of pure 0.05M Doxorubicin.

According to the HPLC data, when 0.3M sodium acetate was added to the Doxorubicin and DNA mixture, the Lu*s increased from 22 Lu*s to 213.8 Lu*s with a standard deviation of +/- 11.61 Lu*s. When converting this using the slope equation from Figure 6, the concentration was found to increase to 36320 ng/mL from 3815 ng/mL. Of the maximum concentration of Doxorubicin, according to the HPLC data, 0.3M sodium acetate was able to recover 74.35% of the initial Doxorubicin.

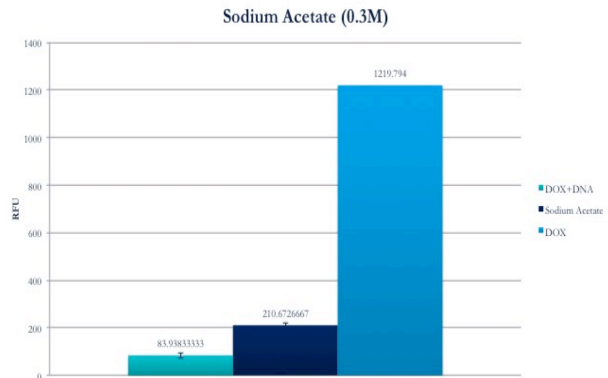


Figure 9: Doxorubicin Extraction With Sodium Acetate (0.3M) (Fluorospectrometer) First column shows a solution of DOX+DNA. The second column shows the solution after 0.3M solution of Sodium Acetate was added. The third column shows a solution of pure 0.05M Doxorubicin.

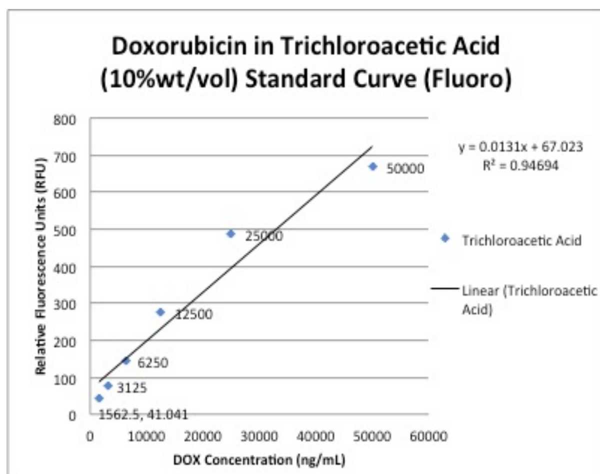


Figure 10: Doxorubicin in Trichloroacetic Acid (10%wt/vol) Standard Curve (Fluorospectrometer).

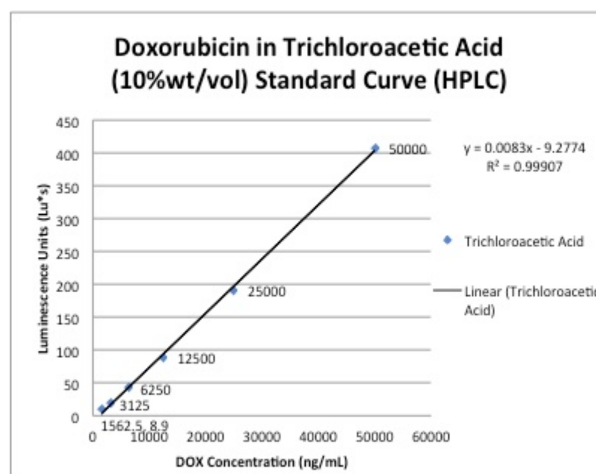


Figure 11: Doxorubicin in Trichloroacetic Acid (10%wt/vol) Standard Curve (HPLC).

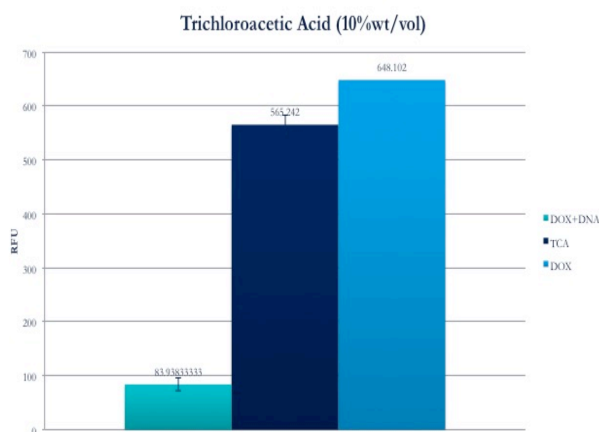


Figure 12: Doxorubicin Extraction With Trichloroacetic Acid (10wt/vol) (Fluorospectrometer) First column shows a solution of DOX+DNA. The second column shows the solution after Trichloroacetic Acid (10wt/vol) was added. The third column shows a solution of pure 0.05M Doxorubicin.

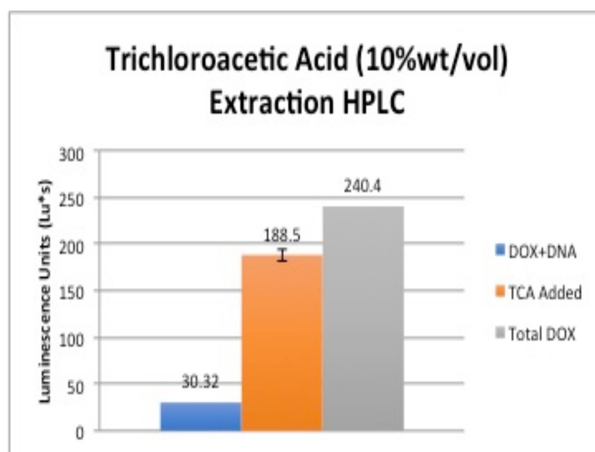


Figure 13: Doxorubicin Extraction With Trichloroacetic Acid (10%wt/vol) (HPLC) First column shows a solution of DOX+DNA. The second column shows the solution after Trichloroacetic Acid (10wt/vol) was added. The third column shows a solution of pure 0.05M Doxorubicin.

As shown in Figure 10, the slope equation from the TCA standard curve obtained from the fluorospectrometer was found to be y=0. 0131x+67.02 with an R^2 of 0.9496. As shown in Figure 11, the slope equation from the TCA HPLC standard curve was found to be y=0.0083x-9.277 with an R^2 of 0.9991.

As shown in Figure 12, after the addition of a 10%wt/vol solution of TCA, the RFU increased from 83.94 RFU to 566.2 RFU with a standard deviation of +/- 16.77 RFU. When

converting RFU using the slope equation from Figure 10, the increase of concentration was found to be from 1291 ng/mL to 38110 ng/mL. Overall, TCA was able to release 85.91% of the initial concentration of Doxorubicin from DNA. As shown in Figure 13, according to HPLC data, the Lu*s increased from 30.32 Lu*s to 188.5 Lu*s with a standard deviation of +/- 6.514 Lu*s and a maximum of 240.4 Lu*s. When converting this using the slope equation found in Figure 11, the concentration of unbound Doxorubicin was found to increase from 4771 ng/mL to 23830 ng/mL. A solution of 10% wt/vol TCA, according to the HPLC data, was able to release of 79.21% bound Doxorubicin from DNA.

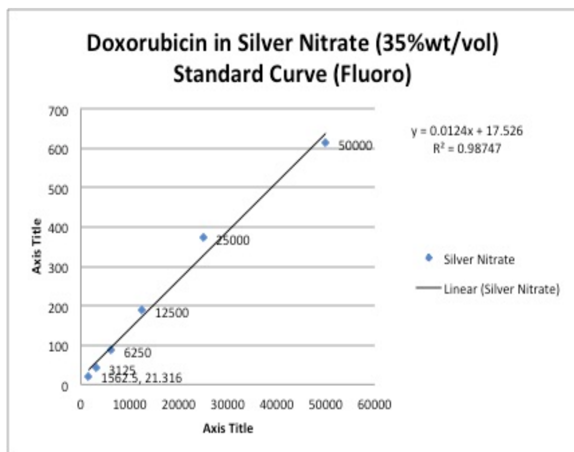


Figure 14: Doxorubicin in Silver Nitrate (35%wt/vol) Standard Curve (Fluorospectrometer).

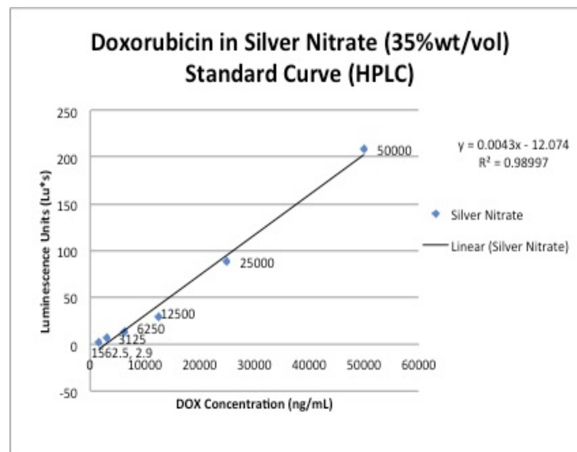


Figure 15: Doxorubicin in Silver Nitrate (35%wt/vol) Standard Curve (HPLC).

As shown in Figure 14, the slope equation obtained from the silver nitrate fluorospectrometer standard curve was found to be y=0.0124x+17.53 with an R^2 of 0.9875. As shown in Figure 15, the slope equation obtained from the silver nitrate HPLC standard curve was found to be y=0.0043x-12.07 with an R^2 of 0.9899.

From Figure 16, it was found that with the addition of a 35%wt/vol solution of silver nitrate to the Doxorubicin and DNA mixture, according to the fluorospectrometer data, the RFU increased from 83.938 RFU to 513.02 RFU with a standard error of +/-11.83 RFU and a maximum of 631.8 RFU. Using the slope equation found in Figure 14, the

concentration was found to increase from 5355 ng/mL to 39960 ng/mL. The addition of Silver nitrate was found release 80.66% of the bound Doxorubicin from DNA. As shown in Figure 17, the HPLC data shows an increase of luminescence from 21.73 Lu*s to 219.7 Lu*s with a standard error of +/- 8.018 Lu*s and a maximum of 225.1 Lu*s. Using the slope equation found in Figure 15, it was found that the concentration of unbound Doxorubicin increased from 7861 ng/mL to 53900 ng/mL. According to the HPLC data, 35% wt/vol of silver nitrate was able to release 98.12% of the initial amount of Doxorubicin bound to DNA.

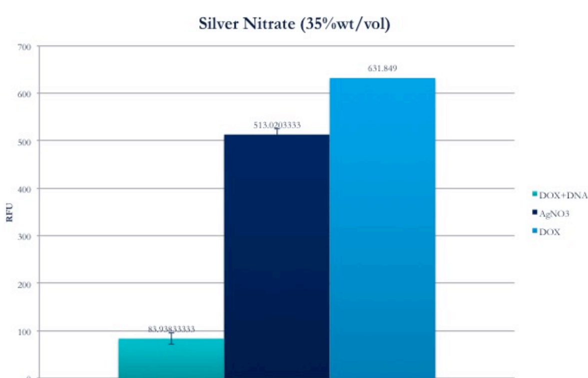


Figure 16: Doxorubicin Extraction With Silver Nitrate (35%wt/vol) (Fluorospectrometer) First column shows a solution of DOX+DNA. The second column shows the solution after Silver Nitrate (35%wt/vol) was added. The third column shows a solution of pure 0.05M Doxorubicin.

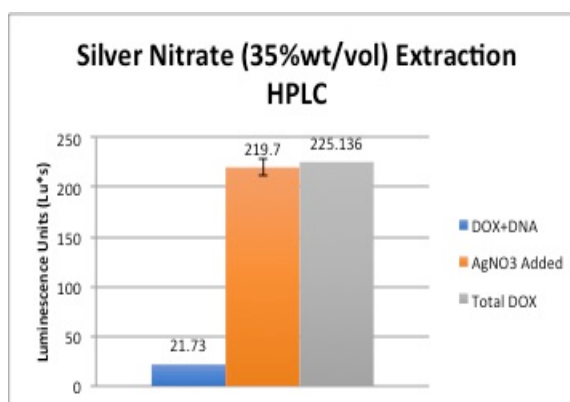


Figure 17: Doxorubicin Extraction With Silver Nitrate (35%wt/vol) (HPLC) First column shows a solution of DOX+DNA. The second column shows the solution after Silver Nitrate (35%wt/vol) was added. The third column shows a solution of pure 0.05M Doxorubicin.

DISCUSSION

	HPLC			Fluorospectrometer		
	Sodium Acetate (0.3M)	TCA (10%wt/vol)	Silver Nitrate (35%wt/vol)	Sodium Acetate (0.3M)	TCA (10%wt/vol)	Silver Nitrate (35%wt/vol)
Trial 1	225.10	194.20	225.20	201.89	558.71	502.51
Trial 2	214.30	189.90	223.40	217.93	552.72	525.84
Trial 3	201.90	181.40	210.50	212.19	584.30	510.72
Average	213.77	188.50	219.70	210.67	565.24	513.02
Standard Error (+/-)	11.61	6.51	8.02	8.13	16.77	11.83
Concentration (ng/mL)	36320	23830	53900	9429	38110	39959
% Doxorubicin Recovery	74.35%	79.21%	98.12%	17.27%	85.91%	80.66%

Figure 18: Summary Table of Extraction Agents with HPLC and Fluorospectrometer Data.

When comparing all of the extraction agents as shown in Figure 18, it was found that TCA was able to release the most Doxorubicin from DNA based on the data obtained

with the fluorospectrometer. Although, when using HPLC data, silver nitrate appeared to have the highest yield for releasing Doxorubicin from DNA. This discrepancy is most likely due to the limitations of the fluorospectrometer. The data obtained using the fluorospectrometer can be greatly influenced by quenching. Quenching is a phenomenon that occurs when a compound emits a photon but is absorbed by another nearby compound and therefore is not detected by the fluorospectrometer. This limitation can be assessed when comparing the sodium acetate data obtained from the fluorospectrometer and the HPLC. According to the fluorospectrometer, only 17.27% of the bound Doxorubicin was released after the addition of sodium acetate but when examining the sodium acetate data obtained from the HPLC, it showed that 74.35% of the bound Doxorubicin was released from DNA. The HPLC is not affected by the quenching phenomenon because it is able to separate compounds such as DNA and Doxorubicin, which can cause quenching, by size and analyzes those compounds at different times, thus making the HPLC a more reliable and accurate measuring device compared to the fluorospectrometer. The compound that a

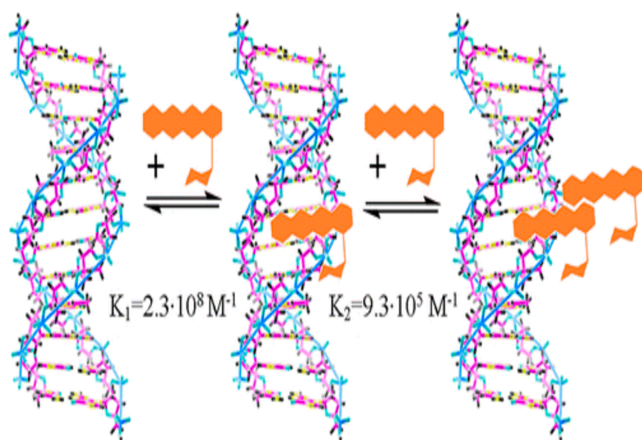


Figure 19: Two-step mechansim of Doxorubicin Binding DNA (11). First a fast step occurs, Doxorubicin binds to the outer AT region on DNA. Second, a slow step occurs, Doxorubicin binds the GC region and intercalates into DNA. When all intercalation sites are filled, Doxorubicin continues to bind to DNA through the first fast step of the two-step binding mechanism.

The concentrations chosen in this paper for the extractions agents were all obtained through papers that successfully precipitated DNA using a 0.3M concentration of sodium acetate (12) or a

10% wt/vol concentration of TCA (10). It was also found that a 30-35%wt/vol concentration of silver nitrate would be sufficient to denature 50mg of genomic DNA (10).

Although silver nitrate is clearly the most effective extraction agent compared to sodium acetate and TCA, it denatures the DNA so that it can no longer be used. Sodium acetate on the other hand appears to remove Doxorubicin through a mechanism that does not damage the DNA. Although sodium acetate was able to release a fair amount of Doxorubicin from DNA, it was limited compared to the other extraction agents because it was unable to extract all of the intercalated Doxorubicin. According to a study done in 2014, Doxorubicin binds to DNA in a two-step process that can be seen in Figure 19 (11). First, a fast step in which the Doxorubicin binds to the outer part of the DNA on the adenine and thymine (AT) region. Second, a slow step in which the Doxorubicin intercalates into the guanine and cytosine (GC) region of DNA. When all the intercalation sites are filled, Doxorubicin binds only to the AT regions of DNA through only the first step of the binding mechanism (11). Sodium acetate appears to be able to release the Doxorubicin bound to the outer portions of DNA but not all the Doxorubicin intercalated in the DNA. Trichloroacetic acid appears to fall under the same limitations as sodium acetate and is not able to fully release all of the intercalated Doxorubicin from DNA.

Future Investigations

A future goal for this project is to attempt use a higher concentration of sodium acetate or a combination of TCA or silver nitrate with sodium acetate to extract a higher concentration of Doxorubicin from DNA. In addition, the current DNA-based chemotherapy device uses iron oxide bound DNA to magnetically bind DNA to the chemotherapy filter

device. In light of this, the proposed assay should first be tested using iron oxide bound DNA to further simulate in-vivo usage of the DNA-based chemotherapy filter.

Conclusion

Ultimately, using a 35% wt/vol solution of silver nitrate was the most efficient extraction agent with the 10% wt/vol solution of TCA being the second best and the 0.3M solution of sodium acetate being the worst. In addition, it was found that HPLC was a much more reliable and accurate measuring device compared to the fluorospectrometer.

This extraction assay may be utilized in a few different ways depending on the goal of the user. Either silver nitrate or TCA could be used to extract Doxorubicin from DNA in order to measure the efficacy of a functionalized DNA-based chemotherapy device. If the user of this assay would like to preserve the functionalize DNA-based chemotherapy filter, 10%wt/vol solution of TCA or a 0.3M solution of sodium acetate may be used to a degree of inaccuracy due to their inability to fully extract intercalated Doxorubicin from DNA. If the user of the assay prefers a more accurate assessment of the amount of Doxorubicin absorbed by the functionalized DNA-based chemotherapy filter and preserving the DNA is unimportant, then a 35% wt/vol solution of silver nitrate can be used along with HPLC to measure the luminescence of the unbound Doxorubicin. Overall, this assay is a simple and quick method for measuring the efficacy of a functionalized DNA-based chemotherapy device.

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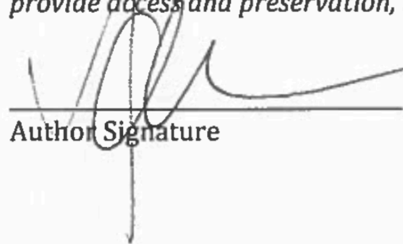

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